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Abstract

During the transition period, dairy cows experience a negative energy balance (**NEB**) caused by the high energy requirement for milk yield, while feed intake is limited. Severity of the NEB has been associated with an increased incidence of metabolic disorders and infectious diseases, inflammation, immunosuppression and oxidative stress. It is known that shortening or omitting the dry period or feeding a glucogenic ration improves the energy balance (**EB**) in dairy cows in early lactation. It can be expected that an improvement of the EB due to shortening or omitting the dry period results in reduced inflammation, immunosuppression and less oxidative stress in dairy cows in early lactation. The first objective of this thesis was to study the effects of dry period length and dietary energy source on immune competence, inflammatory biomarkers and oxidative stress in dairy cows over 2 subsequent lactations. The second objective was to study the consequences of maternal dry period length on colostrum immunoglobulin content and immune competence of calves in the first 12 weeks of life. In the current study, 167 cows were assigned to 3 dry period lengths (0, 30, or 60 d) and 2 early lactation rations (glucogenic or lipogenic). Cows were planned to have the same dry period length and ration over 2 subsequent lactations. Omitting the dry period reduced plasma bilirubin levels compared with a conventional dry period, which is line with the better EB in cows with a 0-d dry period. Effects of dry period length on inflammatory biomarkers, oxidative stress variables and natural antibodies (**NAb**) titers were, however, less consistent. Omitting the dry period increased not only negative acute phase proteins (**APP**) in plasma, but also positive APP, oxidative stress variables in plasma, and NAb in milk. Shortening the dry period to 30-d did not influence inflammatory biomarkers and oxidative stress compared with a conventional dry period of 60-d. Occurrence of clinical health problems did not differ between cows with different dry period lengths. In the current study, changes in positive APP and oxidative stress variables in plasma and NAb in milk could be explained by the occurrence of clinical health problems related to inflammation (clinical mastitis, fever, metritis and retained placenta), rather than a better EB due to a shorter or no dry period. Moreover, a higher titer of IgG binding lipopolysaccharide in plasma was associated with decreased odds of high somatic cell count and occurrence of clinical mastitis. In the first lactation after implementation of dry period length and dietary treatments, feeding a glucogenic ration in early lactation increased NAb titers in milk compared with a lipogenic ration, which could be explained partly by a better EB. In the second lactation after

implementation of dry period length and dietary treatments, feeding a lipogenic ration in early lactation increased cholesterol levels in plasma compared with a glucogenic ration, which could be related to the high fat content in this ration. Cows with a 0-d dry period had a lower colostrum production and less immunoglobulins in colostrum compared with cows with a 30-d or 60-d dry period. After colostrum uptake, NAb titers in plasma of calves from cows with a 0-d dry period were lower during the first week of life compared with calves from cows with a 30-d or 60-d dry period. Levels of specific antibodies in calves, after immunization in week 6 and 10, in calves were not affected by the maternal dry period length. Birth weight of calves from cows with a 0-d dry period was lower compared with calves from cows with a 30-d dry period, but not compared with calves from cows with a 60-d dry period. Growth of calves until 12 weeks of life was not affected by dry period length. In conclusion, although shortening and omitting the dry period improved the EB in early lactation, this did not result in clear consistent effects of dry period length on inflammation or oxidative stress. Changes in inflammation biomarkers, oxidative stress variables and NAb in milk were a reflection of the occurrence of health problems related to inflammation in particular clinical mastitis and compromised uterine health. Furthermore, albeit omitting the dry period compared with shortening or conventional dry period cows resulted in a reduced immunoglobulin content in colostrum and reduced NAb titers in plasma of their calves in the first week of life, but did not affect specific immune response of the calves in the first 12 weeks of life.

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CHAPTER 1

General introduction

1.1 Achievements of the Dairy Industry

In the past decades, continuous progress in genetic selection, diet regimes and technology increased milk production of dairy cows. In the past 20 years, total milk production in the world increased by more than 60 percent (Figure 1.1). Based on FAOstat database (2016), main milk producing countries in the world are India, The United States of America (USA), The Russian Federation, Germany, and Pakistan. The USA, Germany and India were responsible for more than 50 percent of milk production in the world in 2013. Moreover, the Netherlands produced 2 percent of the milk produced by dairy cows in the world in 2013. The average size of dairy herds has continuously increased over recent decades in selected developed countries, where New Zealand has the highest average number of cows per herd (from 200 till 400 cows per herd) when compared to the USA (75 till 150 cows per herd) and Germany (25 till 50 cows per herd) from 1996 till 2014 (Barkema et al., 2015).
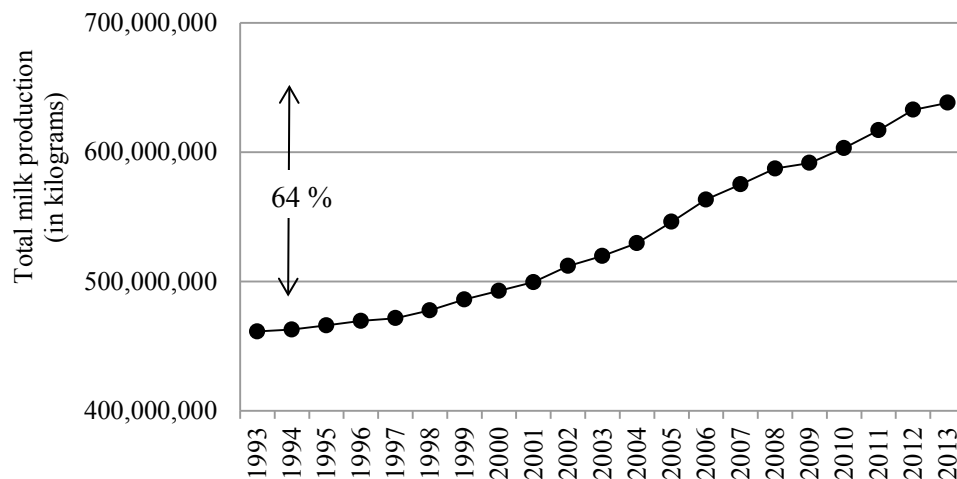

Figure 1.1 Total of milk production from cows in the world over the past 20 years (FAOstat, 2016)

1.2 Health of High-Producing Dairy Cows

High milk production in dairy cows is associated with increased health problems especially during the transition period (Heuer et al., 1999, Fleischer et al., 2001, Walsh et al., 2011). The transition period in dairy cows is defined as 3

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weeks before to 3 weeks after calving (Grummer, 1995, Drackley, 1999) and is considered as a critical time in the lactation cycle of a dairy cow. Most health problems occur during this period. The main health problems in dairy cows are mastitis, lameness and reduced fertility. The prevalence of these 3 major health problems is particularly high during the transition period (Collard et al., 2000, Koeck et al., 2012). Mastitis is an inflammation of the udder and one of the most costly health problems in dairy industry amounting about €61 - €97 per cow on a farm annually (Hogeveen et al., 2011). The risk for clinical mastitis increases with age, and severity of negative energy balance (**NEB**) in early lactation (Leslie et al., 2000) and is associated with a reduced productive life time (Carlén et al., 2004). Lameness represent group of foot and leg problems resulting in poor performance and welfare problems (Whay et al., 1997). Cows with lameness had decreased milk yield (Green et al., 2002, Amory et al., 2008), decreased fertility (Garbarino et al., 2004) and reduced productive life time (Booth et al., 2004). Reduced fertility in high-producing dairy cows is related to reproduction problems like metritis and cystic ovarian disease and manifests by a longer interval from calving until first ovulation and first oestrus, lower conception rates and higher frequency of abnormal oestrus cycles, resulting in an increased calving interval and replacement rate (Lucy et al., 2001, Opsomer et al., 2000, Beam and Butler, 1999).

Mastitis, lameness and reduced fertility in dairy cows are not only related to the start of lactation, but also to metabolic stress related to the high metabolic priority for lactation and the inability of the cow to adapt effectively to the new lactation (Van Knegsel et al., 2014). Failure to adapt to a new lactation is related with the severity of NEB and can result in metabolic and digestive disorders like milk fever, ketosis, fatty liver and left displaced abomasum (Grummer, 1993, Rajala-Schultz et al., 1999). Prevalence of clinical mastitis, lameness, reproductive problems, and ketosis varies widely between studies (Table 1.1). The prevalence of clinical mastitis ranged from 10.1 to 23.0 percent in the first 4 weeks after calving (LeBlanc et al., 2002, Zwald et al., 2004, Santschi et al., 2011). The prevalence of lameness in dairy cows ranged from 3.1 to 12.2 percent in the first 4 weeks after calving (LeBlanc et al., 2002, Zwald et al., 2004). Prevalence of reproduction problems including cystic ovaries, metritis, and retained placenta in dairy cows in the first 4 weeks after calving ranged from 3.0

to 60.4 percent (LeBlanc et al., 2002, Zwald et al., 2004, Santschi et al., 2011). The prevalence of clinical endometritis ranged from 15 to 20 percent in the first 4 weeks after calving (LeBlanc et al., 2002, Sheldon et al., 2009). Ketosis prevalence in dairy cows in the first 4 weeks after calving ranges between 3.1 - 90.3 percent (LeBlanc et al., 2002, Watters et al., 2008, Santschi et al., 2011).

Low fertility and health problems affect performance and culling rates in dairy cows and are the main reason for a short productive life time (Beaudeau et al., 1995). It can be hypothesized that a reduction in the prevalence of health problems in early lactation e.g. by improving adaptation to a new lactation and improving the EB in early lactation, increases productive life time in dairy cows. Earlier, metabolism and energy balance (**EB**) in early lactation have been related with productive life time of dairy cows (Seifi et al., 2011, Roberts et al., 2012, Chiumia et al., 2013). Both a high body condition score at last service in heifers (Chiumia et al., 2013) and an elevated plasma free fatty acid (**FFA**) concentration in early lactation (Seifi et al., 2011) were associated with an increased risk of early culling. A retrospective study showed an elevated FFA and β-hydroxybutyrate (**BHB**) concentrations in plasma and reduced plasma calcium concentrations in early lactation were associated with an increased risk of culling (Roberts et al., 2012).

1.3   Negative Energy Balance During the Transition Period

A NEB in high yielding dairy cows in early lactation is a result of high energy requirement for maintenance and milk production that is not compensated by a sufficient energy intake. Consequently, dairy cows mobilize body fat and to a lesser extend body protein to support energy requirements for maintenance and milk production (Grummer, 1995). The NEB already starts 3 weeks before calving due to high nutrient requirements of the fetus (Bell, 1995), the decreased feed intake during late gestation, and the onset of lacto-genesis in this period (Grummer, 1995). The NEB is most severe during the first weeks after calving, when milk production is high and feed intake is still limited. High producing dairy cows experience a dramatic increase in energy requirements which reaches the peaks between 4 and 8 weeks after calving (Grummer, 1995, Hammon et al., 2009).

1.4 Metabolic Disorders and Infectious Diseases during the Transition Period

Dairy cows experience substantial metabolic and physiological adaptations during the transition from pregnancy to lactation. Metabolic changes during the transition period are characterized by increased FFA and BHB concentrations in plasma (Drackley et al, 2001, Gross et al., 2011) which are related with extensive body fat mobilisation and fatty acid oxidation (Wathes et al., 2007a). In early lactation, the hepatic uptake of FFA exceeds the capacity of the liver to oxidize FFA or to export them from the liver as very low density lipoprotein (**VLDL**). This results in accumulation of tri-acyl-glycerides (**TAG**) in the liver and elevated ketone body concentrations in plasma (Drackley, 1999). High hepatic TAG and high ketone bodies in plasma can result in a fatty liver and ketosis (Veenhuizen et al., 1991, Grummer, 1995). Moreover, cows that experience a severe NEB during transition period show dramatic changes in circulating hormones such as progesterone, estrogen, cortisol, insulin and insulin like growth factor-I (**IGF-I**) (Goff and Horst, 1997). At the onset of lactation, cows with a severe NEB have decreased plasma glucose, insulin and IGF-I concentrations to support elevated endogenous glucose production and transport of glucose and FFA to the mammary gland (Hammon et al., 2009, Gross et al., 2011). As a result, FFA concentration in plasma is increased and cows are more susceptible to ketosis and fatty liver in early lactation (Grummer, 1993). Additionally, low insulin and glucose concentrations in plasma enhance lipolysis in adipose tissue, which is related to reduce responsiveness of the ovaries to gonadotropin stimulation (Butler and Smith, 1989). Another consequence of the high metabolic rate and milk production in early lactation is an increase in mineral requirements such as calcium and magnesium. If the requirements of calcium and magnesium are not met, this results in cows that suffer from clinical hypocalcaemia (milk fever) and hypomagnesaemia (Mulligan and Doherty, 2008).

Table 1.1 Prevalence of health problems during lactation in dairy cows.

Study	Health problem	Time (from calving to ..)	Frequency (percent)	Number of cows in the study (N)
Koeck et al. (2012)[1]	Mastitis	305 d	12.5	54801
	Lameness	305 d	9.0	32172
	Retained placenta	305 d	4.5	75154
	Metritis	150 d	10.8	53327
	Cystic ovaries	150 d	8.6	41144
	Ketosis	100 d	4.4	24237
Collard et al. (2000)[2]	Mastitis	100 d	35.0	140
	Laminitis	100 d	25.7	140
	Reproductive problems (metritis, cystic ovaries, uterus infection, retained placenta, and vaginitis)	100 d	16.4	140
Zwald et al. (2004) [1,2]	Mastitis	28 d	23.4	105029
	Lameness	28 d	12.2	50611
	Cystic ovaries	28 d	2.8	65080
	Metritis	28 d	60.4	97316
	Ketosis	28 d	90.3	52898
LeBlanc et al. (2002) [1,2]	Mastitis	30 d	10.1	21
	Lameness	30 d	3.1	21
	Retained placenta	30 d	14.4	21
	Metritis	30 d	3.0	21
	Endometritis	30 d	14.0	21
	Ketosis	30 d	3.1	21
Watters et al. (2008) [1,2]	Mastitis	300 d	35.0	210
	Lameness	300 d	14.0	210
	Retained placenta	300 d	8.9	210
	Metritis	300 d	19.5	210
	Ketosis	300 d	18.6	210
Santschi et al (2011)[1]	Mastitis	305 d	18.4	190
	Retained placenta	21 d	19.0	190
	Metritis	21 d	7.3	190
	Ketosis	21 d	27.1	190
Santschi et al (2011)[2]	Mastitis	305 d	21.1	238
	Retained placenta	21 d	11.2	238
	Metritis	21 d	4.5	238
	Ketosis	21 d	35.5	238

[1] Measured in primiparous

[2] Measured in multiparous

Severe NEB and occurrence of metabolic disorders during the transition period (Goff and Horst, 1997, Herdt, 2000, Bertoni et al., 2009) may impair the function of the immune system and consequently increase the risk of infectious diseases such as mastitis (Mallard et al., 1998, Sordillo et al., 2009), and metritis (Enevoldsen and Sørensen, 1992, Butler, 2003, Bertoni et al., 2009). Elevated plasma FFA (Dyk, 1995) and ketone body concentrations (LeBlanc, 2010, Suthar et al., 2013) during the prepartum period were associated with retained placenta and high incidence of puerperal metritis (Wathes et al., 2007b). High concentration of plasma BHB in early lactation was related with an increased risk of clinical mastitis (Leslie et al., 2000). Moreover, severe NEB in early lactation was associated with decreased peripheral blood neutrophil functions (Hammon et al., 2006), decreased titers of natural antibodies (**NAb**) in plasma (Van Knegsel et al., 2007a), increased plasma haptoglobin levels (Bionaz et al., 2007), decreased cholesterol levels (Turk et al., 2005) and decreased paraoxonase levels (Turk et al., 2004, Turk et al., 2005). As earlier reviewed the relationship between metabolic status, prevalence of infectious diseases and immune suppression and inflammatory responses during transition period is complex (Esposito et al., 2014). Inflammation and dysregulated immune responses have been proposed as the missing link in the pathobiology of metabolic disorders in transition dairy cows (Esposito et al., 2014). Pro-inflammatory cytokines during the transition period stimulated inflammatory responses, decreased feed intake (Dantzer and Kelley, 2007) and related to metabolic disorders (Trevisi et al., 2010). However, questions remain, whether the NEB is a form of inflammation and cause of immune suppression in early lactation.

1.5   Immune Function and Inflammation

In dairy cows, inability to adapt effectively to a new lactation is not only related to metabolic stress and increased occurrence of infectious diseases but also to impaired function of the immune system (Goff and Horst, 1997, Bertoni et al., 2008, Sordillo and Aitken, 2009). The immune system is also known as the host defence system and acts with a coordinated response when invaded with complex environmental or pathogenic perturbations (Uthaisangsook et al., 2002). Microorganisms that invade host defense system recognize pathogens by a set of germline-encoded pathogen-recognition receptors (**PRRs**) (Akira et al., 2001,

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Akira et al., 2006). During infection, the PRRs like toll-like receptors (**TLR**) and cytoplasmic receptors activate immune cells to mount an inflammatory and immune response (Akira et al., 2006).
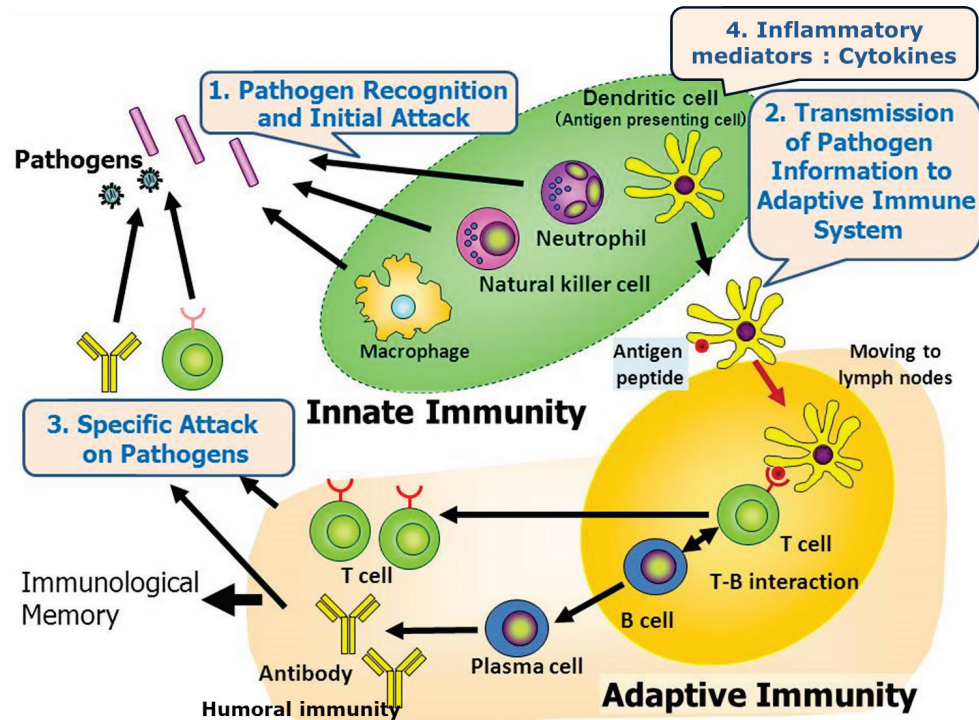
Figure 1.2 Interactions of innate and adaptive immunity. (1) When pathogens invade the body, the innate immune system is activated and participates in the initial attack against the pathogens. (2) Among the cells involved in innate immunity, dendritic cells act as antigen-presenting cells and migrate from the infected tissue to the regional lymph nodes where they present the antigens to T cells. Subsequently, the adaptive immune system is activated, and antibody production and killer T cells are induced. (3) The resulting antibodies and killer T cells specifically attack the pathogens (4) Soluble innate immunity : cytokines acts as inflammatory mediators to response infection or injury (*adapted from Akira, 2011)*

1.5.1 The Innate and Adaptive Immune System

As in humans, the immune system in dairy cows can be divided into the innate and adaptive immune system (Akira, 2011). The innate immune system is nonspecific and present since birth and does not require repeated exposure to potential pathogens (Uthaisangsook et al., 2002). Recently, evidence of non-specific training of innate immunity was obtained in man and mice (Netea, 2013). The adaptive immune system also known as specific immune system is triggered if a pathogen is not completely eliminated by the innate immune system and provide the immunological memory of the infection (Janeway Jr and Medzhitov, 2002). As the adaptive immune system is responsible for the development of immune memory, this system is associated with repeated exposure (O'Leary et al., 2006, Vivier et al., 2011). Both the innate and adaptive immune system play an important role to provide optimal protection from infectious diseases (Sordillo and Streicher, 2002) and they can be measured in cellular and humoral components of the immune system (Beutler, 2004). Cellular adaptive immunity is mediated by T lymphocytes (T cells). Humoral adaptive immunity is involved in the eradication of pathogens present in the blood or fluid by generating antibodies, which are produced by B cells (Akira, 2011). T and B cells express unique T-cell receptors (**TCRs**) and B-cell receptors (**BCRs**), respectively, and recognize enormous numbers of different antigens (Akira, 2011). Components of the innate immune system are mediated by leukocytes (macrophages, neutrophils, and natural killer cells) and certain soluble mediators (cytokines and eicosanoids) (Sordillo, 2005, Lippolis, 2008). Components of humoral innate immunity include the complement cascades and soluble PRRs (Bottazzi et al., 2009). It is known that the innate and adaptive immunity are closely entangled (Rainard and Riollet, 2006) and share many PRRs such as TLRs (Iwasaki and Medzhitov, 2010) and NOD-like receptor (**NLR**) (Fritz et al., 2007). The TLRs and NLR present on both innate cells as well as lymphocytes initiating innate and contributing to specific immune response (Fritz et al., 2007, Iwasaki and Medzhitov, 2010). Furthermore, there is accumulating evidence showing that activation of the innate immune system is a prerequisite for the induction of adaptive immune system (Akira et al., 2001, Fritz et al., 2007, Palm and Medzhitov, 2009).

Innate immune system particularly has been indicated to diminish in dairy cows during transition period by several factors such as pregnancy, parturition,

metabolic stress and hormonal changes in early lactation (Wentink et al., 1997, Lacetera et al. 2002). Pregnancy and parturition are known as a state of immunological depression and consequently increasing the susceptibility to infectious diseases (Mor and Cardenas, 2010). Parturition induces the production of glucocorticoids such as cortisol (Aleri et al., 2016) which induce down regulation of neutrophils and reduce specific immune response capacity (Burton et al., 1995, Preisler et al., 2000, Mallard et al., 2009). During parturition, lacto-genesis contributes to decreased function of neutrophils (Kimura et al., 1999). A high progesterone level before parturition reduced polymononuclear (**PMN**) oxidative burst activity (Moreira da Silva et al., 1997). The phagocytic capacity of macrophages and function of PMN is decreased during parturition and may contribute to increased chronic inflammation and attributed to hormonal changes like interfering the concentration of IGF-1 (Kehrli et al., 1989, Preisler et al., 2000, Kooijman et al., 2012). The changes of diet during transition period affected the number and function of PMN and lymphocytes (Pezeshki et al., 2010). Elevated FFA has been associated with diminished lymphocyte proliferation (Kehrli Jr et al., 1989), and reduced specific immune responsiveness in early lactation (Mallard et al., 1998).

1.5.2 Inflammation

The inflammatory response is a part of the innate immune system. Inflammation is a complex biological response against pathogens, removing injurious agents and initiating the tissue healing process (Sordillo et al., 2009). Inflammation is the result of the release of pro-inflammatory cytokines due to metabolic stress or infection (Grimble., 1990). In response to local injury, primary monocytes secrete a number of pro-inflammatory cytokines such as Interleukin (**IL**)-1, IL-6, and tumor necrosis factor alpha (**TNF α**) into the blood stream to attract effector cells and components to combat infection and to induce the liver to produce acute phase proteins (**APP**) (Gabay and Kushner 1999, Petersen et al., 2004). The liver secretes APP into the blood, which can be used as indicators to assess animal health (Petersen et al., 2004, Gruys et al., 2005). During the infections, levels of APP increase (positive APP, e.g. haptoglobin and

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ceruloplasmin) or decrease (negative APP, e.g. cholesterol and albumin) in plasma (Kushner, 1982, Gruys et al., 1998, Gabay and Kushner, 1999).

Inflammation is categorized as acute or chronic inflammation based on duration of illness, any changes in body condition, the presence or absence of pain and the clinical findings to individual organs or systems (Horadagoda et al., 1999). Acute inflammation is an inflammation which is relatively short in duration from minutes to a few days and characterized by changes in plasma proteins, migration of leukocytes, fever and dullness (Horadagoda et al., 1999). Chronic inflammation is an inflammation which is long in duration (at least a week), and characterized by weight loss and in many cases fever is absent (Horadagoda et al., 1999). In dairy cows, excessive adipose stores and elevated FFA concentration in plasma during transition period have been associated with impaired liver function and increased inflammatory responses (Bionaz et al., 2007, Bertoni et al., 2008) which may result in peroxidative damage of lipids and other macromolecules (Wathes et al., 2009). Inflammatory responses and a dysfunctional immune system can increase the susceptibility to various health problems especially when cows have a high reactive oxygen metabolites (**ROM**) concentrations in plasma (Bernabucci et al., 2005, Sordillo and Aitken, 2009).

1.5.3 *Natural Antibodies*

Natural antibodies (**NAb**) are regarded as a humoral part of the innate immune system (Matter and Ochsenbein, 2008, Vollmers and Brändlein, 2009) and are present in healthy individuals without apparent external antigenic stimulation (Ochsenbein and Zinkernagel, 2000). Natural antibodies play an important role in the activation of innate immune responses against infections through neutralization, complement system activation and pathogen elimination (Ochsenbein et al., 1999 and Zinkernagel, 2012). Most NAb are in IgM isotype with low affinity and have polyspecific binding activity (Boes et al., 1998). In mice and humans, NAb are produced by B1 B-cells (Avrameas et al., 2007). In dairy cows, B1 B-cells producing NAb were not definitely found yet but IgM producing B-cells binding keyhole limpet hemocyanin (**KLH**) were identified (Van Altena, 2016). Natural antibodies have been divided into two classes: overt and cryptic NAb. Overt NAb bind to mostly exogenous antigens that the individual has never encountered before, such as KLH. Cryptic NAb or so-called

natural autoantibodies (**NAAb**) are antibodies that bind to self-antigens or slightly changed self-antigens (neo-epitopes). Natural autoantibodies are thought to be involved in inactivation of cytokines, prevention of inflammation, clearance of metabolic waste (cell waste), and perform various homeostatic roles within the immune response (Cojocaru et al., 2009). The involvement of NAAb in auto immune diseases is unknown. In general, low levels of NAAb in plasma have been related with metabolic and behavioural diseases in man (Arai et al., 2013, Hammer et al., 2014). Moreover, once an individual is stimulated by specific antigens, specific antibodies (**SpAb**) will be produced and the SpAb are generally more restricted in their epitope recognition with high affinity. Both NAb and SpAb can be involved by initial recognition and opsonisation of pathogens to activate complement activity (Ehrenstein and Notley, 2010). In dairy cows, NAb (Van Knegsel et al., 2007a, Ploegaert et al., 2010, Van Knegsel et al., 2012, Thompson-Crispi et al., 2013) and NAAb (Van Knegsel et al., 2012) titers are measurable in both plasma and milk. Reduced levels of NAb binding KLH in plasma (Thompson-Crispi et al., 2013) and reduced levels of NAAb binding transferrin in milk (Van Knegsel et al., 2012) are associated with increased risk of clinical mastitis in dairy cows. In dairy cows, levels of NAb and NAAb were related with health status (Van Knegsel et al., 2012, Thompson-Crispi et al., 2013). Maternal antibodies not only provide passive protection to the neonate, but likely also are involved in shaping the neonatal TCR and BCR repertoire by idiotypes and anti-idiotype interaction (Siegrist, 2001, Adkins et al., 2004). Thus lower levels of antibodies in colostrum might affect the specific immune response in calves.

1.6   Oxidative Stress

Physiological changes and increased occurrence of health problems during the transition period were associated with oxidative stress. During the transition period, the requirement of oxygen increases due to high metabolic demand and consequently an increased production of reactive oxygen species (**ROS**) (Spears and Weiss, 2008). An imbalance between increased production of ROS and the availability of antioxidant defence systems such as ferric reduction antioxidant power (**FRAP**), needed to reduce ROS accumulation, may expose cows to

increased oxidative stress during the periparturient period (Abuelo et al., 2015). Oxidative stress is often associated with numerous pathologies, but it is not always clear if ROS accumulation is the cause or consequence of health problems (Sordillo et al., 2009). Early studies suggested a role of oxidative stress in the aetiology of disorders in dairy cattle since supplementation with certain antioxidants could decrease the severity of a variety of metabolic and infectious diseases (Miller et al., 1993).

1.7 How to Improve Energy Balance and Immune Competence in Early Lactation of Dairy Cows?

1.7.1 Shortening or Omitting the Dry Period

In dairy cows, a non-lactating period before calving, also known as the dry period, is a crucial period affecting milk production and health status in the subsequent lactation. A conventional dry period of 60-d has been applied by farmers since decades to maximize milk production in the first months after calving (Gulay et al., 2003, Grummer and Rastani, 2004, Kuhn et al., 2005). In addition, a dry period of 60-d is used to treat cows with intra-mammary antibiotics to cure existing subclinical mammary infections or to prevent new infections (Smith et al., 1985, Bradley and Green, 2001). The dry period involves mammary involution, a process of cell renewal, followed by a period of mammary epithelial cell proliferation and differentiation (Capuco et al., 1997). In recent years, it is shown that a shortening or omitting the dry period, compared with a conventional dry period of 60-d, improves EB in early lactation. This improvement of the EB due to a short or no dry period is mostly due to a reduced milk production in early lactation (Van Knegsel et al., 2014), sometimes also due to an improvement of dry matter intake (**DMI**) (Rastani et al., 2005). Both shortening the dry period to about 30-d and omitting the dry period completely decreases milk production in the next lactation, as earlier reviewed (Van Knegsel et al., 2013). This meta-analysis showed that across studies, cows with a short dry period produced 1.4 kg/d less milk than cows with a conventional dry period, with an average milk loss of 4.5 percent (range: 3.2 to 13.2 percent). Moreover, cows with no dry period produced 5.9 kg/d less milk than cows with a

conventional dry period, with an average milk loss of 19.1 percent (range: 9.0 to 28.9 percent). Milk production losses in early lactation were partly compensated by additional milk produced in the preceding lactation (Andersen et al., 2005, Rastani et al., 2005). Specifically, this partial shift in milk production from post to pre-calving improves the EB of dairy cows during early lactation (Rastani et al., 2005, Van Knegsel et al., 2014), and consequently an improved body condition in the next lactation (Gulay et al., 2003), improved lymphocyte cell function (Pezeshki et al., 2010), a shorter interval to first ovulation (Gümen et al., 2005, Chen et al., 2015b) and increased incidence of normal resumption of ovarian cyclicity (Chen et al., 2015b) of dairy cows in the subsequent lactation.

The effect of shortening or omitting the dry period on mammary health is not completely clear. A shortened dry period (34 to 40 d) did not result in differences in somatic cell count (**SCC**) (Gulay et al., 2003, Watters et al., 2008, Shoshani et al., 2014), tendency for increased SCC (Rémond et al., 1997, Annen et al., 2004) or tendency for reduced SCC (Rastani et al., 2005) compared with a conventional dry period. A short dry period (34 to 40 d) did not affect the incidence of clinical mastitis, compared with a conventional dry period (55 to 65 d) (Enevoldsen and Sørensen, 1992, Watters et al., 2008). Omitting the dry period resulted in higher SCC values compared with cows with a 56-d dry period in some studies (Klusmeyer et al., 2009, Mayasari et al., 2016, Van Hoeij et al., 2016), but not all (Rastani et al., 2005, Köpf et al., 2014). Omitting the dry period did not affect incidence of clinical mastitis (Rémond et al., 1997, Mayasari et al., 2016, Van Hoeij et al., 2016). Cows with clinical mastitis had increased plasma haptoglobin (Eckersall et al., 2001, Grönlund et al., 2005), increased TNF-α and IL-6 in plasma (Nakajima et al., 1997), reduced NAb IgM in plasma (Thompson-Crispi et al., 2013), and increased ROM in plasma (Sordillo and Aitken, 2009). It can be hypothesized that if omitting or shortening the dry period does not affect occurrence of clinical mastitis, also immune competence, inflammatory biomarkers and oxidative stress may not be affected. To our knowledge, the effects of shortening or omitting the dry period in dairy cows on immune competence variables such as plasma NAb and NAAb levels, inflammation biomarkers and oxidative stress variables are not studied yet. In addition, the

effects of shortening or omitting the dry period over multiple lactations are unknown.

1.7.2 Dietary Energy Source

Several dietary strategies have been studied to improve the EB and reduce the incidence and severity of metabolic disorders in early lactation. Most studies aimed at improving the EB by increasing the energy density of the ration by supplementation with dietary fat or non-fibre carbohydrates (Hough et al., 1990, Castañeda-Gutiérrez et al., 2005, Franklin et al., 2005, Dann et al., 2006, Lake et al., 2006, Nowak et al., 2012). However, dietary fat has been suggested to have potential to depress DMI (Palmquist and Jenkins, 1980), which may be explained by limited palatability of dietary fat (Grummer et al., 1990) or possible effects of dietary fat on ruminal fermentation and gut motility (Allen, 2000). Recent studies introduced alternative approaches to improve EB by altering dietary energy sources like feeding a more glucogenic ration in early lactation. Feeding carbohydrates to increase glucogenic nutrients can result in the production of propionate in the rumen or intestinal digested and absorbed as glucose (Van Knegsel et al., 2007b). Moreover, a glucogenic ration in early lactation improved the EB by decreasing milk fat yield compared with a lipogenic ration (Van Knegsel et al., 2007a). Furthermore, in the same study, cows fed a glucogenic ration had lower plasma BHB and liver TAG concentrations and tended to have fewer days postpartum till first ovulation, compared with cows fed a more lipogenic ration. Moreover, a glucogenic ration enhanced plasma NAb titers in early lactation, compared with a more lipogenic ration (Van Knegsel et al., 2007a). To our knowledge it is unknown what are the effects of dietary energy source during early lactation for cows with different dry period lengths on immune competence and inflammation. It can be hypothesized that if EB is improved by shortening or omitting the dry period, the potential beneficial effect of other management factors to improve the EB and metabolic health like the use of glucogenic rations is reduced.

1.8   Effect of Maternal Dry Period Length on Immune Competence in Calves

Passive transfer of antibodies from cows to their calves plays an important role in immune development and immune competence of calves. Maternal

antibodies present in colostrum provide passive immune protection for the newborn calves, as antibodies are not transferred through the placenta (Pravieux et al., 2007) and the adaptive immune system is still immature in young calves (Hasselquist and Nilsson, 2009). Colostrogenesis or prepartum transfer of immunoglobulins from the maternal circulation into mammary secretions is a distinct stage of mammary gland development and starts several weeks before calving (Barrington et al., 2001). Omitting the dry period, but not shortening the dry period, reduced colostrum immunoglobulin content (Baumrucker et al., 2014, Verweij et al., 2014). Earlier studies showed that shortening the dry period to 4 weeks did not affect colostrum immunoglobulin content (Annen et al., 2004, Rastani et al., 2005, Watters et al., 2008, Klusmeyer et al., 2009), pregnancy length (Santschi et al., 2011) and birth weight of calves (Rastani et al., 2005, Pezeshki et al., 2007), compared with a dry period of 56-d. Omission of the dry period, however, reduced colostrum immunoglobulin content (Annen et al., 2004, Rastani et al., 2005, Klusmeyer et al., 2009, Verweij et al., 2014). Adequate and sufficient passive immune transfer of maternal antibodies via colostrum intake may improve the immune competence of calves (Stelwagen et al., 2009), and would reduce the risk of diseases and infections during the pre-weaning period (Oliveira et al., 2010). To our knowledge, the effect of maternal dry period length on natural (auto) antibodies and specific antibodies in plasma of calves is not studied yet.

1.9 Objectives and Outline of this Thesis

The objectives of this thesis were (1) to investigate effects of dry period length and dietary energy source on immune competence, inflammatory biomarkers and oxidative stress in dairy cows in the transition period from pregnancy to lactation, 2) to investigate the consequences of maternal dry period length on immunoglobulin content in colostrum and the immune competence in calves. For this study, 167 Holstein-Friesian dairy cows were selected from the Dairy Campus research herd (WUR Livestock Research, Lelystad, the Netherlands), blocked according to parity, calving date, milk yield in previous lactation, and body condition score (**BCS**), and randomly assigned to treatments within blocks. Treatments consisted of 3 dry period lengths (0, 30 or 60 d) and 2

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early lactation rations (glucogenic or lipogenic) resulting in a 3 × 2 factorial design. Cows used in this study were clinically healthy at start of the experiment. Plasma and milk samples around the transition period were obtained and analysed for immune competence, inflammatory biomarkers and oxidative stress. Colostrum samples were collected to determine immunoglobulin content. Subsequently the effects of dry period lengths and dietary treatments on health status were assessed. Secondly, humoral immune status and natural (auto) antibodies and specific antibodies in plasma of the calves were determined. The effects of 3 dry period length (0, 30 and 60 d) and 2 early lactation rations (glucogenic or lipogenic) on energy balance (Van Knegsel et al., 2014, Chen et al., 2016) and metabolic status in the first and second subsequent lactations in the current experiment have been reported earlier (Chen et al., 2015a, Chen et al., 2016).

The outline of this thesis is as follows:

Chapter 2 covers the determination of inflammatory biomarkers and oxidative stress variables in plasma in cows in the transition phase from pregnancy to lactation of the first lactation after implementation of dry period length and dietary treatments

In chapter 3 covers the determination of inflammatory biomarkers and oxidative stress in plasma of cows in the second lactation after implementation of dry period length and dietary treatments were related to uterine health.

Chapter 4 covers the estimation of NAb titers and mammary health in dairy cows with different dry period length and dietary treatment for 2 subsequent lactations. In addition, the relationships between NAb titers and mammary health are determined.

Chapter 5 and 6 focus on the effect of maternal dry period length on immunoglobulin content in colostrum and immune competence of calves.

Finally, chapter 7 discusses the biological mechanisms behind the effects of shortening or omitting dry period on immune competence, inflammatory biomarkers and oxidative stress and includes the conclusions of this PhD thesis.

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CHAPTER 2

Effects of dry period length and dietary energy source on inflammatory biomarkers and oxidative stress in dairy cows

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2.1 Abstract

Negative energy balance (**NEB**) in dairy cows in early lactation has been associated with increased inflammation and oxidative stress in these cows. The objective of this study was to evaluate the effects of dry period length and dietary energy source on inflammatory biomarkers and oxidative stress in dairy cows. Holstein-Friesian dairy cows (60 primiparous and 107 multiparous) were assigned randomly to 3×2 factorial design with 3 dry period lengths (0, 30 or 60 d) and 2 early lactation rations (glucogenic or lipogenic). Cows were fed a glucogenic or lipogenic ration from 10 d before the expected calving date. Blood was collected in weeks -3, -2, -1, 1, 2 and 4 relative to calving. Dry period length affected inflammatory biomarkers and oxidative stress especially in week 1 and 2 after calving. Cows with a 0-d dry period had higher levels of ceruloplasmin, cholesterol, reactive oxygen metabolites (**ROM**), and tended to have higher haptoglobin levels compared with cows with a 30-d or a 60-d dry period. Cows with a 0-d dry period had a lower plasma paraoxonase and bilirubin in the first 2 weeks after calving and a lower liver functionality index (**LFI**) compared with cows with a 60-d dry period. Cows with parity > 3 and fed a glucogenic ration had higher cholesterol levels compared with cows fed a lipogenic ration. No interaction between dry period length and ration was present for inflammatory biomarkers or oxidative stress variables. Plasma bilirubin levels for cows with a 0-d dry period were negatively related to EB and metabolic status in these cows. Moreover, occurrence of clinical health problems (fever, mastitis, metritis and retained placenta) was 41 percent, 27 percent and 30 percent for cows with 0-d, 30-d and 60-d dry period, respectively. High plasma levels of ceruloplasmin, cholesterol and ROM in cows with 0-d dry period were related to the occurrence of health problems in these cows. In conclusion, omitting the dry period increased levels of ceruloplasmin, cholesterol, ROM and decreased level of bilirubin and paraoxonase in plasma, independent of ration, compared with cows with a 60-d dry period. These contrasting effects of dry period length on inflammatory status could be explained in part by the improved EB and occurrence of health problems in these cows, but was not related to increased somatic cell count in cows with a 0-d dry period. Cows with a 0-d dry period had better EB, but also had higher levels of oxidative stress compared with cows with a 60-d dry period. Moreover, occurrence of health problems did not differ between cows with different dry period lengths.
Key words: continuous milking, acute phase protein, oxidative stress, energy balance.

2.2   Introduction

During the transition period, which lasts from 3 weeks before to 3 weeks after calving (Drackley, 1999), dairy cows experience negative energy balance (**NEB**) which is accompanied by metabolic disorders and by increased inflammation (Bionaz et al., 2007, Trevisi et al., 2012a), immunosuppression (Mallard et al., 1998) and oxidative stress (Sordillo et al., 2009). Pro-inflammatory cytokines are related to increased inflammation during NEB (Grimble, 1990, Ametaj et al., 2005) by stimulating hepatic synthesis of positive acute phase protein (**APP**), such as haptoglobin and ceruloplasmin, and impairing hepatic synthesis of negative APP such as albumin and cholesterol (Bionaz et al., 2007, LeBlanc, 2012). Cows with NEB and inflammation had a higher bilirubin in plasma due to impairment of hepatic function (Bertoni et al., 2008). Paraoxonase is part of an extensive antioxidative system (Turk et al., 2004) and is considered as a negative APP (James and Deakin, 2004). In the early postpartum period, a lower plasma paraoxonase activity is positively associated with lipid metabolic disorders. Negative energy balance and high plasma free fatty acid (**FFA**) levels contribute to the development of fatty liver, which is in turn a contributing factor to other health concerns (e.g. metritis and retained placenta; Kaneene et al., 1997) and periparturient immunosuppression in the postpartum period (Lacetera et al., 2005, Kehrli et al., 2006).

Shortening or omitting the dry period during early lactation in dairy cows has been shown to improve energy balance (**EB**) (Van Knegsel et al. 2014) and metabolic status (Rastani et al., 2005, Chen et al., 2015) of dairy cows in the subsequent lactation. However, earlier studies showed variable effects of dry period length on mammary health (Pezeshki et al., 2007, Watters et al., 2008, Mayasari et al., 2016). A shortened dry period (34 to 40 d) compared with a conventional dry period (55 to 65 d) did not result in differences in SCC (Gulay et al., 2003, Watters et al., 2008, Shoshani et al., 2014) or clinical mastitis (**CM**) (Enevoldsen and Sørensen, 1992, Watters et al., 2008, Shoshani et al., 2014). Other studies have shown that a shortened dry period was associated with a tendency for higher SCC (Rémond et al., 1997, Annen et al., 2004) or tendency for lower SCC (Rastani et al., 2005) compared with a conventional dry period. Some studies (Klusmeyer et al., 2009, Mayasari et al., 2016) concluded that omitting the dry period resulted in higher SCC values compared with cows with a 56-d dry period, but some studies showed no differences on SCC between cows with 0-d and 56-d dry period (Rastani et al., 2005, Kopf et al., 2014). Another study showed omitting the dry period had a tendency for higher SCC (Rémond et al., 1997) compared with a conventional dry period. To our knowledge,

omitting the dry period did not affect CM (Rémond et al., 1997, Mayasari et al., 2016). The effects of shortening or omitting the dry period in dairy cows on inflammatory biomarkers, liver functionality (paraoxonase and bilirubin levels), oxidative stress [reactive oxygen metabolites (**ROM**) and ferric-reducing antioxidant power (**FRAP**) levels], and creatinine as markers for mobilization of body muscle have not been reported.

We can hypothesize that when EB is improved by shortening or omitting the dry period, the beneficial effects of other management strategies, such as feeding a more glucogenic ration to improve EB and metabolic health are reduced. Earlier, we reported that cows in the current experiment cows fed a glucogenic ration compared with a lipogenic ration had improved EB (Van Knegsel et al., 2014) and decreased plasma β-hydroxybutyrate (**BHB**) concentration (Chen et al., 2015a), independent of dry period lengths. In other studies, the reduced FFA and BHB concentrations were associated with increased cholesterol and reduced haptoglobin levels in plasma (Bionaz et al., 2007, Trevisi et al., 2012) and reduced plasma ROM levels (Bernabucci et al., 2005, Trevisi et al., 2009). Thus, we could expect that feeding a glucogenic ration would reduce inflammation and oxidative stress of cows with different dry period lengths.

Our hypothesis was that improving EB and metabolic status by omitting or shortening of the dry period would reduce the inflammatory response (i.e. higher levels of negative APP, lower levels of positive APP, and higher liver functionality) and oxidative stress (lower ROM and higher FRAP levels in plasma) during transition period. Moreover, if omitting the dry period resulted in increased SCC but no occurrence of CM, inflammatory biomarkers and oxidative stress may not be affected. The objective of this study was to evaluate effects of dry period length and dietary energy source on inflammatory biomarkers and oxidative stress in plasma from dairy cows.

2.3   Materials and Methods

2.3.1 Experimental Design and Animals

The Institutional Animal Care and Use Committee of Wageningen University (Wageningen, the Netherlands) approved the experimental protocol (registration number 2010026). The experimental design, dry period length and dietary contrast were described by Van Knegsel et al. (2014). In summary, 167 Holstein-Friesian dairy (60 primiparous cows and 107 multiparous cows) were

selected from the Dairy Campus research herd (WUR Livestock Research, Lelystad, the Netherlands), blocked according to parity, calving date, milk yield in previous lactation, and body condition score (**BCS**), and randomly assigned to treatments within blocks. In total, 28 blocks were used and each block consisted of 6 cows, 1 cow/treatment (28 blocks × 6 cows = 168 cows; 1 cow was excluded because she received the wrong ration at drying off). Before the experiment began, all cows had SCC < 250,000 cells/mL at the last and second last monthly test-day recordings. Treatments consisted of 3 dry period lengths (0, 30 or 60-d) and 2 early lactation rations (glucogenic or lipogenic) resulting in a 3 × 2 factorial design. Cows used in this study were clinically healthy at start of the experiment. Cows were housed in a freestall with slatted floor and cubicles, and milked twice daily (0500 and 1630 h). The drying-off protocol was as follows: cows with a 30-d or a 60-d dry period received a far-off ration 7 d before drying-off, were milked once daily 4 d before drying-off, and all were treated with an intramammary antibiotic at drying off (Supermastidol; Virbac Animal Health, Barneveld, the Netherlands).

2.3.2 Rations

Ration composition was described earlier (Van Knegsel et al., 2014). Prepartum, cows with a 30-d and 60-d dry period received a dry cow ration, and cows with a 0-d dry period received a lactating cow ration supporting 25 kg of milk yield per day. From 10 d before expected calving onwards, cows of all treatments were fed 1 kg/d glucogenic or lipogenic concentrate, which was increased postcalving in steps of 0.5 kg/d until the concentrate supply reached 8.5 kg/d. The main ingredient for the glucogenic concentrate was corn, the main ingredients for the lipogenic concentrate were sugar beet pulp, palm kernel, and rumen-protected palm oil. A computerized feeder located in the freestall provided experimental concentrates. Cows in the parlor received 1 kg/d of standard lactation concentrate. Forage composition did not differ among rations and was supplied ad libitum and consisted prepartum of grass silage, corn silage, wheat straw, and a protein source (rapeseed meal or soybean meal) in a ratio of 39:25:25:11 (DM basis). Postpartum, forage consisted of grass silage, corn silage, straw, and a protein source (rapeseed meal or soybean meal) in a ratio of 51:34:2:13 (DM basis). Rations were isocaloric (net energy basis: Dutch net energy evaluation (VEM system; Van Es, 1975), and contained equal amounts of intestinal digestible protein and degraded protein balance (DVE/OEB system; Tamminga et al., 1994). Concentrate and forage were supplied separately.

2.3.3 Milk Yield and Composition, Energy Balance, Dry Matter Intake and Somatic Cell Count

Milk sampling and EB calculation were described previously by Van Knegsel et al. (2014). In short, milk yield was recorded daily. Milk samples for fat, protein, lactose, and SCC analysis (ISO 9622, 2013 Qlip NV, Zutphen, the Netherlands) were collected 4 times per week (Tuesday afternoon, Wednesday morning, Wednesday afternoon, and Thursday morning). Dry matter intake was recorded weekly. Energy balance was calculated according to the VEM system (Van Es, 1975, CVB, 2005) as the difference between VEM supplied with feed and VEM required for maintenance and milk production. Animal maintenance requirements are 42.4 VEM/kg0.75·d (1,000 VEM = 6.9 MJ of net energy). The VEM required for milk production is 442 VEM/kg of fat and protein-corrected milk (Van Es, 1975).

2.3.4 Blood Sampling

Blood sampling was described previously by Chen et al. (2015). In short, blood samples from 92 cows were taken weekly from the tail vein at 3 h before the morning feeding from weeks −3 to 8 relative to calving date. Blood was collected in evacuated tubes (Vacuette, Greiner BioOne, Kremsmunster, Austria) containing NaF for glucose, EDTA for insulin, FFA, BHB, and urea analysis, or litium-heparin for IGF-I, haptoglobin, ceruloplasmin, cholesterol, albumin, bilirubin, paraoxonase, ROM, FRAP, and creatinine. Samples were kept cold on ice for a maximum of 2 h until they were centrifuged at 3,000 × g for 15 min at 4°C. Plasma was decanted, aliquoted, and frozen at −20°C until analysis.

2.3.5 Laboratory Analysis

Plasma samples for metabolite and hormones determination were analyzed at the Veterinary Physiology group of the Vetsuisse Faculty, University of Bern (Bern, Switzerland). Concentrations of glucose and urea were measured using commercial kits no. 61269 and no. 61974 from BioMérieux (Marcy l'Etoile, France), as described previously (Graber et al., 2012). Concentrations of FFA and BHB were measured enzymatically using kit no. 994–75409 from Wako Chemicals (Neuss, Germany) and kit no. RB1007 from Randox Laboratories (Ibach, Switzerland), as described previously (Graber et al., 2012). Insulin-like growth factor-1 and insulin were measured using RIA, as described previously (Vicari et al., 2008).

Inflammatory biomarkers and oxidative stress were measured at the Istituto di Zootecnica of the Università Cattolica del Sacro Cuore (Piacenza, Italy), following the procedures described previously by Bionaz et al. (2007), Calamari et al. (2016) and Jacometo et al. (2015) using a clinical auto-analyzer (ILAB 650, Instrumentation Laboratory, Lexington, MA). In short, total cholesterol (catalog no. 0018250540), albumin (catalog no. 0018250040), total bilirubin (catalog no. 0018254640), and creatinine (catalog no. 0018255540) were measured using the IL Test purchased from Instrumentation Laboratory Spa (Werfen Co., Milan, Italy). The haptoglobin was determined with the method described by Skinner et al. (1991) and Owen et al. (1960), adapted to ILAB 650 condition. The method based on peroxidase activity of methaemoglobin-haptoglobin complex measured by the rate of oxidation of guaiacol (hydrogen donor) in the presence of hydrogen peroxide (oxidizing substrate). The ceruloplasmin was determined with the method described by Sunderman and Nomoto (1970), adapted to ILAB 650 conditions. The test is based on measurement of the colour which originates from the oxidation of the p-phenylenediamine dihydrochloride induced by the ceruloplasmin. The ROM was measured using commercial kits (kit d-ROMs-test cod. MC003, Diacron International s.r.l., Grosseto, Italy) adapted to the ILAB 650 conditions. Antioxidant potential was assessed as ferric reducing antioxidant power (FRAP) using the colorimetric method of Benzie and Strain (1996). Plasma paraoxonase activity was measured by adapting the method of Ferré et al. (2002) to the ILAB 650 conditions.

2.3.6 Statistical Analysis

The MIXED procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC, Littell et al., 1996) for repeated measures analysis was used to analyze the effects of dry period length and ration on inflammatory biomarkers and oxidative stress. The fixed effects were dry period length (0, 30 or 60 d), ration (glucogenic or lipogenic), parity (2, 3 or > 3), week (-3, -2, -1, 1, 2 and 4 relative to calving), and their 2-way interactions (model 1). Cow was considered as the repeated subject. All variables approximated normality of residuals by examining whether skewness and kurtosis were in a range of -2 to 2. Four covariance structures were tested: compound symmetry, heterogeneous compound symmetry, first-order autoregressive [AR (1)], and unstructured covariance structure. Covariance structure that resulted in the smallest Akaike information criterion was used. For comparison of dry period length effects, *P*-values are presented after a Tukey-Kramer adjustment. Preliminary analysis showed that effects of dry period length

were more pronounced in the first 2 weeks after calving, therefore data were analyzed for total transition period (-3, -2, -1, 1, 2 and 4 relative to calving) and specifically for the first 2 weeks after calving, separately. The [AR (1)] covariate structure covariance structure was the best fit and was used to account for within-cow variation. Preliminary result showed that the levels of bilirubin for all samples was relatively low compared earlier studies (Bionaz et al., 2007, Bertoni et al., 2008, Trevisi et al., 2012a). The animal experiment was performed from 2010 until 2013, and the prolonged storage condition of the plasma samples may have affected the level of bilirubin. However, in the current study the dynamic of bilirubin levels by week around calving followed the typical pattern of change observed in the peripartum period in earlier studies (Ametaj et al., 2005, Bionaz et al., 2007, Trevisi et al., 2012a). All the plasma parameters have been setted with calibration curves, and data were standardized using quality control standards (internal and purchased by Instrumentation Laboratory, Lexington, MA).

Liver functionality index (**LFI**) was calculated according to Trevisi et al. (2012a). Albumin, cholesterol, and bilirubin data were used to calculate the LFI. The LFI calculation is carried out in 2 steps; the first step considers the values of the 3 parameters observed in week 1 and their changes between week 1 and 4. In the second step, these partial indexes were standardized according to average values observed in ''healthy'' cows. The higher value of LFI, the better adaptation of the cows to transition period; lower value of LFI indicates the presence of inflammation and metabolic disorder (Trevisi et al., 2016). The LFI was calculated according to the following formula:

$$LFI = [(albumin\ index – 17.71)/1.08 + (cholesterol\ index-2.57)/0.43 – (bilirubin\ index - 6.08)/2.17)]$$

These partial indices were categorized to 4 quartiles of LFI [low (LO), intermediate low (IN-LO), intermediate up (IN-UP) and high (UP)]. To determine the 4 quartiles, we ranked cows by LFI value from the smallest to the highest, and then we divided cows into 4 groups with equal number of cows in each. The high LFI value was taken to define "healthy" and "well adapted" cows and low values were associated with a large inflammatory response and clinical health problem. Clinical health problems were defined as occurrence of metritis, fever, mastitis or retained placenta per cow per week, these are diseases that cause an inflammatory response. Healthy cows were defined as cows not treated for disease. The frequency of clinical health problem was determined in the 4 quartiles of LFI from week 1 to 4 postcalving. Statistical analysis for LFI was performed in the first 4

weeks after calving with model 1. Because there was only one LFI value per cow, week was excluded from the model. The AR (1) covariance structure was the best fit and was used to account for within-cow variation. Statistical analysis for milk yield, EB, SCC, plasma metabolites and hormones were performed in the first 2 weeks after calving with model 1. Cow was considered as the repeated subject. The AR (1) covariate structure was the best fit, and was used to account for within-cow variation. For comparison of dry period length effects *P*-values are presented after a Tukey-Kramer adjustment. The result for milk yield, EB, SCC, plasma metabolites and hormones in the first 2 weeks after calving are presented in the Appendix Table 2. A1.

To analyze whether EB, SCC or clinical health problems (yes or no) explained the differences in inflammatory biomarkers and oxidative stress between different dry period lengths in the first 2 weeks after calving were included as fixed effect in model 1 in separate analyses. Cow was considered as the repeated subject. The AR (1) covariate structure was the best fit, and was used to account for within-cow variation. For comparison of dry period length effects *P*-values are presented after a Tukey-Kramer adjustment. Values are presented as least squares means (LSM) with their pooled standard errors of the mean (SEM), unless otherwise stated.

2.4   Results

2.4.1 *Effects of Dry Period Length and Ration on Inflammatory Biomarkers in Plasma of Cows*

During transition period from weeks -3 to 4 relative to calving, cows with a 0-d dry period had higher ceruloplasmin and cholesterol levels in plasma compared with cows with a 30-d or a 60-d dry period (Table 2.1). Cows with a 60-d dry period had higher bilirubin levels compared with cows with a 30-d dry period. Plasma bilirubin levels did not differ among cows with a 0-d dry period and those with a 30-d or 60-d dry period. The effects of dry period length on inflammatory biomarkers were more pronounced in the first 2 weeks after calving (Figure 2.1). In the first 2 weeks after calving, cows with a 0-d dry period had higher levels of ceruloplasmin, cholesterol and tended to have higher haptoglobin levels in plasma compared with cows with a 30-d or a 60-d dry period (Table 2.2). In addition, in the first 2 weeks after calving, cows with a 0-d dry period had lower plasma paraoxonase and bilirubin levels compared with cows with a 60-d dry period. From

weeks -3 to 4 relative to calving, cows with parity ≥ 3 had higher ceruloplasmin and lower albumin levels in plasma compared with cows with parity 2 (1.7 vs. 1.8 vs. 1.8 $\pm$ 0.1 μmoL/L and 37.8 vs. 36.9 vs. 36.8 $\pm$ 0.2 g/L for parity 2 vs. 3 vs. > 3 for ceruloplasmin and albumin, respectively). From weeks -3 to 4 relative to calving, we detected an interaction between ration and parity for cholesterol. Cows with parity > 3 fed a glucogenic ration had higher cholesterol in plasma compared with cows fed a lipogenic ration. In contrast, cows with parity 2 fed a lipogenic ration had higher cholesterol in plasma compared with cows fed a glucogenic ration.

2.4.2 Effects of Dry Period Length and Ration on Liver Functionality Index in Plasma of Cows

Cows with a 0-d dry period had lower LFI compared with cows with a 30 or 60-d dry period (Table 2.1). Significant interactions between dry period × ration and dry period × parity were found for LFI. We ranked the cows by LFI and divided them into 4 groups with an equal number of cows, resulting in the 4 quartiles of LFI [low (-1.70 to 0.70), intermediate-low (0.86 to 2.32), intermediate-high (2.36 to 3.34), and high (3.36 to 12.00)]. Cows with low LFI had a higher frequency of clinical health problem (mastitis, metritis, retained placenta and fever, 17 occurrences) compared with intermediate-low, intermediate-high and high LFI (7 vs. 1 vs. 8 occurrences, respectively, $P < 0.01$).

2.4.3 Effects of Dry Period Length and Ration on Oxidative Stress in Plasma of Cows

Cows with a 0-d dry period had higher level of ROM in plasma compared with cows with a 30-d or a 60-d dry period (Table 2.1). The contrasts between dry period length for ROM levels were more pronounced in the first 2 weeks after calving. We detected no effect of dry period length on FRAP levels in plasma of cows but did detect an interaction between dry period length and parity for FRAP. Cows with parity > 3 cows had lower FRAP compared with cows with parity ≤ 3 (171.6 vs. 171.2 vs. 158.9 $\pm$ 4.0 μmol/L for parity 2 vs. 3 vs. > 3) in the first 2 weeks after calving.

2.4.4 Effects of Dry Period Length on Creatinine, Milk Yield, DMI, EB, SCC, Plasma Metabolites and Hormones

There was no effect of dry period length or ration on creatinine levels in plasma of cows (Table 2.1 and 2.2). However, there was an interaction between dry period lengths and parity for creatinine. Cows with parity ≥ 3 had higher creatinine in plasma when they had a 0-d dry period compared with cows with parity 2. Contrary, cows with parity > 3 had lower creatinine in plasma when they had a 30-d or a 60-d dry period compared with cows with parity 2 and 3. In the first 2 weeks after calving, cows with a 0-d dry period produced less milk, had better EB, higher SCC in milk, lower FFA, higher IGF-I, higher glucose and higher insulin concentrations in plasma compared with cows with a 30-d or 60-d dry period (see Table 2.A1). In addition, DMI did not differ among dry period lengths treatments in the first 2 weeks after calving ($P = 0.95$).

2.4.5 Relationship between Energy Balance and Somatic Cell Count with Inflammatory Biomarkers and Oxidative Stress

When EB was included as a covariate, low bilirubin levels were related to better EB in the first 2 weeks after calving. Bilirubin levels between dry period length were similar and not significant (Table 2.3).

Level of SCC was not related to levels of inflammatory biomarkers and oxidative stress, except for ceruloplasmin. The relation of SCC with ceruloplasmin was dependent on parity (Table 2.4).

2.4.6 Relationship of Clinical Health Problems with Inflammatory Biomarkers and Oxidative Stress

Occurrence of clinical health problems was related to high ceruloplasmin, low albumin, low bilirubin, and high ROM, and tended to be related to high levels of haptoglobin in plasma (Table 2.5). The contrast between dry period length remained whether excluding or including clinical health problems in the model. In the first 2 weeks after calving, occurrence of clinical health problems related to inflammation (fever, mastitis, metritis and retained placenta) was 41, 27 and 30 percent for cows with 0-d, 30-d and 60-d dry period, respectively (Table 2.6). Dry period length did not affect occurrence of clinical health problems ($P = 0.23$).

Table 2.1 Inflammatory biomarkers (haptoglobin, ceruloplasmin, albumin and cholesterol), index of liver function (paraoxonase and bilirubin), oxidative stress (reactive oxidative metabolites (ROM) and ferric reducing antioxidant power (FRAP)), creatinine and liver functionality index in plasma of dairy cows with varying dry period lengths (0, 30 or 60 d) and fed either a glucogenic (G) or a lipogenic (L) ration (LSM ± pooled SEM) from week -3 till 4 relative to calving.

Item	Dry period lengths (d)			SEM	Ration		SEM	P-values[1]							
	0	30	60		G	L		D	R	P	W^2	D × R	D × P	D × W	P × R
Cows (n=89)	29	31	29		44	45									
Haptoglobin (g/L)	0.44	0.43	0.38	0.03	0.41	0.43	0.02	0.35	0.62	0.50	<0.01	0.15	0.22	0.20	0.63
Ceruloplasmin (µmol/L)	1.98[a]	1.68[b]	1.67[b]	0.07	1.81	1.74	0.06	<0.01	0.31	0.05	<0.01	0.88	0.49	<0.01	0.90
Albumin (g/L)	36.98	36.95	37.67	0.40	37.02	37.38	0.31	0.35	0.38	0.03	<0.01	0.36	0.06	0.28	0.13
Cholesterol (mmol/L)	3.70[a]	3.26[b]	2.91[b]	0.11	3.39	3.20	0.08	<0.01	0.09	<0.01	<0.01	0.68	0.23	<0.01	0.01
Bilirubin (µmol/L)	0.80[ab]	0.73[a]	0.93[b]	0.06	0.79	0.85	0.03	0.04	0.13	0.29	<0.01	0.04	0.34	<0.01	0.88
Paraoxonase, U/mL	39.50	45.60	44.41	2.02	43.41	42.92	1.62	0.08	0.83	0.64	<0.01	0.20	0.72	0.02	0.87
ROM (mg H_2O_2/100mL)	16.79[a]	15.03[b]	14.39[b]	0.33	15.57	15.24	0.27	<0.01	0.37	0.23	<0.01	0.82	0.53	<0.01	0.26
FRAP (µmol/L)	171.02	167.10	164.05	5.25	168.39	166.39	3.67	0.66	0.61	0.01	<0.01	0.60	0.07	0.54	0.44
Creatinine (µmol/L)	101.74	104.49	105.78	1.74	103.10	104.99	1.38	0.25	0.30	0.02	<0.01	0.67	0.01	0.35	0.24
Cows (n=82)	29	28	25		40	42									
Liver functionality index	1.92[a]	2.49[b]	2.85[b]	0.16	2.39	2.46	0.13	<0.01	0.70	<0.01	NA	<0.01	0.02	NA	0.08

[a,b] Values within dry period lengths in the same row with different superscripts differ (P < 0.05).

[1] D = dry period; R = ration; P = parity, NA = not applicable

[2] -3, -2, -1, 1, 2 and 4 relative to calving.

Table 2.2 Inflammatory biomarkers (haptoglobin, ceruloplasmin, albumin and cholesterol), index of liver function (paraoxonase and bilirubin), oxidative stress (reactive oxidative metabolites (ROM) and ferric reducing antioxidant power (FRAP)), and creatinine in plasma of dairy cows with varying dry period lengths (0, 30 or 60 d) and fed either a glucogenic (G) or a lipogenic (L) ration in weeks 1 and 2 postpartum (LSM ± pooled SEM) in the first 2 weeks after calving.

Item	Dry period lengths (d)			SEM	Ration		SEM	P-values[1]							
	0	30	60		G	L		D	R	P	W[2]	D × R	D × P	D × W	P × R
Cows (n=89)	29	31	29		44	45									
Haptoglobin (g/L)	0.74	0.66	0.53	0.06	0.62	0.66	0.05	0.07	0.64	0.91	<0.01	0.53	0.21	0.41	0.51
Ceruloplasmin (µmol/L)	2.39[a]	1.84[b]	1.83[b]	0.10	2.09	1.96	0.08	<0.01	0.27	0.67	0.80	0.95	0.66	0.60	0.82
Albumin (g/L)	35.73	36.75	36.75	0.52	36.44	36.11	0.43	0.38	0.58	0.36	0.01	0.64	0.41	0.09	0.78
Cholesterol (mmol/L)	3.03[a]	2.49[b]	2.20[b]	0.12	2.64	2.52	0.10	<0.01	0.40	<0.01	<0.01	0.99	0.43	0.02	0.10
Bilirubin (µmol/L)	1.22[a]	1.33[ab]	1.83[b]	0.15	1.49	1.44	0.13	0.02	0.78	0.65	<0.01	0.25	0.51	0.84	0.39
Paraoxonase, U/mL	33.44[a]	44.66[b]	44.01[b]	2.37	42.32	39.08	1.62	<0.01	0.24	0.63	0.75	0.12	0.66	0.34	0.44
ROM (mg H_2O_2/100mL)	18.95[a]	16.13[b]	15.18[b]	0.44	17.01	16.49	0.36	<0.01	0.30	0.46	0.95	0.93	0.29	0.32	0.22
FRAP (µmol/L)	169.38	163.28	168.64	9.40	271.63	162.57	7.70	0.88	0.41	0.64	0.57	0.51	0.53	0.24	0.83
Creatinine (µmol/L)	100.02	102.79	105.78	2.14	101.38	104.35	1.75	0.17	0.23	0.68	<0.01	0.73	0.02	0.22	0.47

[a,b] Values within dry period lengths in the same row with different superscripts differ ($P < 0.05$).

[1] D = dry period; R = ration; P = parity

[2] Weeks 1 and 2 postcalving.

Table 2.3 Inflammatory biomarkers (haptoglobin, ceruloplasmin, albumin and cholesterol), index of liver function (paraoxonase and bilirubin), oxidative stress markers (reactive oxidative metabolites (ROM) and ferric reducing antioxidant power (FRAP)), and creatinine in plasma of dairy cows dry period lengths (0, 30 or 60 d) and fed either a glucogenic (G) or a lipogenic (L) ration when energy balance (EB) was added in the model (LSM ± pooled SEM).

Item	Dry period lengths (d)			SEM	Ration		SEM	P-values[1]						
	0	30	60		G	L		EB	D	R	P	W^2	EB × R	EB × W
Cows (n=89)	29	30	26		45	40								
Haptoglobin (g/L)	0.83	0.65	0.48	0.09	0.65	0.65	0.07	0.11	0.17	0.23	0.65	0.05	0.14	0.91
Ceruloplasmin (µmol/L)	2.55	1.88	1.78	0.14	2.14	2.00	0.11	0.25	0.18	0.08	0.67	0.17	0.14	0.44
Albumin (g/L)	35.76	36.15	37.28	0.72	36.47	36.32	0.55	0.97	0.25	0.42	0.61	0.09	0.25	0.21
Cholesterol (mmol/L)	3.00[a]	2.50[b]	2.18[b]	0.16	2.64	2.48	0.13	0.77	0.02	0.30	0.25	0.28	0.05	<0.01
Bilirubin (µmol/L)	1.30	1.29	1.54	0.18	1.36	1.39	0.14	<0.01	0.94	0.52	0.74	<0.01	0.38	0.14
Paraoxonase, U/mL	31.56[a]	45.16[b]	47.31[b]	3.34	42.64	40.05	1.62	0.18	0.01	0.08	0.72	0.83	0.01	0.62
ROM (mg H_2O_2/100mL)	19.13[a]	16.19[b]	15.16[b]	0.62	17.14	16.51	0.46	0.68	<0.01	0.06	0.09	0.94	0.12	0.75
FRAP (µmol/L)	174.35	164.62	160.89	15.23	173.81	159.43	11.16	0.76	0.92	0.88	0.48	0.48	0.40	0.52
Creatinine (µmol/L)	100.85	102.13	104.09	2.94	100.90	100.82	2.20	0.36	0.58	0.22	0.51	<0.01	0.42	0.34

[a,b] Values within dry period lengths in the same row with different superscripts differ ($P < 0.05$).

[1] D = dry period; R = ration; P = parity. EB × P, EB × P, EB × R, D × R, D × P, D × W and P × R interactions were also included in the model, but not significant.

[2] Weeks 1 and 2 postcalving.

Table 2.4 Inflammatory biomarkers (haptoglobin, ceruloplasmin, albumin and cholesterol), index of liver function (paraoxonase and bilirubin), oxidative stress (reactive oxidative metabolites (ROM) and ferric reducing antioxidant power (FRAP)), and creatinine in plasma of dairy cows with dry period lengths (0, 30 or 60 d) and fed either a glucogenic (G) or a lipogenic (L) ration when somatic cell count (SCC) was added in the model (LSM ± pooled SEM).

Item	Dry period lengths (d)				Ration			P-values[1]							
	0	30	60	SEM	G	L	SEM	SCC	D	R	P	W^2	SCC × P	D × P	D × W
Cows (n=89)	29	30	26		45	40									
Haptoglobin (g/L)	0.73	0.63	0.41	0.19	0.57	0.61	0.12	0.56	0.52	0.98	0.97	0.02	0.26	0.20	0.54
Ceruloplasmin (μmol/L)	2.34[a]	1.92[b]	1.85[ab]	0.27	2.11	1.96	0.17	0.81	0.02	0.37	0.77	0.50	0.71	0.84	0.65
Albumin (g/L)	35.82	36.30	33.70	1.37	35.45	35.09	0.86	0.22	0.27	0.52	0.40	0.17	0.55	0.44	0.02
Cholesterol (mmol/L)	3.03[a]	2.52[b]	1.70[b]	0.30	2.48	2.35	0.20	0.36	<0.01	0.59	<0.01	<0.01	0.90	0.55	0.09
Bilirubin (μmol/L)	1.28	1.34	1.69	0.46	1.43	1.44	0.28	0.73	0.19	0.98	0.76	0.02	0.89	0.38	0.54
Paraoxonase, U/mL	32.44[a]	46.21[b]	48.16[b]	6.59	43.93	40.61	4.06	0.55	0.01	0.26	0.65	0.69	0.79	0.83	0.64
ROM (mg H_2O_2/100mL)	28.73[a]	16.36[b]	13.94[b]	1.22	16.65	16.03	0.76	0.72	<0.01	0.46	0.74	0.87	0.04	0.30	0.99
FRAP (μmol/L)	176.95	164.39	151.69	31.02	169.50	159.19	19.02	0.78	0.76	0.36	0.40	0.87	0.83	0.42	0.14
Creatinine (μmol/L)	100.46	103.33	103.51	5.56	100.51	104.36	3.46	0.92	0.52	0.23	0.36	<0.01	0.85	0.01	0.09

[a,b] Values within dry period lengths in the same row with different superscripts differ ($P < 0.05$).

[1] D = dry period; R = ration; P = parity; SCC= somatic cell count. SCC × D, SCC × R, SCC × W, D × R, SCC × W, D × R, and P × R interactions were also included in the model, but not significant.

[2] Weeks 1 and 2 postcalving.

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Table 2.5 Inflammatory biomarkers (haptoglobin, ceruloplasmin, albumin and cholesterol), index of liver function (paraoxonase and bilirubin), oxidative stress (reactive oxidative metabolites (ROM) and ferric reducing antioxidant power (FRAP)), and creatinine in plasma of dairy cows with varying dry period lengths (0, 30 or 60 d) and fed either a glucogenic (G) or a lipogenic (L) ration when clinical health problem (CHP) in weeks 1 and 2 postcalving was added in the model (LSM ± pooled SEM).

Item	Health problem[1]			Dry period lengths (d)				Ration			P-values[1,4]						
	No	Yes	SEM	0	30	60	SEM	G	L	SEM	CHP	D	R	P	W²	CHP × W	D × W
Cows (n=85)	61	24		29	30	26		45	40								
Hapto-globin (g/L)	0.62	0.88	0.09	0.92	0.73	0.59	0.11	0.80	0.69	0.09	0.06	0.20	0.33	0.61	0.83	0.15	0.36
Cerulo-plasmin (µmol/L)	1.97	2.37	0.11	2.59ᵃ	1.85ᵇ	2.07ᵇ	0.14	2.33	2.10	0.11	0.02	<0.01	0.04	0.59	0.48	0.68	0.99
Albumin (g/L)	36.42	34.84	0.54	34.82	36.40	35.66	0.74	35.57	35.69	0.57	0.05	0.24	0.87	0.28	0.01	0.13	0.01
Cholesterol (mmol/L)	2.59	2.37	0.11	2.87ᵇ	2.47ᵃᵇ	2.09ᵇ	0.15	2.55	2.41	0.13	0.12	0.01	0.43	0.01	0.06	0.03	<0.01
Bilirubin (µmol/L)	1.51	0.97	0.18	0.79	1.26	1.68	0.24	1.23	1.26	0.19	0.05	0.07	0.91	0.44	<0.01	0.18	0.39
Paraoxo-nase, U/mL	40.40	40.40	2.71	34.74ᵃ	47.25ᵇ	39.20ᵃᵇ	3.60	40.78	40.01	2.75	1.00	0.02	0.84	0.39	0.93	0.86	0.47
ROM (mg H₂O₂/100mL)	16.54	18.69	0.51	20.21ᵃ	16.42ᵇ	16.21ᵇ	0.67	18.33	16.89	0.51	0.01	<0.01	0.04	0.64	0.14	0.23	0.84
FRAP (µmol/L)	166.81	178.56	13.48	174.01	181.76	162.28	17.20	171.15	174.22	12.74	0.55	0.71	0.86	0.97	0.15	0.13	0.26
Creatinine (µmol/L)	102.88	100.23	2.13	98.15	103.84	102.67	2.90	100.55	102.56	2.25	0.35	0.31	0.52	0.91	<0.01	0.46	0.07

Continued to the next page

Table 2.5 continued

[a,b] Values within dry period lengths in the same row with different superscripts differ ($P < 0.05$).

[1] D = dry period, R = ration, P = parity, CHP=clinical health problem (metritis, mastitis, retained placenta, or fever). CHP × D, CHP × R, CHP × P, D × R, D × P, and P × R interactions were also included in the model, but not significant

[2] Weeks 1 and 2 postcalving.

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Table 2.6 Distribution of cows with different clinical health problems in weeks 1 and 2 postcalving.

Dry period lengths	n	Fever	Mastitis	Metritis	Retained Placenta	Total	Percentages
0 d	56	2	8	2	11	23	41%
30 d	55	2	5	1	7	15	27%
60 d	56	4	2	1	10	17	30%
	167	8	15	4	28	55	33%

2.5   Discussion

2.5.1 Effect of Dry Period Length on Inflammatory Biomarkers

In the current study, cows with a 0-d dry period, compared with a 30-d or a 60-d dry period, had higher cholesterol, ceruloplasmin and tended to have higher haptoglobin levels in plasma especially in the first 2 weeks after caving, independent of dietary treatment. Previous studies have shown that a quicker increase in cholesterol (Kaneene et al., 1997, Trevisi et al., 2009) has been associated with a better EB and improved metabolic status in dairy cows in early lactation. In another study, the reduced levels of haptoglobin and ceruloplasmin were associated with better EB (Bionaz et al., 2007). The increase of haptoglobin was earlier related to high production of liver macrophages (known as Kupffer cells) during inflammation (Ametaj et al., 2005, Trebicka et al., 2011). The increase of ceruloplasmin was associated with health problems in cows in early lactation (Conner et al., 1988, Chassagne et al., 1998, Sheldon et al., 2001).
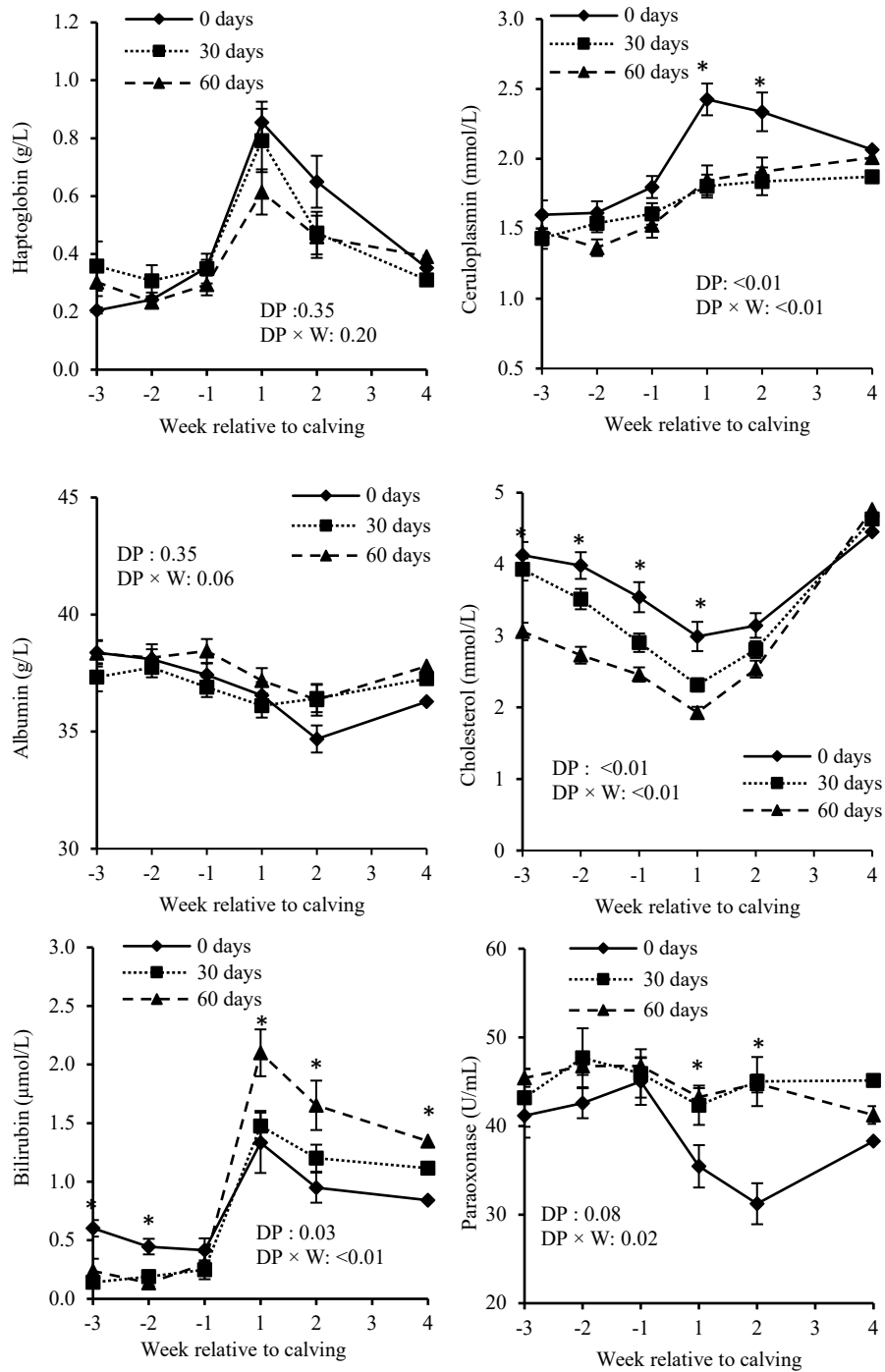
Continued to the next page
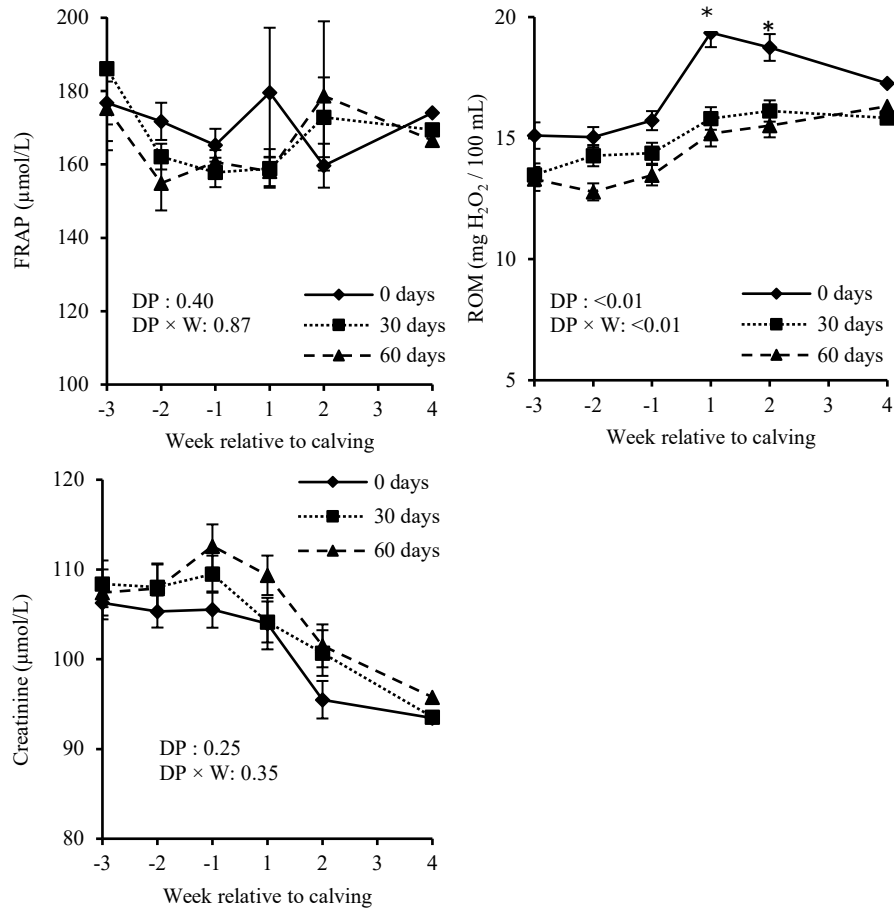
Figure 2.1 Inflammatory biomarkers (haptoglobin, ceruloplasmin, albumin and cholesterol), index of liver function (paraoxonase and bilirubin), oxidative stress markers [reactive oxidative metabolites (ROM) and ferric reducing antioxidant power of plasma (FRAP)], and creatinine in plasma of dairy cows per dry period lengths (0, 30 or 60-d) per week (Mean ± SEM). *P*-values of dry period (DP) and interaction between DP × week (W) for each parameter are shown. Asterisks indicate significant (*P* < 0.05) differences between dry period lengths within week.

2.5.2 Effect of Dry Period Length on Liver Functionality

In the current study, cows with a 0-d dry period had lower levels of bilirubin and paraoxonase in plasma compared with cows with a 60-d dry period, especially in the first 2 weeks after calving. Bilirubin and paraoxonase are commonly used biomarkers for liver functionality status around calving (Bertoni et al., 2008, Bertoni and Trevisi, 2013). Previous studies have shown that reduced bilirubin levels indicated better clearance of secretory enzymes in the liver and are associated with a better EB and improved metabolic status in dairy cows in early lactation (Assenat et al., 2004, Bertoni et al., 2008). In the current experiment, omitting the dry period reduced milk yield, improved the EB, reduced total milk and fat- and protein-corrected milk yields, and yield of lactose, fat, and protein yields but did not affect DMI, compared with cows with a 60 dry period (Van Knegsel et al., 2014), and not in the first weeks of lactation which was the focus of the current study (Table 2.A1). In the current study, DMI was negatively related to bilirubin levels ($r = -0.35$, $P < 0.01$) while milk yield was positively related to bilirubin levels ($r = 0.22$, $P < 0.01$).

Paraoxonase, known as a liver protein with hydrolase activity is considered as a marker of liver activity (Bionaz et al., 2007) and known as a negative APP (James and Deakin, 2004). Previous studies showed that a reduced paraoxonase levels in plasma were associated with lipid metabolic disorders (Aviram and Rosenblat, 2004, Turk et al., 2004) and chronic liver damage in dairy cows (Ferré et al., 2002). In the current study, the reduced bilirubin levels in plasma after a 0-d dry period might be associated with better functionality of the hepatocytes related to better EB. Moreover, the decreased paraoxonase was not associated with EB. Previously, reduced level of paraoxonase in plasma was associated with increased level of bilirubin in plasma (Bionaz et al., 2007, Trevisi et al., 2012b). However, in the current study the reduced paraoxonase levels in plasma of cows with 0-d dry period was accompanied by an increased haptoglobin levels ($r = -0.28$, $P = 0.03$), which could be related to a more severe inflammatory condition (Bionaz et al., 2007, Bertoni and Trevisi, 2013) of these cows in the first 2 weeks after calving. Our study indicated that reduced paraoxonase was related to inflammation and low liver functionality of dairy cows with a 0-d dry period at least in the first 2 weeks after calving.

Results of this study indicate that cows with a 0-d dry period had better clearance of waste products in the liver as shown by a reduced level of bilirubin in plasma immediately after calving. However, during the 4 weeks after calving, the

levels of bilirubin, albumin, and cholesterol in plasma of cows with a 0-d dry period resulted in lower LFI compared with cows with 30-d or 60-d dry period. The LFI defines a good liver functionality when the levels of bilirubin decrease, and cholesterol and albumin increase between week 1 and 4 in lactation (Trevisi et al., 2012a). When liver functionality, according to LFI, is poor, these levels stabilize during weeks 1 to 4 in lactation. Trevisi et al. (2012a) suggested that low LFI indicates lower liver function immediately after calving because of severe inflammation and cows have problems adapting to their inflammatory challenges have a low response of negative APP in the first month of lactation. In the current study, cows with a 0-d dry period had high cholesterol both at calving and after calving (but with a slight increase in the first month of lactation), compared with cows with a 60-d dry period, which showed a marked increase from week 1 to 4. In addition, cows with a 0-d dry period had a low bilirubin at calving and a limited decrease after calving. Thus, levels of cholesterol are high but stable, levels of bilirubin are low and stable for cows with a 0-d dry period, whereas levels of albumin showed a reduction after calving. This might imply that indeed recovery of the liver is poor for cows with a 0-d dry period between week 1 and 4 of lactation, which is partly related to the high liver status at calving.

2.5.3 Effect of Dry Period Length on Oxidative Stress

In the first 2 weeks after calving, omitting the dry period increased ROM levels in plasma compared with a 60-d dry period. High ROM levels indicate conditions of high oxidative stress (Esposito et al., 2014), which is associated with severe NEB (Bernabucci et al., 2005, Pedernera et al., 2010). In the current study, the better EB, due to lower milk yield, in cows with a 0-d dry period was not associated with increased ROM concentration. Osorio et al. (2014) suggested that oxidative stress is triggered by the imbalance between the production of ROM and the neutralizing capacity of antioxidant mechanisms (including production of FRAP) in tissues and in blood. A previous study showed that increased levels of ROM in plasma were associated with reduced levels of paraoxonase as part of a huge anti-oxidative system in plasma (Trevisi et al., 2012b). In the current study, omitting the dry period compared with a 30-d or 60-d dry period increased levels of ROM and reduced paraoxonase levels without affecting FRAP levels. Our results indicate that the increased of ROM levels in cows with a 0-d dry period might be related to the reduced paraoxonase levels (r = -0.31, P = 0.02), which indicated a more severe oxidative stress, compared with cows with a 60-d dry period.

In the current study, cows with a 0-d dry period increased pro- and anti-inflammatory markers, oxidative stress, and lower liver functionality compared with cows with 60-d dry period. Inflammation and oxidative stress are associated with reduced mammary health (Pyörälä, 2003), NEB, and the occurrence of clinical health problems (Drackley, 1999, Trevisi et al., 2014). To our knowledge, only one study (Trevisi et al., 2010) has reported the relationship between inflammatory biomarkers and SCC or health status in cows with different dry period lengths from 47 to 71 d. However, a research about the relationship between SCC, EB, or clinical health problems and inflammatory biomarkers or oxidative stress in cows with different dry period length (0, 30, or 60-d) is lacking. It is not clear whether the increase of pro-inflammatory biomarkers and oxidative stress in the current study was directly due to dry period length effects, or related to differences in SCC, EB or clinical health problems.

2.5.4 Effect of Somatic Cell Count on Inflammatory Biomarkers and Oxidative Stress

In the current study, the effect of dry period length on inflammatory biomarkers and oxidative stress variables could not be explained by the effect of dry period length on SCC. A previous study showed that mammary gland cell differentiation and proliferation, and oxidative stress might causes inflammation during transition period (Trevisi et al., 2010). Subclinical mastitis, defined as an elevated SCC without clinical signs, was associated with increased levels of haptoglobin in plasma (Safi et al., 2009). Occurrence of clinical mastitis was associated with increased haptoglobin levels (Pyörälä, 2003) and bilirubin levels (Minuti et al., 2015) and decreased paraoxonase levels (Turk et al., 2012). As earlier reported, in the current experiment, omitting the dry period increased SCC in milk in the subsequent lactation but did not affect the occurrence of CM compared with cows with a 30-d or a 60-d dry period (Mayasari et al., 2016). This is in line with the present results that high SCC in cows with 0-d dry period was not associated with inflammatory biomarkers and oxidative stress.

2.5.5 Effect of Energy Balance on Inflammatory Biomarkers and Oxidative Stress

In the current study, low plasma bilirubin levels were associated with better EB, independent of dry period length. In addition, DMI was negatively related to haptoglobin levels ($r = -0.38$, $P < 0.01$), cholesterol levels ($r = -0.26$, $P <$

0.01) and bilirubin levels (r = -0.35, P < 0.01). Milk yield was positively related to bilirubin levels (r = 0.22, P < 0.01), independent of dry period length. Previous studies showed that NEB in dairy cows is related to fatty liver, potentially affecting liver function (Ametaj et al., 2005, Bertoni et al., 2008). Bilirubin is commonly used as a biomarker for liver status around calving (Bionaz et al., 2007, Bertoni et al., 2008). Hence, it can be suggested that the effect of dry period length on bilirubin levels was associated with alterations in EB and metabolic status in the first 2 weeks after calving. Moreover, liver functionality was associated with fatty acid uptake and secretion of cholesterol from triglycerides (Bell, 1995). In contrast, the lower bilirubin levels in cows with a 0-d dry period immediately after calving suggests a better clearance in the liver.

2.5.6 *Effect of Clinical Health Problems on Inflammatory Biomarkers and Oxidative Stress*

In the current study, the occurrence of clinical health problems was related to high levels of ceruloplasmin, low albumin levels, low bilirubin levels, high ROM levels, and a tendency for high haptoglobin levels in plasma, independent of dry period length. In addition, the levels of ceruloplasmin and ROM before calving are higher for cows with clinical health problem. This is in line with previous studies that the occurrence of clinical health problems was associated with consequences of severe or prolonged inflammations before calving (Trevisi et al., 2011, Trevisi et al., 2012a) and oxidative stress (Trevisi et al., 2010). Our results indicate that the dry period length may not directly be a causative factor for inflammation and stress. The changes in inflammatory biomarkers after calving are related to the occurrence of health problems, but many of these begin before calving and several remain subclinical.

2.5.7 *Effect of Ration and Parity on Inflammatory Biomarkers and Oxidative Stress*

Parity influenced the levels of ceruloplasmin, albumin, cholesterol, creatinine and FRAP. Albumin levels were lower in cows with parity > 3 compared with cows with parity 2, independent of dry period length. Ceruloplasmin levels were higher in cows with parity > 3 compared with cows with parity 2, independent of dry period length. In addition, cows with parity > 3 fed a glucogenic ration had higher cholesterol levels compared with cows fed a lipogenic ration. In the current study, the differences in cholesterol levels among dry period

lengths were present from week -3 to 1 relative to calving but did not differ after week 1. Previously, it has been suggested that different levels of cholesterol might be affected by ration composition during the dry period (Bertoni and Trevisi, 2013, Newman et al., 2016). In the current experiment, during prepartum, cows with a 0-d and 30-d dry period had higher DMI and energy intake compared with cows with a 60-d dry period (Van Knegsel et al., 2014). Indeed, during prepartum, cows with a 0-d dry period received a lactating ration and produced milk, whereas cows with a 30-d and 60-d dry period received a dry cow ration and did not produce milk. It is likely that the differences in cholesterol levels between dry period lengths are explained by the intake and the different rations before calving.

The changes in concentration of APP were affected by internal and external challenges such as infection and stress (Murata et al., 2004, Ceciliani et al., 2012), which can occur in young or old animals. During the first 2 weeks after calving, cows with parity > 3 cows compared with cows with parity 2 had higher creatinine and FRAP levels in plasma when the dry period was omitted. Creatinine, an indicator of body muscle mass, typically decreases around calving due to milk production (Kokkonen et al., 2005, Osorio et al., 2014). In addition, FRAP is an indicator of antioxidant status (Jacometo et al., 2015, Konvičná et al., 2015), and acts to neutralize the production of reactive intermediates caused by oxidative stress in early lactations (Esposito et al., 2014). Previous studies from Annen et al. (2004) and Santschi et al. (2011) showed a reduction in milk yield after shortening or omitting the dry period for cows with parity 2, but not for cows with parity > 3. In our study, during the first 2 weeks after calving, cows with parity > 3 cows showed less reduction in milk yield when the dry period was omitted compared with cows with parity 2. Therefore, it seems that the high creatinine and high FRAP levels in plasma of cows with parity > 3 in the first 2 weeks of lactation compared with cows with parity 2 indicate high mobilization of body muscle mass after calving and less stress perhaps explained by lesser reduction of milk yield when the dry period is omitted.

2.6   Conclusions

Omitting the dry period resulted in higher level of cholesterol (negative APP), ceruloplasmin (positive APP), ROM and lower bilirubin, paraoxonase, liver functionality index, and tended to result in higher haptoglobin levels in plasma, compared with cows with a 60-d dry period. Omission of the dry period length is related to a better EB and improvement of metabolic status in the early lactation

due to less milk yield, which could be also contribute to a reduction of the inflammation and oxidative stress. Low bilirubin is associated with better EB, independent of dry period length. In addition, omission of the dry period is associated with the increase of positive APP and oxidative stress, which could be explained partly by occurrence of clinical health problems.

2.7   Acknowledgements

Table 2.A1. Milk yield, dry matter intake, energy balance, somatic cell count and plasma metabolites and metabolic hormones of dairy cows with different dry period lengths (0, 30 or 60 d) and fed either a glucogenic (G) or a lipogenic (L) ration (LSM ± pooled SEM).

Item	Dry period length (d)			SEM	Ration		SEM	P-values[1]							
	0	30	60		G	L		D	R	P	W[2]	D×R	D×P	D×W	P×R
Cows (n=89)	29	31	29		44	45									
Milk yield[2] (kg/d)[2]	24.83[a]	30.30[b]	35.20[b]	0.74	29.89	30.33	0.61	<0.01	0.61	<0.01	<0.01	0.99	0.12	0.84	0.67
FPCM (kg/d)[2]	30.71[a]	38.07[b]	42.61[c]	1.01	37.13	37.13	0.82	<0.01	1.00	<0.01	<0.01	0.91	0.03	0.03	0.38
Lactose (%)[2]	4.22[a]	4.38[b]	4.32[ab]	0.04	4.32	4.29	0.03	0.01	0.58	0.05	<0.01	0.92	0.92	0.04	0.86
Fat (%)[2]	5.23	5.15	5.16	0.10	5.21	5.15	0.08	0.81	0.61	0.15	<0.01	0.38	0.18	0.73	0.90
Protein (%)[2]	4.64[c]	4.27[c]	4.08[c]	0.07	4.33	4.33	0.06	<0.01	0.93	0.03	<0.01	0.42	0.10	0.01	0.72
Lactose (kg/d)[2]	1.08[a]	1.33[b]	1.53[b]	0.04	1.31	1.31	0.03	<0.01	0.88	<0.04	<0.04	0.97	0.14	0.78	0.77
Fat (kg/d)[2]	1.30[a]	1.55[b]	1.81[b]	0.05	1.55	1.56	0.04	<0.01	0.92	0.04	<0.01	0.70	0.08	0.52	0.59
Protein (kg/d)[2]	1.09[a]	1.28[b]	1.43[b]	0.03	1.26	1.27	0.02	<0.01	0.75	<0.01	<0.01	0.60	0.10	0.02	0.51
DMI (kg/day)[2]	17.09	17.19	17.05	0.33	17.16	17.06	0.27	0.95	0.78	0.78	<0.01	0.27	0.54	0.04	0.81
Energy balance (kj/kg[0.75]x d)[2,3]	-111.53[a]	-291.53[a]	-451.62[b]	25.15	-269.97	-299.81	20.52	<0.01	0.31	<0.01	0.01	0.67	0.06	0.03	0.32
SCC (x10^{3})[2,5]	843.06[a]	252.50[b]	224.85[b]	118.80	451.20	429.08	96.66	<0.01	0.98	0.10	<0.01	0.63	0.32	0.04	0.48
FFA (mmol/L)[2]	0.34[a]	0.45[ab]	0.55[b]	0.05	0.42	0.47	0.04	0.01	0.40	0.16	0.09	0.37	0.20	0.53	0.24
BHB (mmol/L)[2,4]	0.68	0.63	0.67	0.05	0.64	0.68	0.04	0.72	0.44	0.87	0.17	0.16	0.35	0.29	0.12
Glucose (mmol/L)[2]	3.93[a]	3.43[b]	3.32[b]	0.09	3.51	3.61	0.08	<0.01	0.36	0.07	<0.01	0.51	0.10	0.37	0.72
Urea (mmol/L)[2]	2.78	2.67	2.59	0.14	2.49	2.87	0.12	0.67	0.03	0.52	0.01	0.70	0.41	0.42	0.66
IGF-1(ng/L)[2]	110.40[a]	74.50[b]	58.24[b]	7.48	80.88	81.22	6.14	<0.01	0.97	<0.02	0.78	0.42	0.14	0.04	0.62
Insulin (µIU/mL)[2]	21.20[a]	11.00[b]	7.50[b]	1.79	14.12	12.35	1.47	<0.01	0.40	0.07	0.02	0.86	0.25	0.25	0.93

[a-c] Values within a row (across dry period lengths) with different superscripts differ ($P < 0.05$)
[1] D = dry period; R = ration, P = parity, [2] Week 1 and 2 postcalving for milk yield, fat- and protein-corrected milk (FPCM), dry matter intake (DMI), energy balance (EB), somatic cell count (SCC), concentration of plasma free fatty acid (FFA), β-hydroxybutyrate (BHB), glucose, urea, insulin growth factor-1 (IGF-I), and insulin; [3]Energy balance calculated with the VEM system (Van Es, 1975); [4]P-value based on the natural logarithm of plasma BHB, [5]P-value based on the natural logarithm of plasma SCC.

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CHAPTER 3

Relationships between inflammatory biomarkers and oxidative stress with uterine health in dairy cows with different dry period lengths

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3.1 Abstract

The objective of this study was to determine relationships between inflammatory biomarkers and oxidative stress with uterine health in dairy cows with different dry period lengths. Holstein-Friesian dairy cows (N = 130) were assigned randomly to 1 of 3 dry period lengths (0, 30, or 60 d). Part of the cows which were planned for a 0-d dry period dried themselves off and were attributed to a new group (0→30-d dry period), which resulted in total in 4 dry period groups. Cows were fed a glucogenic or lipogenic ration from 10 d before the expected calving date. Blood was collected (N = 110) in week -3, -2, -1, 1, 2 and 4 relative to calving to determine inflammatory biomarkers and oxidative stress. Uterine health status (**UHS**) was monitored by scoring vaginal discharge (**VD**) based on a 4-point scoring system (0, 1, 2 or 3) in week 2 and 3 after calving. Cows (N = 91) were classified as having a healthy uterine environment (**HU**, VDS = 0 or 1 in both week 2 and 3), non- recovering uterine environment (**NRU**, VDS = 2 or 3 in week 3) or a recovering uterine environment (**RU**, VDS = 2 or 3 in week 2 and VDS = 0 or 1 in week 3). Cows with NRU had higher haptoglobin and a lower paraoxonase levels in plasma in the first 4 weeks after calving and lower liver functionality index (**LFI**) compared with cows with HU, independent of dry period length. Cows with NRU had lower albumin and creatinine levels in plasma compared with cows with a RU, but not compared with cows with HU. Independent of UHS, cows a 0→30-d dry period had higher bilirubin levels in plasma compared with cows with 0-d, 30-d, or 60-d dry period. Independent of UHS, cows a 60-d dry period had lower creatinine levels in plasma compared with cows with 0→30-d or 30-d dry period, but not compared with cows with a 0-d dry period. Cows with RU and fed a lipogenic ration had higher levels of albumin in plasma compared with cows with NRU and fed a lipogenic ration. In conclusion, uterine health was related with variables for inflammation (haptoglobin, albumin) and paraoxonase in dairy cows in early lactation. Independent of UHS, cows in the 0→30-d dry period had higher bilirubin and creatinine levels, which was possibly related to a more severe NEB in these cows. Inflammatory biomarkers in dairy in early lactation are related with uterine health in this period.

Key words: continuous milking, uterine health, oxidative stress, inflammation.

3.2 Introduction

Shortening and omitting the dry period is of interest because it has the potential to improve the energy balance (**EB**) (Rastani et al., 2005, de Feu et al., 2009, Van Knegsel et al., 2014), metabolic status (Rastani et al., 2005, Chen et al., 2015a) and fertility (de Feu et al., 2009, Chen et al., 2015b) of dairy cows in the next lactation. The improvement of the EB is mainly due to a reduction in milk yield in the subsequent lactation (Van Knegsel et al., 2014), and sometimes also to an improvement of the dry matter intake (**DMI**) in the peripartum period (Rastani et al., 2005). Earlier we reported that cows with a 0-d dry period had increased levels of ceruloplasmin, cholesterol, reactive oxygen metabolites (**ROM**) and had decreased levels of bilirubin and paraoxonase in plasma, and had decreased liver functionality index (**LFI**) compared with cows with a 60-d dry period (Mayasari et al., 2017). The effects of dry period length on inflammatory status could partly be explained by the improved EB and occurrence of health problems in cows with a 0-d dry period (Mayasari et al., 2017). In this earlier study, clinical health problems included clinical mastitis, fever, metritis and retained placenta. It can be hypothesized that also other inflammation-related disorders, like metritis or endometritis, could clarify alterations in inflammatory and oxidative stress variables in dairy cows in early lactation.

In early lactation, a severe negative energy balance (**NEB**) has been related with a greater risk for metritis or endometritis (Hammon et al., 2006, Manimaran et al., 2016). During infection of the uterus, pro-inflammatory cytokines alter the acute phase protein (**APP**) concentration in plasma (Baumann and Gauldie, 1994) and negatively affect the uterine immunity (Manimaran et al., 2016). Cows with endometritis have an increased culling rate and impaired fertility by reduced pregnancy rate (LeBlanc et al., 2002). Uterus palpation and monitoring of vaginal discharge (**VD**) has been widely used to diagnose clinical endometritis in dairy cows (LeBlanc et al., 2002, Gilbert et al., 2005, Williams et al., 2005, Prunner et al., 2014). Vaginal discharge has been related to high plasma haptoglobin levels (Huzzey et al., 2009, Dubuc et al., 2010, Ametaj et al., 2014). The relationship between uterine health status (**UHS**), as indicated by VD, and oxidative stress is unclear. Previous studies showed that metritis and VD were associated with increased oxidative stress (Kizil et al., 2010, Magata et al., 2016), but not all (Bicalho et al., 2014). To our knowledge the relationships between inflammatory biomarkers and oxidative stress with uterine health in dairy cows with different dry period lengths is unknown. The objective of this study was to determine the

relationships between inflammatory biomarkers and oxidative stress with uterine health in dairy cows in early lactation after different dry period lengths.

3.3 Materials and Methods

3.3.1 Animals and Experimental Design

The Institutional Animal Care and Use Committee of Wageningen University approved the experimental protocol. The registration number of the experimental protocol was 2010026. The experimental design, dry period lengths and dietary contrast were described earlier (Van Knegsel et al., 2014). Holstein-Friesian dairy cows (N = 167) were selected from the Dairy Campus research herd (WUR Livestock Research, Lelystad, the Netherlands), blocked according to parity, calving date, milk yield in the previous lactation, and body condition score (**BCS**), and randomly assigned to treatments within blocks. Treatments consisted of 3 dry period lengths (0, 30, or 60 d) and 2 early lactation rations (glucogenic or lipogenic) resulting in a 3 × 2 factorial design. Results of the first lactation after implementation of dry period lengths and dietary treatments have been reported for EB and milk yield (Van Knegsel et al., 2014), metabolites and metabolic hormones (Chen et al., 2015a) and fertility (Chen et al., 2015b). After the first lactation, 37 cows were excluded due to non-pregnancy and health problems. Therefore, the second lactation started with 130 cows: 39 cows with a 0-d dry period, 41 cows with a 30-d dry period, and 50 cows with a 60-d dry period. Moreover, 19 cows in the 0-d dry period group were attributed before the second lactation to a new group (0→30-d dry period; actual days dry: 67 ± 8 d) because these cows had a milk yield of < 4 kg/d at least 30 d before expected calving date and were dried off without use of intramammary antibiotics. Blood was collected from 110 cows. Vaginal discharge was collected and scored in week 2 and 3 after calving from 91 cows according to Williams et al. (2005). Cows were housed in a freestall with slatted floor and cubicles, and milked twice daily (0500 and 1630 h). The drying-off protocol was as follows: cows with a 30-d or a 60-d dry period received a far-off ration 7 d before drying-off, were milked once daily 4 d before drying-off, and were treated with an intramammary antibiotic at drying off (Supermastidol Virbac Animal Health, Barneveld, the Netherlands). Ration composition was described earlier (Van Knegsel et al., 2014). Prepartum, cows with a 30-d and 60-d dry

period received a dry cow ration, and cows with a 0-d dry period received a lactating cow ration supporting 25 kg of milk yield per day. From 10 d before expected calving onwards, cows of all treatments were fed 1 kg/d glucogenic or lipogenic concentrate, which was increased postcalving in steps of 0.5 kg/d until the concentration supply reached 8.5 kg/d. The main ingredient for the glucogenic concentrate was corn, the main ingredients for the lipogenic concentrate were sugar beet pulp, palm kernel, and rumen-protected palm oil. A computerized feeder located in the freestall provided experimental concentrates. In the milking parlor, cows received 1 kg/d of standard lactation concentrate. Forage composition did not differ among rations and was supplied ad libitum and consisted prepartum of grass silage, corn silage, wheat straw, and a protein source (rapeseed meal or soybean meal) in a ratio of 39:25:25:11 (DM basis). Postpartum, forage consisted of grass silage, corn silage, wheat straw, and a protein source (rapeseed meal or soybean meal) in a ratio of 51:34:2:13 (DM basis). Rations were isocaloric (net energy basis: Dutch net energy evaluation (**VEM)** system; Van Es, 1975), and contained equal amounts of intestinal digestible protein and degraded protein (DVE/OEB system; Tamminga et al., 1994). Concentrate and forage were supplied separately.

3.3.2 Blood Sampling and Analysis

Blood sampling was described earlier by Chen et al. (2015a). In short, blood samples (N = 110) were taken weekly from the tail vein at 3 h before the morning feeding from week -3 to 4 relative to calving. Blood was collected in evacuated tubes (Vacuette, Greiner BioOne, Kremsmunster, Austria) containing lithium-heparin for haptoglobin, ceruloplasmin, cholesterol, albumin, bilirubin, paraoxonase, ROM, FRAP, and creatinine. Samples were kept cold on ice for a maximum of 2 h until they were centrifuged at 3,000 × g for 15 min at 4°C. Plasma was decanted, aliquoted, and frozen at −20°C until analysis.

3.3.3 Laboratory Analysis

Determination of inflammatory biomarkers and oxidative stress variables was described earlier by Mayasari et al. (2017). In short, inflammatory biomarkers and oxidative stress were measured at the Istituto di Zootecnia of the Università Cattolica del Sacro Cuore (Piacenza, Italy), following the procedures described previously by Bionaz et al. (2007), Calamari et al. (2016) and Jacometo et al. (2015)

using a clinical auto-analyzer (ILAB 650, Instrumentation Laboratory, Lexington, MA). In short, total cholesterol (catalog no. 0018250540), albumin (catalog no. 0018250040), total bilirubin (catalog no. 0018254640), and creatinine (catalog no. 0018255540) were measured using the IL Test purchased from Instrumentation Laboratory Spa (Werfen Co., Milan, Italy). The haptoglobin was determined with the method described by Skinner et al. (1991) and Owen et al. (1960), adapted to ILAB 650 condition. The method based on peroxidase activity of methaemoglobin-haptoglobin complex measured by the rate of oxidation of guaiacol (hydrogen donor) in the presence of hydrogen peroxide (oxidizing substrate). The ceruloplasmin was determined with the method described by Sunderman and Nomoto (1970), adapted to ILAB 650 conditions. The test is based on measurement of the colour which originates from the oxidation of the *p*-phenylenediamine dihydrochloride induced by the ceruloplasmin. The ROM was measured using commercial kits (kit d-ROMs-test cod. MC003; Diacron International s.r.l., Grosseto, Italy) adapted to the ILAB 650 conditions. Antioxidant potential was assessed as ferric reducing antioxidant power (FRAP) using the colorimetric method of Benzie and Strain (1996). Plasma paraoxonase activity was measured by adapting the method of Ferré et al. (2002) to the ILAB 650 conditions.

3.3.4 Vaginal Discharge Scoring

Vaginal discharge collection and scoring has been described earlier (Chen et al., submitted). Vaginal discharge was scored based on a 4-point scoring system according to Williams et al. (2005): score 0 = clear translucent mucus, score 1 = clear translucent mucus with discrete flecks of purulent exudate present, score 2 = discharge containing < 50 percent white or off-white mucopurulent material, and score 3 = discharge containing > 50 percent purulent material.

3.3.5 Statistical Analyses

The MIXED procedure of SAS (SAS version 9.2; SAS Institute Inc., Cary, NC, (Littell et al., 1996) for repeated measures analysis was used to analyze the effects of dry period lengths and rations on inflammatory biomarkers and oxidative stress (model 1). Fixed effects in model 1 were dry period length (0, 30, 60, or 0 → 30 d), ration (glucogenic or lipogenic), parity (3, 4 or > 4), week relative to calving (-3, -2, -1, 1, 2 and 4), and their 2-way interactions. Preliminary result showed that

the levels of bilirubin for all samples was relatively low compared earlier studies (Bionaz et al., 2007, Bertoni et al., 2008, Trevisi et al., 2012a). The animal experiment was performed from 2010 until 2013, and the prolonged storage condition of the plasma samples may have affected the level of bilirubin. However, in the current study the dynamic of bilirubin levels by week around calving followed the typical pattern of change observed in the peripartum period in earlier studies (Ametaj et al., 2005, Bionaz et al., 2007, Trevisi et al., 2012a). All the plasma parameters have been setted with calibration curves, and data were standardized using quality control standards (internal and purchased by Instrumentation Laboratory, Lexington, MA). Liver functionality index was calculated according to Trevisi et al. (2012). Albumin, cholesterol, and bilirubin data were used to calculate the LFI. Statistical analysis for LFI was performed in the first 4 weeks after calving with model 1.

The MIXED procedure of SAS was used to analyze the relation of uterine health status (**UHS**) with inflammatory biomarkers and oxidative stress. Cows were classified as having a healthy uterine environment (**HU**, VDS = 0 or 1 in both week 2 and 3), non-recovering uterine environment (**NRU**, VDS = 2 or 3 in week 3) or a recovering uterine environment (**RU**, VDS = 2 or 3 in week 2 and VDS = 0 or 1 in week 3). To analyze the relation between inflammatory biomarkers and oxidative stress with UHS, the MIXED procedure of SAS was used (model 2). Fixed effects in model 2 were uterine health status (HU, NRU or RU), dry period lengths, ration, parity, week, and their 2-way interactions in separate analyses one by one. In model 1 and 2, cow was considered as the repeated subject. Preliminary analysis showed that number of cows per category of UHS × dry period length was small (range 1 to 15), thus this interaction was not included in model 2. The [**AR (1)**] covariate structure was the best fit, and was used to account for within-cow variation. For comparison of dry period length effects *P*-values are presented after a Tukey-Kramer adjustment. Values are presented as least squares means (**LSM**) with their pooled standard errors of the mean (**SEM**), unless otherwise stated.

3.4   Results

3.4.1   Effects of Dry Period Length and Ration on Inflammatory Biomarkers, Liver Functionality and Oxidative Stress in Plasma of Cows

Cows with a 0-d dry period had higher levels of ceruloplasmin in plasma compared with cows with a 0→30-d, 30-d or 60-d dry period from 3 weeks before calving until 4 weeks after calving (Table 3.1). Cows with a 0-d dry period had higher levels of ROM in plasma compared with cows with a 30-d or 60-d dry period from 3 weeks before calving until 4 weeks after calving, but not compared with cows with a 0→30-d dry period. Plasma bilirubin levels did not differ among cows with a 0-d, 30-d or 60-d dry period. Cows with a 0→30-d dry period had greater bilirubin levels in plasma compared with cows with a 0-d, 30-d or 60-d dry period especially in the first 2 weeks after calving. Cows with a 0-d dry period had a higher LFI compared with cows with a 30-d, 0→30-d and 60-d dry period in the first 4 weeks after calving. Cows with a 0→30-d dry period had higher creatinine levels in plasma compared with cows with a 60-d dry period, but not compared with cows a 0-d and 30-d dry period. Dry period length had no effect on levels of haptoglobin or cholesterol in plasma. Rations had no effect on levels of bilirubin or paraoxonase in plasma. Cows fed a lipogenic ration had higher cholesterol levels in plasma and higher LFI compared with cows fed a glucogenic ration. There was an interaction between dry period × ration for FRAP and creatinine. Cows with a 0-d dry period fed a lipogenic ration had higher FRAP and creatinine levels in plasma compared with cows with a 0-d dry period and fed a glucogenic ration. There was an interaction between dry period × parity for albumin. Cows with 0→30 d dry period had higher albumin levels in plasma in cows with parity 4 compared with cows with parity 3. Contrary, cows with a 0-d, a 30-d or a 60-d dry period had lower albumin levels in plasma in cows with parity ≥ 4 compared with cows with parity 3.

3.4.2   Relationships of Uterine Health Status with Inflammatory Biomarkers and Oxidative Stress

Cows with NRU had higher haptoglobin levels in plasma compared with cows with HU, but not compared with cows with RU, independent of dry period length (Table 3.2). Cows with NRU had lower paraoxonase levels in plasma and

LFI compared with cows with HU or RU, independent of dry period length. In addition, cows with NRU had lower albumin and creatinine levels in plasma compared with cows with a RU, but not compared with cows with HU. There was an interaction between UHS × ration and dry period length × parity for plasma albumin levels (Figure 3.1). Cows with RU and fed a lipogenic ration had higher levels of albumin in plasma compared with cows with NRU and fed a lipogenic ration. Cows with a 30-d dry period had higher levels of albumin in plasma in cows with parity 3 compared with cows with parity > 4. There was an interaction between UHS × week for haptoglobin (Figure 3.2). In addition, cows with NRU had higher levels of haptoglobin, lower albumin, paraoxonase and creatinine in plasma compared with cows with UH or RU especially in the first 4 weeks after calving.

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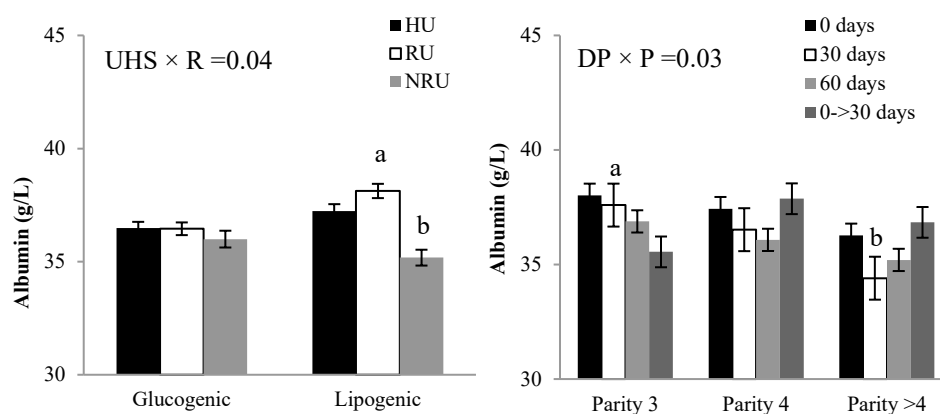
Figure 3.1 Interaction between ration (glucogenic vs. lipogenic) and uterine health status (UHS; a healthy uterine environment (HU), a recovering uterine environment (RU) and a non-recovering uterine environment (NRU)) and interaction between dry period length (0, 30, 0→30 or 60 d dry period) × parity (3, 4 or > 4) for albumin (Mean ± SEM). Bar with different superscripts within dry period length (DP), ration (R), or parity (P) class differ ($P < 0.05$).
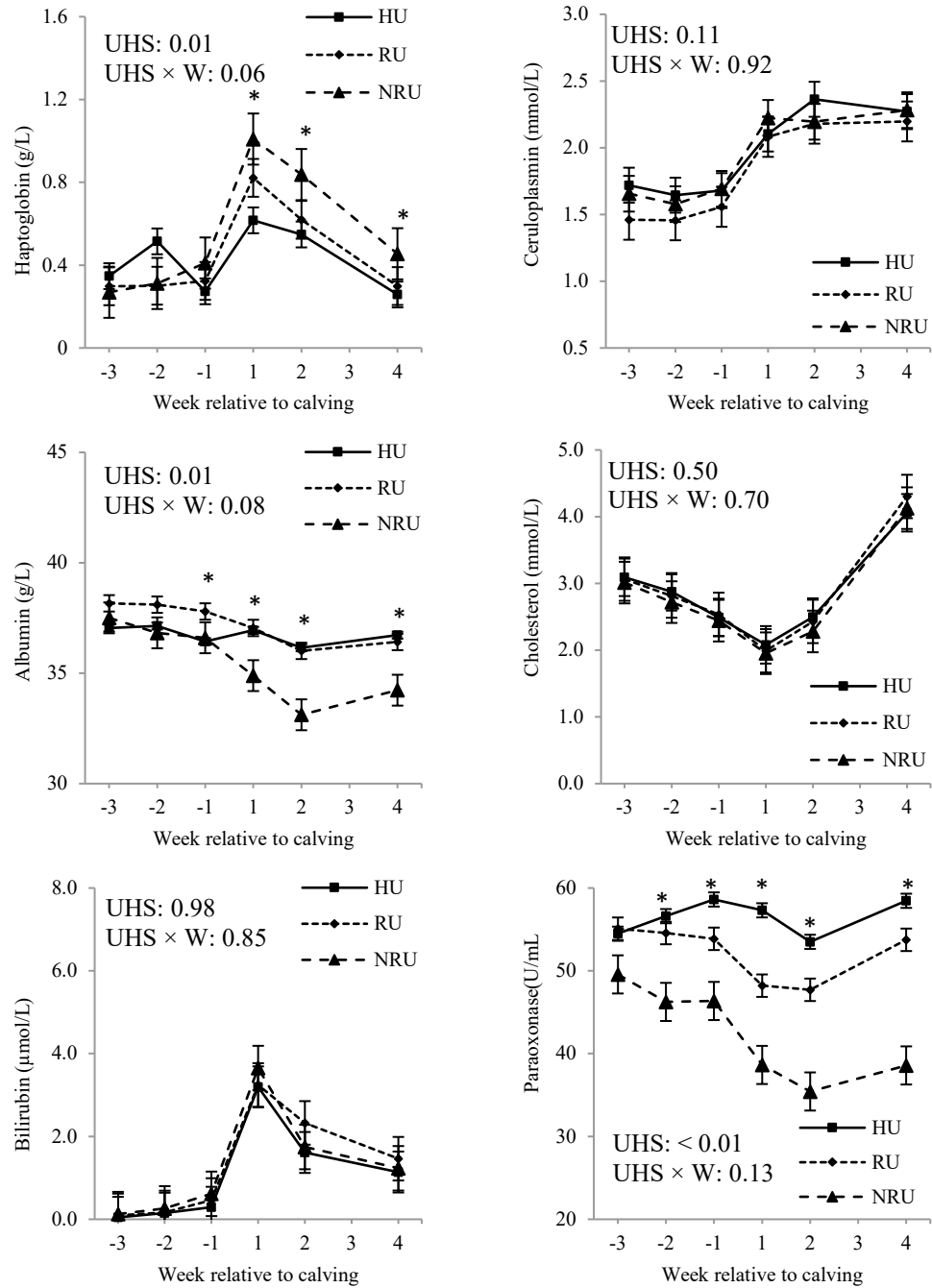
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Figure 3.2 Inflammatory biomarkers, oxidative stress (reactive oxidative metabolites (ROM) and ferric reducing antioxidant power (FRAP)) and creatinine in plasma per week per 3 group of uterine health status (UHS); a healthy uterine environment (HU; vaginal discharge score (VDS: 0 or 1 in week 2 and score 0 or 1 in week 3 (N = 17), a recovering uterine environment (RU; VDS: 2 or 3 in week 2 and score 0 or 1 in week 3 (N = 31), and a non-recovering uterine environment (NRU; VDS: 2 or 3 in week 3 (N = 43)) (Mean ± SEM). Significant UHS group differences within a week are indicated by * (*P* < 0.05).

Table 3.1 Inflammatory biomarkers (haptoglobin, ceruloplasmin, albumin and cholesterol), index of liver function (paraoxonase and bilirubin), oxidative stress (reactive oxidative metabolites (ROM) and ferric reducing antioxidant power (FRAP)), and creatinine in plasma of dairy cows with different dry period lengths (0, 30, 60, or 0→30[1] d) and fed either a glucogenic (G) or a lipogenic (L) ration (LSM ± SEM).

Item	Dry period length (d)					Ration			P-values[2]							
	0	30	60	0→30[1]	SEM	G	L	SEM	D	R	P	W[3]	D×R	D×P	D×W	P×R
Cows (n=110)	15	32	44	19		56	54									
Haptoglobin (g/L)	0.47[a]	0.45	0.46	0.51	0.04	0.49	0.45	0.03	0.65	0.45	0.76	<0.01	0.58	0.65	0.12	0.94
Ceruloplasmin (µmol/L)	2.01[a]	1.75[b]	1.80[b]	1.81[b]	0.09	1.89	1.79	0.06	0.04	0.45	0.15	<0.01	0.42	0.96	0.11	0.30
Albumin (g/L)	37.37	36.12	36.09	36.86	0.53	36.21	37.01	0.37	0.20	0.21	0.08	<0.01	0.34	0.04	0.34	0.19
Cholesterol (mmol/L)	3.02	2.93	2.65	2.65	0.11	2.75	2.88	0.07	0.13	0.04	<0.01	<0.01	0.71	0.65	0.34	0.26
Bilirubin (µmol/L)	0.94[a]	1.10[a]	1.14[a]	1.92[b]	0.15	1.22	1.33	0.08	<0.01	0.22	0.74	<0.01	0.52	0.99	<0.01	0.21
Paraoxonase, U/mL	46.54	48.05	47.00	51.68	2.76	47.48	49.16	1.94	0.50	0.36	0.08	<0.01	0.08	0.75	0.01	0.64
ROM[4] (mg H_2O_2/100mL)	16.17[a]	14.84[b]	14.79[b]	15.24[ab]	0.39	15.52	15.01	0.27	0.01	0.20	0.07	<0.01	0.74	0.46	0.04	0.24
FRAP[4] (µmol/L)	156.44	147.76	151.51	148.34	3.15	152.90	149.12	2.39	0.29	0.06	<0.01	<0.01	0.03	0.14	0.62	0.07
Creatinine (µmol/L)	106.21[ab]	103.92[ab]	98.54[a]	109.83[b]	2.13	104.02	105.23	1.39	<0.01	0.61	0.04	0.04	<0.01	0.55	0.32	0.44
LFI[4]	1.41[a]	0.38[b]	0.55[b]	-0.05[b]	0.21	-0.22	1.37	0.15	<0.01	<0.01	<0.01	NA	<0.01	<0.01	NA	0.02

[a,b] Values within dry period length in the same row with different superscripts differ ($P < 0.05$). [1] In the second subsequent lactation, 19 cows which were planned to have a 0-d dry period, had a milk production of < 4 kg/d for at least 30 d before the expected calving date and were allowed to go dry; [2] D = dry period; R = ration; P = parity; [3] Weeks -3, -2, -1, 1, 2 and 4 relative to calving; [4] ROM = Reactive oxygen metabolites; FRAP = Ferric-reducing antioxidant power; LFI = Liver functionality

Table 3.2 Inflammatory biomarkers (haptoglobin, ceruloplasmin, albumin and cholesterol), index of liver function (paraoxonase and b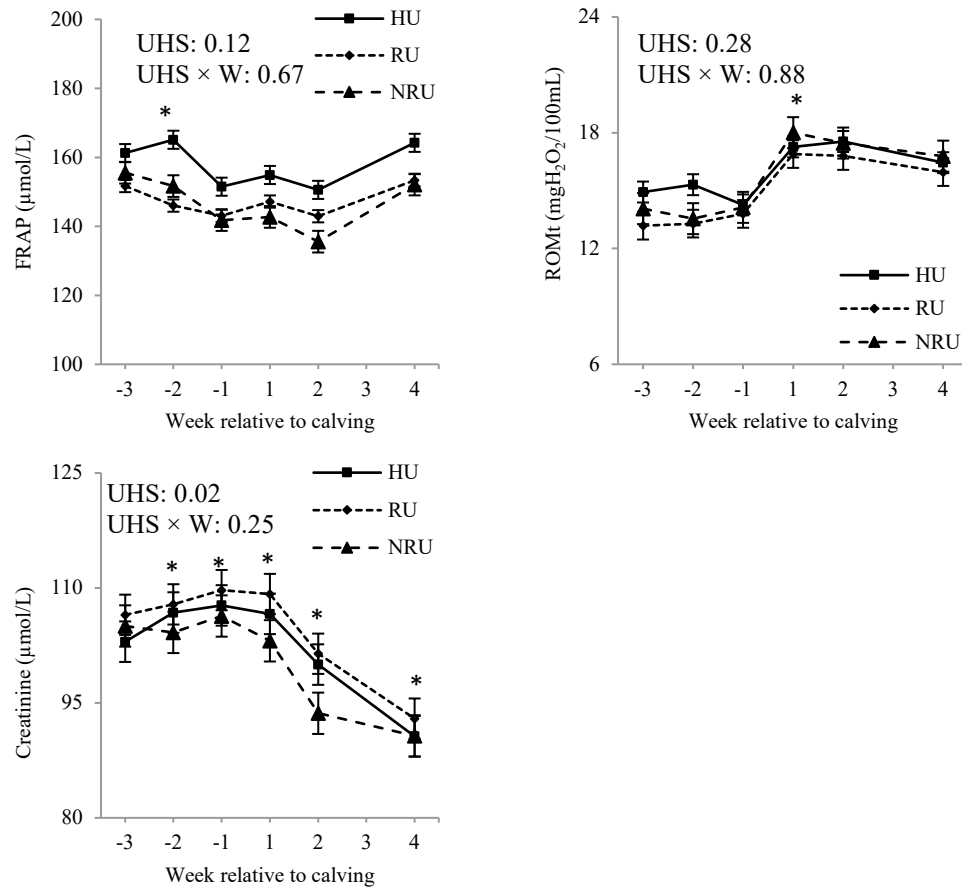ilirubin), oxidative stress (reactive oxidative metabolites (ROM) and ferric reducing antioxidant power (FRAP)), and creatinine in plasma of dairy cows with varying dry period lengths (0, 30, 60, or 0→30¹ d) and fed either a glucogenic (G) or a lipogenic (L) ration categorized according to uterine health status (UHS) (LSM ± pooled SEM).

Item	UHS²			SEM	Dry period length (d)				SEM	P-values²				
	HU	RU	NRU		0	30	60	0→30¹		UHS	D	R	P	W
Cows (n=91)	17	31	43		12	29	36	14						
Haptoglobin (g/L)	0.41[b]	0.44[ab]	0.56[a]	0.04	0.48	0.46	0.47	0.46	0.05	0.01	0.99	0.78	0.84	<0.01
Ceruloplasmin (μmol/L)	2.00	1.86	1.97	0.09	2.09	1.83	1.95	1.91	0.10	0.39	0.38	0.85	0.28	<0.01
Albumin (g/L)	37.22[ab]	37.28[b]	35.62[a]	0.54	36.44	36.70	36.39	37.30	0.62	0.01	0.65	0.88	0.26	<0.01
Cholesterol (mmol/L)	2.90	2.97	2.78	0.15	3.25	2.86	2.75	2.68	0.17	0.50	0.22	0.37	<0.01	<0.01
Bilirubin (μmol/L)	1.22	1.25	1.27	0.15	0.73[a]	1.22[a]	1.17[a]	1.86[b]	0.18	0.98	<0.01	0.39	0.35	<0.01
Paraoxonase, U/mL	59.83[a]	52.40[b]	43.30[b]	2.73	50.38	51.92	48.24	56.83	3.14	<0.01	0.15	0.06	0.12	<0.01
ROM³ (mg H₂O₂/100mL)	15.69	15.22	15.85	0.36	16.70	15.13	15.27	15.25	0.41	0.28	0.13	0.53	0.03	<0.01
FRAP³ (μmol/L)	157.35	148.03	145.87	3.49	152.55	147.75	152.86	148.51	4.01	0.09	0.57	0.34	<0.01	<0.01
Creatinine (μmol/L)	104.40[ab]	106.84[b]	100.45[a]	1.95	101.49[ab]	105.57[a]	98.80[b]	109.73[a]	2.25	0.02	<0.01	0.53	0.52	<0.01
LFI²	1.19[a]	1.32[a]	-0.34[b]	0.22	1.91[a]	0.36[b]	0.58[b]	0.04[b]	0.26	<0.01	<0.01	<0.01	<0.01	NA

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Table 3.2 continued

Item	P-values[2]			
	D × P	D × W	P × R	UHS × R
Cows (n=91)				
Haptoglobin (g/L)	0.66	0.35	0.93	0.93
Ceruloplasmin (μmol/L)	0.31	0.03	0.43	0.43
Albumin (g/L)	0.62	0.48	0.02	0.02
Cholesterol (mmol/L)	0.83	0.73	0.89	0.89
Bilirubin (μmol/L)	0.71	<0.01	0.08	0.08
Paraoxonase,U/mL	0.26	0.49	0.73	0.73
ROM[3] (mg H_2O_2/100mL)	0.69	0.08	0.57	0.57
FRAP[3] (μmol/L)	0.16	0.58	0.41	0.41
Creatinine (μmol/L)	0.60	0.31	0.02	0.02
LFI[2]	<0.01	NA	<0.01	<0.01

[a,b] Values within dry period length in the same row with different superscripts differ ($P < 0.05$); [1] In the second subsequent lactation, 19 cows which were planned to have a 0-d dry period, had a milk production of < 4 kg/d for at least 30 d before the expected calving date and were allowed to go dry; [2] D = dry period; R = ration; P = parity; LFI; liver functionality index; UHS=uterine health status; HU= healthy uterine environment: vaginal discharge score (VDS) = 0 or 1 in both week 2 and 3; RU= a recovering uterine environment: VDS = 2 or 3 in week 2 and score = 0 or 1 in week 3; NRU= non-recovering uterine environment: VDS: 2 or 3 in week 3; [3]ROM = Reactive oxygen metabolites; FRAP = Ferric-reducing antioxidant power; [4] D × R, UHS × W and UHS × P are included in the model, but not significant. UHS × D are excluded from the model because of fewer animals in the group.

3.5   Discussion

In the current study, independent of dry period length or ration, cows with NRU had higher haptoglobin levels in plasma compared with cows with HU, but not compared with cows with RU. Cows with NRU had lower paraoxonase levels in plasma and LFI compared with cows with HU or RU, independent of dry period length. In addition, cows with NRU had lower albumin levels in plasma compared with cows with a RU, but not compared with cows with HU. Our finding is in line with a previous study where cows with an uterine infection had lower albumin, lower paraoxonase and higher haptoglobin levels in plasma compared with cows with healthy uterus (Schneider et al., 2013). Cows with retained placenta and endometriosis had lower levels of plasma albumin as negative APP (Fleck, 1989) compared with healthy cows (Green et al., 2009, Burke et al., 2010). Paraoxonase is mainly synthesized in the liver, considered as negative APP and a marker for liver functionality (Feingold et al., 1998). Low plasma paraoxonase level was associated with increased occurrence of metritis in early lactation (Bionaz et al., 2007). Previous studies showed that cows with uterine infection had high haptoglobin levels in plasma (Chan et al., 2010, Hassanpour et al., 2011, Pohl et al., 2015). Plasma haptoglobin increased in cows with retained fetal membranes (Mordak, 2009). Retained fetal membranes is a key risk factor for uterine infection (Mordak, 2009). Total bilirubin, albumin and vitamin A have been proposed as possible indicators for retained fetal membranes (Trevisi et al., 2008). In the current study, we found no evidence that haptoglobin is an indicator or a consequence of uterine infection. Recently, the level of haptoglobin in plasma has been considered as biomarker to predict uterine infection in early lactation (Williams et al., 2005, Huzzey et al., 2009). In the current study, levels of haptoglobin in plasma were associated with uterine health.

In the present study the increase of ceruloplasmin and ROM was affected by both dry period length and uterine health status. In this study, levels of ceruloplasmin were positively associated with level of ROM in plasma ($r = 0.83$, $P < 0.01$), independent of dry period length. In addition, levels of ROM were negatively associated with level of paraoxonase in plasma ($r = -0.19$, $P < 0.01$). Cows with acute puerperal metritis had higher oxidative stress, indicated by high malondialdehyde concentrations compared with healthy cows (Kizil et al., 2010, Magata et al., 2016). Increased plasma malondialdehyde concentration which is a thiobarbituric acid reactive species, indicate oxidative status (Bernabucci et al., 2005). Cows with endometritis had higher ceruloplasmin levels in plasma as

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positive APP compared with healthy cows (Kaya et al., 2016). During inflammation, copper binding ceruloplasmin mediated formation of reactive oxygen species and may promote oxidative pathology (Shukla et al., 2006). This study suggests that ROM is positively related with ceruloplasmin.

In the current study, cows with a 0-d dry period had higher ceruloplasmin and higher ROM levels in plasma compared with cows with a 60-d dry period, independent of UHS. In our earlier study, high levels of ceruloplasmin and ROM in plasma of cows with a 0-d dry period could partly be explained by the occurrence of clinical health problems related to inflammation such as clinical mastitis, metritis, retained placenta and fever (Mayasari et al., 2017). Earlier in this experiment, dry period length did not affect UHS (Chen et al., submitted). Albeit, VD is related with inflammation in the uterus (Ametaj et al., 2005, Bionaz et al., 2007, Trevisi et al., 2012) and might result in changes of inflammatory biomarkers and oxidative stress variables, VD was not affected by dry period length treatment in the current study. This result indicated that high levels of ceruloplasmin and ROM in plasma of cows with 0-d dry period could not be explained by UHS but may be caused by other specific diseases related with inflammation in these cows.

Plasma bilirubin levels did not differ among dry period lengths (0-d vs. 30-d or 60-d dry period). Cows with $0\rightarrow30$–d dry period had higher plasma bilirubin levels compared with cows with 0-d, 30-d or 60-d dry period, independent of UHS. Cows with a $0\rightarrow30$–d dry period had a more severe NEB compared with cows with a 0-d or 30-d dry period (Chen et al., 2016). Moreover, cows with 0-d dry period had a less pronounced improvement of EB and metabolic status in early lactation in the current study, compared with the first lactation after implementation of a short or no dry period strategy (Chen et al., 2016). In the first subsequent lactation, a better EB was related with low plasma bilirubin levels (Mayasari et al., 2017). The limited differences in EB among dry period groups in the second lactation may explain the lack of effect of dry period length on bilirubin in the second lactation, compared with the first lactation after implementation of dry period length treatments.

In the present study, cows with NRU had lower creatinine levels compared with cows with RU, but not compared with HU. In contrast with earlier studies that cows with endometritis had higher creatinine in plasma compared with cows with a healthy uterus (Sattler and Fürll, 2004, Kaya et al., 2016). In our study, cows with a $0\rightarrow30$–d dry period had higher creatinine levels in plasma compared with cows with a 60-d dry period, independent of UHS. A previous study showed that cows

with high BCS group (BCS ≥ 3.75) had higher plasma creatinine and severe NEB compared with cows with low BCS group (BCS ≤ 2.5) (Pires et al., 2013). For this experiment, cows with a 0→30–d dry period had higher BCS (4.3 ± 0.3) compared with cows with a 30-d (3.4 ± 0.2) or 60-d (3.2 ± 0.1) dry period during 3 weeks before calving. In addition, cows with a 0→30–d dry period had a more severe NEB compared with cows with a 0-d or 30-d dry period (Chen et al., 2016). This result indicated that high plasma creatinine levels in cows with a 0→30–d dry period was related to high precalving BCS and a more severe NEB in these cows, but could not be explained by uterine health status in these cows.

In the current study, cows fed a lipogenic ration had increased cholesterol levels in plasma compared with cows fed a glucogenic ration, independent of UHS or dry period length. In addition, cows with RU and fed a lipogenic ration had higher levels of albumin in plasma compared with cows with NRU and fed a lipogenic ration. A previous study showed that cows with a dietary fat supplementation of 5 percent in ration showed increased plasma cholesterol levels in plasma compared with cows not receiving fat supplementation (Carroll et al., 1990). Cholesterol is known as negative APP (i.e. apolipoproteins) and also a precursor for steroid hormone synthesis and it may increase in plasma by feeding supplemental fat to dairy cows (Grummer and Carroll, 1991). Albumin is not only associated with fat infiltration in the liver, but also as risk for uterine diseases (Bobe et al., 2004), and an important protein to transport long-chain fatty acids from adipocyte triacylglycerol stores to muscle tissue (Spector, 1986). Therefore, it is likely that high albumin levels in plasma in cows with RU and fed a lipogenic ration may be due to intense transport of fat in these cows, compared with cows with NRU. In any case, the higher concentration of albumin in plasma during the peripartum reflects a less inflammation of the cows.

As we suggested earlier, the increased positive APP (ceruloplasmin) and ROM in plasma in early lactation could partly be explained by many factors such as dry period length, ration and occurrence of clinical health problem related inflammation. In the current study, increased haptoglobin and reduced paraoxonase and albumin were related with uterine infection, independent of dry period length. This suggests that the inflammatory status attributed to NEB should be disentangled in various concurrently operating inflammatory responses. This implies that uterine health status that considered as one of clinical health problem related with inflammation may explain the changes in inflammatory biomarkers.

3.6   Conclusions

Cows with a NRU had higher levels of haptogloblin and lower paraoxonase in plasma compared with cows with a HU, independent of dry period length. Cows with a RU had higher levels of albumin and creatinine in plasma in the first 4 weeks after calving and a higher liver functionality index compared with cows with a NRU. Cows fed lipogenic had higher levels of albumin in plasma in the cows with RU compared with cows with NRU. This study shows that uterine health is associated with variables of inflammation (haptoglobin and albumin) and paraoxonase, independent of dry period length. Cows with 0→30-d dry period had high plasma bilirubin, which is related with severe NEB in the early lactation in comparison to other groups. In addition, omission of the dry period is associated with the high positive APP and oxidative stress that could partly be explained by poor uterine health status indicated by vaginal discharge the first 3 weeks after calving.

3.7   Acknowledgements

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CHAPTER 4

The effects of dry period length and dietary energy source on natural antibody titers and mammary health in dairy cows

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4.1 Abstract

In earlier studies, natural antibodies (**NAb**) were related not only to the energy balance (**EB**) of dairy cows, but also to somatic cell count (**SCC**) and clinical mastitis. The first objective of our study was to evaluate the effects of dry period length and dietary energy source on titers of NAb binding keyhole limpet hemocyanin (**KLH**) and lipopolysaccharide (**LPS**) in plasma and milk, SCC and clinical mastitis occurrence in dairy cows in 2 subsequent lactations. Our second objective was to study the relationship between NAb levels and mammary health. Holstein-Friesian dairy cows (N = 167) were randomly assigned to 3 dry period lengths (0, 30 or 60 d) and 2 early lactation rations (glucogenic or lipogenic). Treatments were repeated during 2 subsequent lactations. In the second lactation after implementation of dry period length treatments, 19 cows which were planned to have 0-d dry period dried off naturally and were assigned to an additional group 0→30-d dry period. In the first lactation after implementation of dry period length treatments, cows with a 0-d dry period had a higher SCC, a higher titer of immunoglobulin G (IgG) binding LPS in plasma, and higher titers of IgG and IgM binding KLH and LPS in milk compared with cows with a 30-d or 60-d dry period. In the second lactation after implementation of dry period length treatments, cows with a 60-d dry period had a lower SCC than cows with a 30-d and 0→30-d dry periods. In the second lactation after implementation of dry period length treatments, dry period length did not affect NAb titers in plasma or milk. The clinical mastitis occurrence was 17 percent in the first lactation and 25 percent in the second lactation, and did not differ according to dry period lengths or rations. For both lactations, an increasing titer of IgG binding LPS in plasma was associated with decreased odds of a high SCC and decreased odds of clinical mastitis occurrence. Also up to 3 weeks before the clinical mastitis occurrence, an increasing titer of IgM binding KLH and LPS in plasma was associated with a decreased odds of clinical mastitis occurrence. In conclusion, omitting the dry period increased SCC, NAb titers in milk and IgG binding LPS in plasma compared with a short (30-d) or conventional (60-d) dry period. The effects on NAb titers, however, were only present in the first lactation after omitting the dry period and disappeared after repeated omitting the dry period. Moreover, an increasing titer of IgG binding LPS in plasma was associated with decreased odds of high SCC and clinical mastitis occurrence.

Key words: continuous milking, nutrition, somatic cell count, clinical mastitis.

4.2   Introduction

Natural antibodies (**NAb**) are a humoral part of innate immunity (Matter and Ochsenbein, 2008, Vollmers and Brändlein, 2009) and are present in healthy individuals without apparent antigenic stimulation (Ochsenbein and Zinkernagel, 2000). Natural antibodies are polyreactive and have a low affinity binding with various antigens (Casali and Notkins, 1989). In human and mice, NAb are produced by CD5^{+} B-1 cells, and mainly consist of immunoglobulin M (**IgM**), and to a lesser extent immunoglobulin G (**IgG**) and immunoglobulin A (**IgA**) (Kohler et al., 2003, Avrameas, 2006). Natural antibodies act as a first line of defense against infection through neutralization, complement system activation and pathogen elimination (Ochsenbein et al., 1999, Zinkernagel, 2012). For dairy cows, NAb titers are present in both plasma and milk (Van Knegsel et al., 2007, Ploegaert, 2010). In previous research, we reported higher titers of NAb binding keyhole limpet hemocyanin (**KLH**) in plasma for cows with an improved energy balance (**EB**) in early lactation (Van Knegsel et al., 2007). In this earlier work, the EB was improved by feeding a more glucogenic ration, compared with a lipogenic ration. In other studies, also shortening or omitting the dry period has been shown to improve EB of dairy cows in the subsequent lactation (Rastani et al. 2005, Van Knegsel et al. 2014). Hence, it can be hypothesized that shortening or omitting the dry period improves the EB in dairy cows in early lactation, also NAb titers in plasma increase.

Earlier studies showed variable effects of dry period length on mammary health (Annen et al., 2004, Watters et al., 2008, Köpf et al., 2014). A shortened dry period (34-40 d) did not result in differences in somatic cell count (**SCC**) (Watters et al., 2008, Bernier-Dodier et al., 2011, Shoshani et al., 2014) or clinical mastitis (Enevoldsen and Sørensen, 1992, Watters et al., 2008, Shoshani et al., 2014), compared with a conventional dry period (55 to 65 d). From some studies (Annen et al., 2004, Klusmeyer et al., 2009) it was concluded that omitting the dry period resulted in higher SCC values compared with cows with a 56-d dry period, but in other studies (Rastani et al., 2005, Köpf et al., 2014) no effect of omitting the dry period on SCC was present. No dry period lowered the odds of new infections during the dry period, but it has been hypothesized that no dry period also imposes the odds of an increased SCC because cows cannot be treated with long-acting intramammary antibiotics (Annen et al., 2004). When the dry period is reduced from 56 d to 0 d, Rastani et al. (2005) reported an increase in clinical mastitis occurrence (9.5 percent vs. 14.3 percent), whereas

Schlamberger et al. (2010) reported a decrease in clinical mastitis occurrence (41.7 percent vs. 25.0 percent). Both studies, however, had a limited number of animal (44 and 36 cows, respectively), which justifies evaluation of effects of dry period omission on clinical mastitis occurrence in a larger study.

It has been suggested that NAb play a role in the prevention of clinical mastitis or lead to a high SCC depending on the NAb isotype and antigen-specificity (Ploegaert, 2010). Thompson-Crispi et al. (2013) showed that an increasing titer of IgM binding KLH in plasma was associated with decreased odds of clinical mastitis occurrence (unadjusted odds ratio of 0.96), while no relationship was found with levels of IgG binding KLH. In the study of Thompson-Crispi et al. (2013), NAb titers were related to the clinical mastitis occurrence at anytime during the study period. We hypothesized that if NAb play a role in the development or prevention of clinical mastitis occurrence, alterations in NAb titers are already detectable days or weeks prior to the clinical mastitis occurrence. This hypothesis was tested for NAb in milk against auto-antigens (Van Knegsel et al., 2012). In this study, a higher NAb binding transferrin tended to have an association with decreased odds of clinical mastitis occurrence in the week of clinical mastitis diagnosis but not earlier.

The first objective of this study was to evaluate the effects of dry period length and dietary energy source on titers of NAb-binding KLH and LPS in plasma and milk, SCC in milk, and clinical mastitis occurrence in dairy cows in 2 subsequent lactations. The second objective was to study the relationship between NAb and mammary health as characterized by a high SCC and clinical mastitis occurrence.

4.3   Materials and Methods

4.3.1 Experimental Design and Animals

The Institutional Animal Care and Use Committee of Wageningen University approved the experimental protocol. The registration number of the experimental protocol is 2010026. The experimental design, dry period lengths and dietary contrast were described by Van Knegsel et al. (2014). In summary, 167 Holstein-Friesian dairy cows were selected from the Dairy Campus research herd (WUR Livestock Research, Lelystad, the Netherlands), blocked according to parity, calving date, milk yield in previous lactation, and body condition score (**BCS**), and randomly assigned to treatments within blocks. In total, 28 blocks were

used and each block consisted of 6 cows, 1 cow for each treatment (28 blocks × 6 animals = 168 cows, 1 cow was excluded because she received the wrong diet at drying off). Only cows were selected which had SCC < 250,000 cells/mL at the last and second last monthly test day recordings. Treatments consisted of 3 dry period lengths (0, 30 or 60 d) and 2 early lactation rations (glucogenic or lipogenic) resulting in a 3 × 2 factorial design. The same treatments were imposed on cows for 2 subsequent lactations. Cows used in this study were clinically healthy. Cows were housed in a free stall with slatted floor and cubicles, and milked twice daily (at 0500 and 1630 h). The drying-off protocol for both lactations was as follows: cows with a 30-d or a 60-d dry period received a far-off ration 7 d before drying-off, were milked once daily 4 d before drying-off, and all were treated with an intramammary antibiotic treatment at drying off (Supermastidol, Virbac Animal Health, Barneveld, the Netherlands). This treatment aims to prevent clinical mastitis occurrence during or after the dry period. In the first lactation after implementation of dry period length treatments, all cows which were planned to have a 0-d dry period were milked till calving. In the second lactation after implementation of dry period length treatments, 19 cows of the 0-d dry period group had a milk yield of less than 4 kg/d for at least 30 d before the expected calving date and were assigned to the 0→ 30-d dry period group. These cows were allowed to go dry without use of antibiotics, and received a dry cow ration during the dry period.

The first lactation started with 167 Holstein-Friesian dairy cows (60 primiparous and 107 multiparous cows). The second lactation started with 130 cows, 37 cows had been removed from the study because 18 cows did not become pregnant (8 cows with a 0-d dry period, 8 cows with a 30-d dry period and 2 cows with a 60-d dry period), 8 cows had leg problems (3 cows with a 0-d dry period, 3 cows with a 30-d dry period and 2 cows with 60-d dry period), 3 cows with 0-d dry period had udder problems, 8 cows had been removed for other reasons such as abortion and pneumonia (3 cows with 0-d dry period, 3 cows with 30-d dry period, 2 cows with 60-d dry period).

4.3.2 Rations

Ration composition was described earlier (Van Knegsel et al., 2014). Prepartum, dry cows received a dry cow ration, and lactating cows received a lactating cow ration supporting 25 kg of milk yield per day. From 10 d before expected calving onwards, cows of all treatments were fed 1 kg/d glucogenic or

lipogenic concentrate, which was increased postcalving in steps of 0.5 kg/d until the concentration supply reached 8.5 kg/d. The main ingredient for the glucogenic concentrate was corn, the main ingredients for the lipogenic concentrate were sugar beet pulp, palm kernel, and rumen protected palm oil. Forage composition did not differ among dry cow rations, was supplied ad libitum, and consisted of grass silage, corn silage, wheat straw, and a protein source (rapeseed meal or soybean meal) in the ratio of 39:25:25:11 (DM basis). Postpartum, forage consisted of grass silage, corn silage, straw, and a protein source (rapeseed meal or soybean meal) in the ratio 51:34:2:13 (DM basis). Rations were isocaloric (net energy basis: Dutch net energy evaluation (VEM) system, Van Es, 1975), and contained equal amounts of intestinal digestible protein and degraded protein balance (DVE/OEB system, Tamminga et al., 1994).

4.3.3 Milk and Plasma Sample Collection

Blood samples were taken weekly from week -3 till 9 relative to calving from 92 cows in the first lactation after implementation of dry period length treatments, and 114 cows in the second lactation after implementation of dry period length treatments. The number of blood samples in the first lactation is lower than in the second lactation, animal numbers were lower because 37 cows were removed from the study. Moreover, at start of second lactations some cows from 0-d dry period dried themselves off, which resulted in an extra treatment group (0→30 group). As soon as it was clear this extra treatment group was present, it was decided to take blood samples from all cows in the second lactation. This was short after the start of second lactation, resulting in a blood sample collection of 114 cows for the second lactation. In both lactations, blood samples were taken from the tail vein in Vacutainer tubes (10 mL, Greiner Bio-One GmbH, Kremsmunster, Austria) containing heparin as an anticoagulant. Blood samples were centrifuged at 3000 × g at 4°C, for 15 min, and within 2 h of collection. Plasma was harvested and stored at -20°C until analysis. Milk samples were collected from 163 cows in the first lactation and 120 cows in the second lactation. Milk samples for NAb measurement were collected weekly on Fridays. Composite milk samples for SCC determination (Qlip NV, Zutphen, the Netherlands) were collected from individual cows 4 times a week (on Tuesday afternoon, Wednesday morning, Wednesday afternoon, and Thursday morning). Milk samples (10 mL, VWR International Ltd., Leighton Buzzard, UK) were stored at -20°C until analysis. High SCC values in milk was defined as being at least 250,000 cells/mL,

and low SCC in milk as less than 250,000 cells/mL. Clinical mastitis was defined as cows with abnormalities in milk (flakes, clots or watery appearance), abnormalities associated with the udder (heat, swelling, or sensitivity to touch) and treated with intramammary antibiotic. The clinical mastitis occurrence was recorded at cow level per week.

4.3.4 Laboratory Analysis

Milk and plasma samples were thawed to room temperature before analysis. Titers of NAb binding *Megathura crenulata*-derived KLH (Sigma, H7017 Sigma Aldrich Co., St Louis, MO) and *Escherichia coli* 055:B5 LPS (Sigma) in plasma and milk of the cows were measured by an indirect enzyme-linked immunosorbent assay (ELISA) technique, as described by Van Knegsel et al. (2007). Natural antibodies in plasma and milk of IgG isotype were detected using respectively, 1:20,000 and 1:40,000, diluted sheep anti-bovine IgG-heavy chain conjugated to horseradish peroxidase (Cat. No. E10-118P, Bethyl Laboratories). Natural antibodies of the IgM isotype in plasma and milk samples were detected using diluted rabbit anti-bovine IgM-whole molecule conjugated to horseradish peroxidase (Cat. No. A10-100P, Bethyl Laboratories) in the ratio 1:20,000. Four-step serial dilutions for both IgG and IgM in plasma and milk samples started with the ratio 1:40. After washing, a substrate containing tetra methyl benzidine (Sigma) and 0.05 percent H_2O_2 was added, and incubated for 10 min at room temperature. The reaction was stopped by adding 1.25 M sulfuric acid. Extinctions were measured with a Multiskan reader (Lab Systems, Helsinki, Finland) at a wavelength of 450 nm. Titers were defined as log2 values of the dilutions that gave an extinction closest to 50 percent of Emax, where Emax represents the highest mean extinction of a standard positive (pooled) serum present on each microtiter plate (Ploegaert et al., 2007).

The increase of 1 titer of NAb implies that 1 observation increases 2^1 compared with the observations the titer is compared with. In other words, "1 titer" difference is doubling the levels of antibodies.

4.3.5 Statistical Analysis

As several successive measurements were taken per cow, observations within cows cannot be considered independent. Therefore, repeated measurement analyses were performed.

The NAb titers of IgG and IgM binding KLH or LPS in plasma or milk,

and SCC in milk, were analyzed using PROC MIXED in SAS 9.2 (SAS Inst. Inc. Cary, NC). A repeated measurement analysis with cows as the subject was used applying a first order auto-regressive [**AR (1)**] variance-covariance structure determined to be the best fit according to Akaike's corrected information criterion (Aikake, 1997). Data from the first and second lactation after implementation of dry period length treatments were analyzed separately. The fixed class effect of dry period length (0, 30, or 60 d for the first lactation; 0, 30, 60, or 0 → 30 d for the second lactation), ration (glucogenic or lipogenic), parity (2 (N = 60), 3 (N = 53) or > 3 (N = 54) for the first lactation and 3 (N = 53), 4 (N = 41) or > 4 (N = 36) for the second lactation), week (-3 to 9 weeks relative to calving for NAb titers in plasma, and 1 - 14 weeks relative to calving for NAb titers in milk), and their 2-way interactions were assessed. Cow was considered as the repeated subject (Model 1). To approximate normality, the natural logarithm of SCC values used in the analysis. All other variables approximated normality of residuals by examining whether skewness and kurtosis were in a range of -2 until 2. Multiple comparisons adjustments were performed using the Bonferroni adjustment method. Values are presented as least squares means (**LSM**) with their pooled standard errors of the mean (**SEM**), unless otherwise stated.

High SCC and clinical mastitis occurrence were analyzed in PROC GENMOD in SAS 9.2 with a binary distribution and a logit link. A repeated measurement analysis (GEE model) was used with cows as the subject applying an AR(1) variance-covariance structure determined to be the best fit according to Akaike's corrected information criterion. The same fixed effects as described for model 1 were assessed (Model 2).

To assess the association of NAb titers in milk or in plasma with the odds of a high SCC or the clinical mastitis occurrence, each individual NAb titers was assessed in separate models as a continuous fixed effect added to the fixed effects described in model 2 with all possible 2- way interactions (Model 3). Linearity to the logit of the continuous fixed effects was confirmed using the Box-Tidwell method. Preliminary analysis showed that the relationships between NAb titers and high SCC or clinical mastitis occurrence were similar for both lactations. Therefore, data in both lactations were analyzed simultaneously. The relationships between NAb titers and the odds of a high SCC or clinical mastitis occurrence, were analyzed with; NAb titers as continous variable, dry period length (0, 30, 60, or 0→30 d), ration (glucogenic or lipogenic), parity (≤ 3 or > 3), week (-3 to 9 weeks relative to calving for NAb titers in plasma; 1-14 weeks relative to calving

for NAb titers in milk), with their interactions included as fixed effects (Model 3). The AR (1) was the best fit, and was used to account for within-cow variation. A backward elimination procedure was performed using a stay-in *P*-value of < 0.05 except for dry period length that was forced in all models. Confounding was monitored as described by Santman-Berends et al. (2012). To study the relationships between NAb titers in milk or plasma with high SCC or clinical mastitis occurrence during the week of high SCC or clinical mastitis occurrence and in the 3 weeks before high SCC or clinical mastitis occurrence, a separate model (Model 4) for each week was built. Analyses showed there were no relationships between NAb titers in milk with high SCC or clinical mastitis occurrence during the week of high SCC or clinical mastitis occurrence, nor in the 3 weeks before. Therefore, these results are not shown. Results of the effect of NAb titers in plasma for high SCC or clinical mastitis occurrence are presented as the relative risk (odds ratio).

4

4.4 Results

In the first lactation after implementation of dry period length treatments, the average number of days dry were 61 ± 1, 30 ± 1, and 2 ± 1 d dry (means ± SEM) for cows with a 60-d, a 30-d or a 0-d dry period, respectively. In the second lactation after implementation of dry period length treatments, the actual number of days dry were: 64 ± 2, 40 ± 5, 0 ± 0 d dry (means ± SEM) for cows with a 60-d, a 30-d or a 0-d dry period, respectively. Cows in the 0→30-d group had an average dry period of 67 ± 8 d.

4.4.1 Effects of Dry Period Length and Ration on Natural Antibodies in Plasma (Model 1)

In the first lactation after implementation of dry period length treatments, but not in the second lactation, cows with a 0-d dry period had higher titer of IgG binding LPS in plasma compared with cows with a 60-d dry period (Table 4.1). Both in the first and second lactations after implementation of dry period length treatments, dry period length did not affect plasma NAb titers binding KLH or IgM binding LPS. In both lactations after implementation of dry period length and dietary treatments, ration did not affect NAb titers in plasma. There was an interaction between parity × ration for IgM binding KLH and LPS in the first lactation after implementation of dry period length treatments and IgG and IgM binding LPS in the second lactation (Figure 4.1). In short, older cows (parity > 3

and parity > 4 for the first and second lactations, respectively) fed the lipogenic diet had higher NAb titers in plasma, compared with older cows fed the glucogenic diet. On the contrary, younger cows (parity ≤ 3 and parity 4 for the first and second lactations, respectively) fed the lipogenic diet had lower NAb titers in plasma, compared with younger cows fed the glucogenic diet.
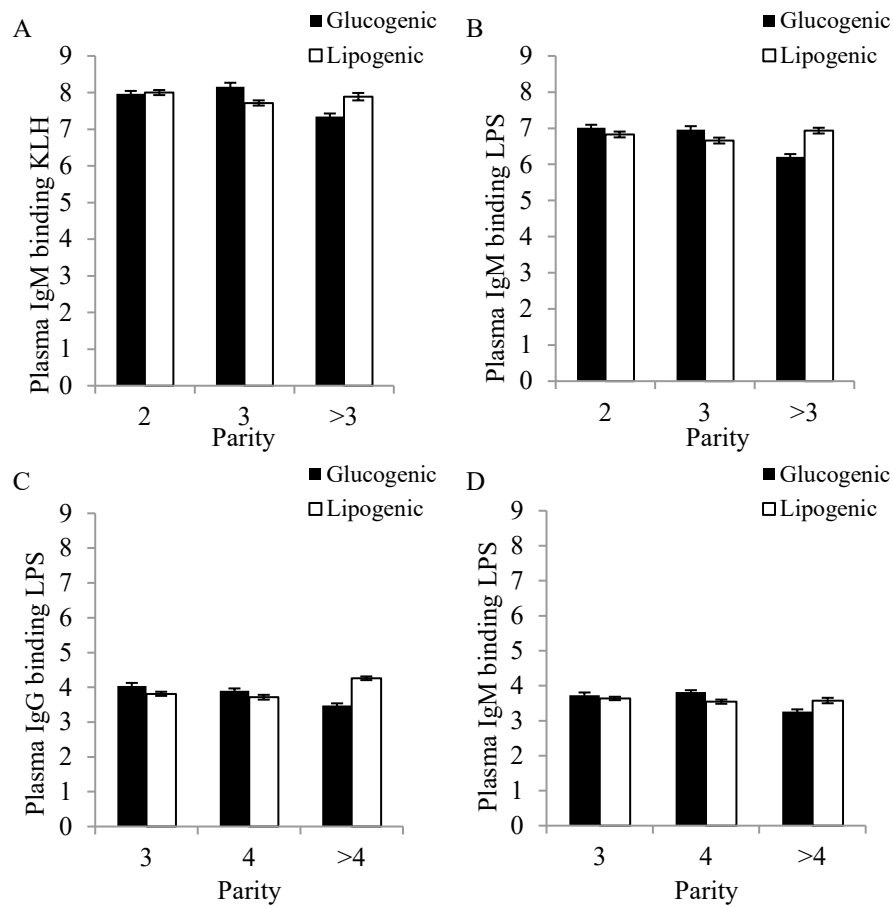
Figure 4.1 Plasma natural antibodies titers (IgG and IgM) binding keyhole limpet hemocyanin (KLH) and lipopolysaccharide (LPS) for cows from wk -3 till wk 9 relative to calving in the first lactation (A and B) and in second lactation (C and D) per ration per parity class. Values represent means per ration per parity class (± SEM).

Figure 4.2 Milk natural antibodies titers (IgG and IgM) binding keyhole limpet hemocyanin (KLH) and lipopolysaccharide (LPS) for cows from week 1 till week 14 relative to calving in the first lactation (A, B and C) and in the second lactation (D and E), and somatic cell count in the second lactation after implementation of dry period length treatments (F) per ration per parity class. Values represent means per ration per parity class (± SEM).

Table 4.1 Plasma natural antibody titers[a] (IgG and IgM) binding keyhole limpet hemocyanin (KLH) and lipopolysaccharide (LPS) of dairy cows with varying dry period lengths (0, 30 or 60 days or 0→30 group[b]) and fed either a glucogenic (G) or a lipogenic (L) ration during 2 subsequent lactations[b] (LSM ± pooled SEM)

Item	Dry period length (d)				SEM	Ration		SEM	P-values[c,d]				
	0	30	60	0→30[b]		G	L		D	R	P	W[e]	R × P
1st Lactation[b] Cows (n)	30	32	30			46	46						
NAb IgG-KLH	4.79	4.95	4.74	NA[f]	0.18	4.78	4.87	0.14	0.65	0.64	0.87	<0.01	0.16
NAb IgM-KLH	7.87	7.99	7.71	NA[f]	0.11	7.85	7.87	0.09	0.20	0.86	0.09	<0.01	0.01
NAb IgG-LPS	7.99[x]	7.98[xy]	7.41[y]	NA[f]	0.17	7.88	7.71	0.14	0.03	0.35	0.89	<0.01	0.62
NAb IgM-LPS	6.87	6.76	6.74	NA[f]	0.09	6.74	6.84	0.08	0.58	0.35	0.06	<0.01	<0.01
2nd Lactation[b] Cows (n)	17	34	44	19		58	56						
NAb IgG-KLH	4.45	4.35	4.07	4.14	0.16	4.21	4.29	0.12	0.25	0.62	0.12	<0.01	0.19
NAb IgM-KLH	5.29	5.39	5.32	5.36	0.11	5.39	5.29	0.07	0.87	0.29	0.05	0.03	0.37
NAb IgG-LPS	5.92	5.70	5.47	5.62	0.19	5.59	5.78	0.17	0.23	0.24	0.85	<0.01	0.01
NAb IgM-LPS	3.59	3.72	3.53	3.58	0.11	5.58	3.63	0.07	0.41	0.59	0.02	<0.01	0.02

[x, y, z] Values within dry period length in the same row with different superscripts differ ($P < 0.05$); [a] Natural antibody titers are log2 values of the dilutions that gave an extinction closest to 50 percent of Emax where Emax represents the highest mean extinction of a standard positive (pooled) serum present on each microtiter plate (Ploegaert et al., 2007); [b] The first and second lactation after implementation of dry period length treatments; in the second lactation, 19 cows which were planned to have a 0-d dry period, had a milk production of < 4 kg/d for at least 30 d before the expected calving date and were allowed to go dry; [c] D = dry period; R = ration; P = parity; [d] Other included interactions were D × R, D × P and D × W; [e] Weeks -3, -2..., 9 relative to calving; [f] NA= not applicable.

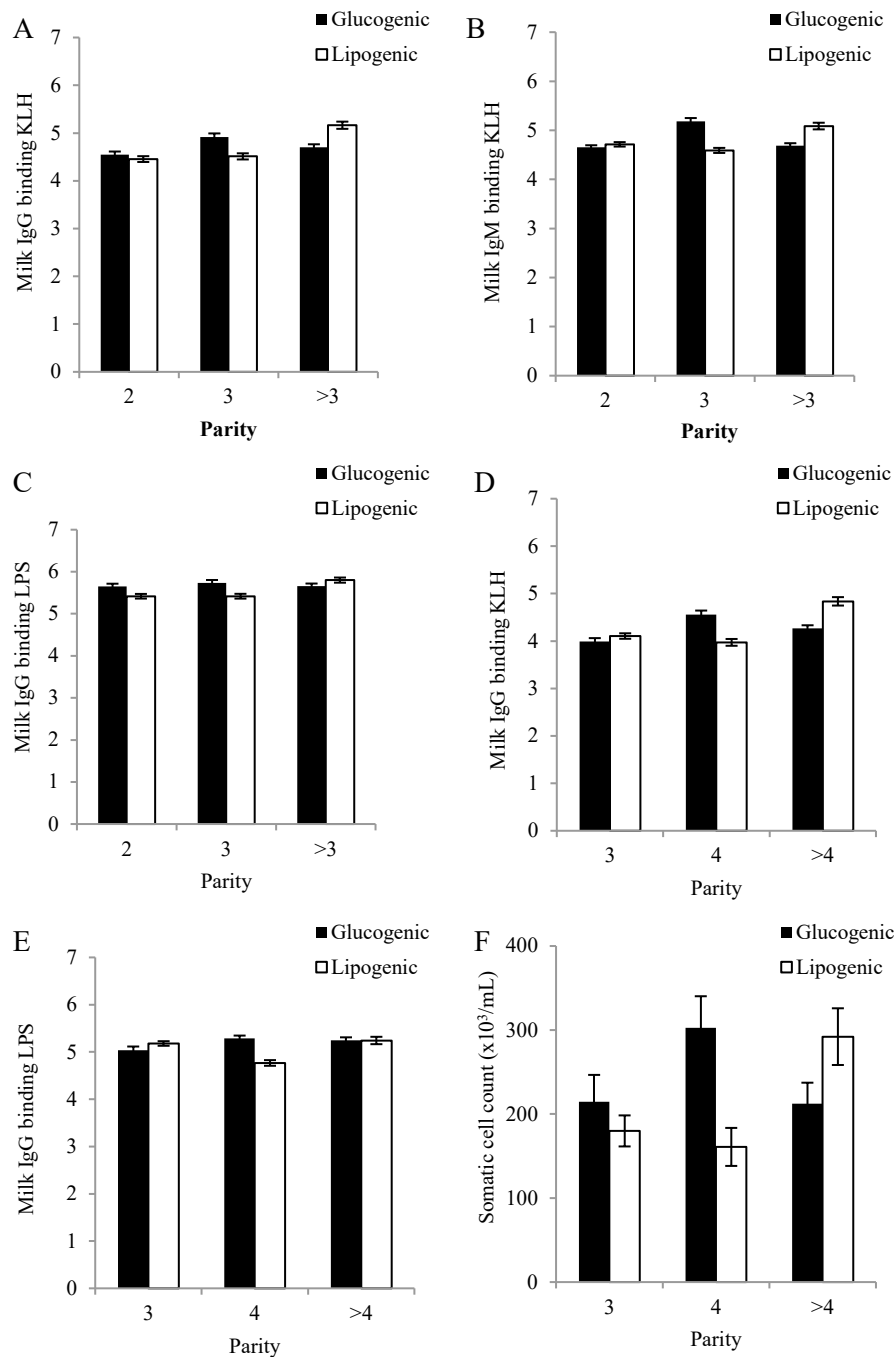**Table 4.2** Milk natural antibody titers[a] (IgG and IgM) binding keyhole limpet hemocyanin (KLH) and lipopolysaccharide (LPS) and somatic cell count (SCC) of dairy cows with varying dry period lengths (0, 30 or 60 days or 0→30 group[b]) and fed either a glucogenic (G) or a lipogenic (L) ration during 2 subsequent lactations[b] (LSM ± pooled SEM)

Item	Dry period length (d)				SEM	Ration		SEM	P-values[c,d]				
	0	30	60	0→30[b]		G	L		D	R	P	W[e]	R × P
1st Lactation[b]													
Cows (n)	55	52	56			81	82						
NAb IgG-KLH	5.08[x]	4.66[y]	4.65[y]	NA[f]	0.09	4.80	4.80	0.07	<0.01	0.98	0.01	<0.01	0.01
NAb IgM-KLH	5.23[x]	4.87[y]	4.63[z]	NA[f]	0.07	4.95	4.87	0.05	<0.01	0.30	0.04	<0.01	<0.01
NAb IgG-LPS	6.04[x]	5.65[y]	5.39[y]	NA[f]	0.07	5.77	5.62	0.06	<0.01	0.08	0.25	<0.01	0.08
NAb IgM-LPS	5.12[x]	4.76[y]	4.59[y]	NA[f]	0.06	4.92	4.72	0.05	<0.01	<0.01	0.93	<0.01	<0.01
SCC[g] (× 10³ cells/mL)	285[x]	147[y]	138[y]	NA[f]	18	178	205	15	<0.01	0.71	<0.01	<0.01	0.68
2nd lactation[b]													
Cows (n)	17	35	50	18		60	60						
NAb IgG-KLH	4.46	3.32	4.38	4.28	0.13	4.36	4.37	0.09	0.83	0.75	<0.01	<0.01	0.01
NAb IgM-KLH	4.79	4.75	4.58	4.63	0.12	4.69	4.69	0.08	0.44	0.99	<0.01	<0.01	0.11
NAb IgG-LPS	5.21	5.40	5.08	5.10	0.12	5.26	5.14	0.08	0.08	0.32	0.33	<0.01	0.03
NAb IgM-LPS	5.12	5.12	4.94	4.98	0.11	5.05	5.03	0.08	0.42	0.86	0.16	<0.01	0.19
SCC[g] (× 10³ cells/mL)	270[xy]	252[x]	183[y]	308[x]	17	251	220	17	0.01	0.31	0.01	<0.01	0.01

[x, y, z] Values within dry period length in the same row with different superscripts differ (*P* < 0.05); [a] Natural antibody titers are log2 values of the dilutions that gave an extinction closest to 50 percent of Emax where Emax represents the highest mean extinction of a standard positive (pooled) serum present on each microtiter plate (Ploegaert et al., 2007); [b] The first and second lactation after implementation of dry period length treatments; in the second lactation, 19 cows which were planned to have a 0-d dry period, had a milk production of < 4 kg/d for at least 30 days before the expected calving date and were allowed to go dry; [c] D = dry period; R = ration; P = parity; [d] Other included interactions were D × R, D × P and D × W; [e] Weeks 1, 2,…,14 relative to calving; [f] NA= not applicable.; [g] SCC values represented means per dry period or per ration, *P*-values are based on the natural logarithm of SCC.

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4.4.2 Effects of Dry Period Length and Ration on Natural Antibodies in Milk (Model 1)

In the first lactation after implementation of dry period length treatments, cows with a 0-d dry period had higher titers of IgG and IgM-binding KLH or LPS in milk compared with cows with a 30-d or a 60-d dry period (Table 4.2). In the second lactation after implementation of dry period length treatments, dry period treatment did not affect NAb titers in milk. In line with NAb titers in plasma, there was an interaction between parity × ration for IgG and IgM binding KLH and IgG binding LPS in the first lactation and IgG binding KLH and LPS in the second lactation (Figure 4.2). In short, older cows (parity > 3 and parity > 4 for the first and second lactations after implementation of dry period length and dietary treatments, respectively) fed the lipogenic diet had higher NAb titers in milk, compared with older cows fed the glucogenic diet. On the contrary, younger cows (parity ≤ 3 and parity 4 for the first and second lactations after implementation of dry period length and dietary treatments, respectively) fed the lipogenic diet had lower NAb titers in milk, compared with younger cows fed the glucogenic diet.

4.4.3 Effects of Dry Period Length and Ration on Somatic Cell Count and Clinical Mastitis (Model 2)

In the first lactation after implementation of dry period length treatments, there were 71 out of 163 cows with high SCC and the average cases of high SCC per affected cow was 3.4 (from week -3 until 14 relative to calving). In the second lactation after implementation of dry period length treatments, there were 82 out of 120 cows with high SCC and the average cases of high SCC per affected cow is 4.3 (from week -3 until 14 relative to calving). In the first lactation after implementation of dry period length treatments, cows with a 0-d dry period had a higher SCC in milk compared with cows with a 30-d or a 60-d dry period. In the second lactation after implementation of dry period length treatments, cows with a 60-d dry period had a lower SCC in milk, compared with cows with a 30-d or a 0 → 30-d dry period, but did not differ from cows with a 0-d dry period (Table 4.2). In the first and second lactations after implementation of dry period length and dietary treatments, there was no ration effect on SCC in milk. However, there was an interaction between parity × ration for SCC in the second lactation (Figure 4.2). A glucogenic ration resulted in a lower SCC in milk in older cows (parity > 4), but resulted in a higher SCC in younger cows (parity = 4). In the first lactation after implementation of dry period length treatments, the clinical mastitis occurrence

was 17 percent (29 cows out of 167) and did not differ among dry period lengths or rations. In the second lactation after implementation of dry period length treatments, the clinical mastitis occurrence was 25 percent (32 cows out of 130) and did not differ among dry period lengths or rations. Eight cows had clinical mastitis occurrence in both first and second lactations after implementation of dry period length treatments (2 cows with a 0-d dry period and glucogenic ration, 1 cow with a 30-d dry period and lipogenic ration, 1 cow with a 30-d dry period and glucogenic ration, 2 cows with a 60-d dry period and glucogenic ration, and 2 cows with a 60-d dry period and lipogenic ration).

Table 4.3 Effects of natural antibody[a] (NAb) titers (IgG and IgM) binding keyhole limpet hemocyanin (KLH) and lipopolysaccharide (LPS) in plasma and milk on the odds of a high somatic cell count.

	Mean	Minimum	Maximum	Median	n	Odds Ratio	95% Confidence Limits for Odds Ratio	
Plasma[b]								
IgG_KLH	4.86	0.40	11.20	4.20	206	0.90	0.77	1.04
IgM_KLH	6.44	1.10	12.40	6.30	206	0.91	0.82	1.00
IgG_LPS	6.58	1.40	12.04	6.34	206	0.87	0.77	0.98
IgM_LPS	4.99	4.50	10.60	1.60	206	0.91	0.81	1.01
Milk[c]								
IgG_KLH	4.51	1.20	10.80	4.30	283	1.28	1.16	1.43
IgM_KLH	4.70	1.70	11.40	4.55	283	1.31	1.16	1.49
IgG_LPS	5.40	1.90	10.60	5.30	283	1.26	1.15	1.39
IgM_LPS	4.81	1.20	9.40	4.70	283	1.24	1.10	1.41

The working correlations (rho) using AR (1) varied from 0.42 - 0.51 between the 8 models

[a] Natural antibody titers are log2 values of the dilutions that gave an extinction closest to 50 percent of Emax where Emax represents the highest mean extinction of a standard positive (pooled) serum present on each microtiter plate (Ploegaert et al., 2007).

[b] Plasma samples were taken weekly during weeks -3 to 9 relative to calving.

[c] Milk samples were taken weekly during weeks 1 to 14 relative to calving.

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4.4.4 Titers of Natural Antibodies and the Association with Somatic Cell Count and Clinical Mastitis (Model 3 and 4)

All relationships between NAb titers and a high SCC or clinical mastitis occurrence were independent of dry period length. All odds ratios for NAb titers in plasma were < 1 and for NAb titers in milk > 1 (Table 4.3 and 4.4, Model 3). Increasing titers of IgM binding KLH and IgG binding LPS in plasma were associated with a decreased odds of a high SCC (Table 4.3). On the contrary, increasing titers of IgG and IgM binding KLH and LPS in milk were associated with an increased odds of high SCC. For clinical mastitis occurrence, increasing titer of IgG binding LPS in plasma was associated with a decreased odds of occurrence (Table 4.4). On the contrary, increasing titers of IgG and IgM binding KLH and LPS in milk were associated with a significantly increased odds of clinical mastitis occurrence.

A week before the occurrence of high SCC, increasing titer of IgM binding KLH in plasma was associated with a decreased odds of high SCC occurrence (Table 4.5, Model 4). Three weeks before the incidence of diseases, increasing titers of IgM binding KLH and LPS in plasma were associated with a decreased odds of clinical mastitis occurrence. A week before the incidence of disease, increasing titer of IgG binding LPS in plasma was associated with a decreased odds of clinical mastitis occurrence.

Table 4.4 Effects of natural antibody[a] (NAb) titers (IgG and IgM) binding keyhole limpet hemocyanin (KLH) and lipopolysaccharide (LPS) in plasma and milk on the odds of clinical mastitis occurrence.

	Mean	Minimum	Maximum	Median	n	Odds Ratio	95% Confidence Limits for Odds Ratio	
Plasma[b]								
IgG_KLH	4.86	0.40	11.20	4.20	206	0.81	0.53	1.23
IgM_KLH	6.44	1.10	12.40	6.30	206	0.84	0.65	1.02
IgG_LPS	6.58	1.40	12.04	6.34	206	0.76	0.61	0.94
IgM_LPS	4.99	4.50	10.60	1.60	206	0.88	0.69	1.12
Milk[c]								
IgG_KLH	4.51	1.20	10.80	4.30	283	1.24	1.04	1.48
IgM_KLH	4.70	1.70	11.40	4.55	283	1.30	1.03	1.64
IgG_LPS	5.40	1.90	10.60	5.30	283	1.32	1.08	1.62
IgM_LPS	4.81	1.20	9.40	4.70	283	1.28	1.01	1.63

The working correlations (rho) using AR (1) varied from 0.28 - 0.37 between the 8 models

[a] Natural antibody titers are log2 values of the dilutions that gave an extinction closest to 50 percent of Emax where Emax represents the highest mean extinction of a standard positive (pooled) serum present on each microtiter plate (Ploegaert et al., 2007).

[b] Plasma samples were taken weekly during weeks -3 to 9 relative to calving.

[c] Milk samples were taken weekly during weeks 1 to 14 relative to calving.

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Table 4.5 Effects of natural antibody titers[a] (IgG and IgM) binding keyhole limpet hemocyanin (KLH) and lipopolysaccharide (LPS) in plasma in the 3 weeks before the occurrence of diseases on the odds of high somatic cell count (SCC) or clinical mastitis (CM).

	N	Odds Ratio	95% Confidence Limits For Odds Ratio	
1 week before high SCC occurrence				
IgG_KLH	206	0.94	0.82	1.02
IgM_KLH	206	0.89	0.79	1.00
IgG_LPS	206	0.91	0.81	1.01
IgM_LPS	206	0.92	0.83	1.03
2 weeks before high SCC occurrence				
IgG_KLH	206	0.96	0.84	1.10
IgM_KLH	206	0.93	0.84	1.03
IgG_LPS	206	0.93	0.83	1.04
IgM_LPS	206	0.95	0.85	1.07
3 weeks before high SCC occurrence				
IgG_KLH	206	1.03	0.90	1.18
IgM_KLH	206	0.91	0.82	1.01
IgG_LPS	206	0.94	0.84	1.04
IgM_LPS	206	0.94	0.84	1.06
1 week before CM occurrence				
IgG_KLH	206	0.86	0.66	1.12
IgM_KLH	206	0.74	0.60	0.91
IgG_LPS	206	0.83	0.70	0.99
IgM_LPS	206	0.81	0.67	0.97
2 weeks before CM occurrence				
IgG_KLH	206	0.83	0.62	1.12
IgM_KLH	206	0.78	0.64	0.93
IgG_LPS	206	0.86	0.70	1.09
IgM_LPS	206	0.81	0.66	1.00
3 weeks before CM occurrence				
IgG_KLH	206	1.05	0.79	1.38
IgM_KLH	206	0.71	0.56	0.90
IgG_LPS	206	0.87	0.69	1.10
IgM_LPS	206	0.81	0.66	0.99

[a] Natural antibody titers are log2 values of the dilutions that gave an extinction closest to 50 percent of Emax where Emax represents the highest mean extinction of a standard positive (pooled) serum present on each microtiter plate (Ploegaert et al., 2007).

4.5 Discussion

The current study demonstrates that in the first lactation after implementation of dry period length treatments, cows with a 0-d dry period had higher titers of IgG binding LPS in plasma, and higher titers of IgG and IgM binding KLH or LPS in milk compared with cows with a 60-d dry period. In addition, parity 2 cows fed a glucogenic ration had higher titer of IgM binding LPS in milk, and tended to have higher titers of IgG binding LPS in milk compared with cows fed a lipogenic ration in early lactation. In a previous study, higher titers of NAb binding KLH in plasma were associated with a less negative EB in dairy cows in early lactation (Van Knegsel et al., 2007). In this earlier work, the less negative EB was related to feeding the cows a more glucogenic ration, compared with a lipogenic ration. In the current experiment, cows had improved EB both by feeding a glucogenic ration and by shortening or omitting the dry period, as earlier reported (Van Knegsel et al., 2014). Specifically, omitting the dry period resulted in a less negative EB in the subsequent lactation as a consequence of lower daily milk yield at a similar dry matter intake (**DMI**).

Hence, it can be suggested that the higher NAb titers for cows in plasma and in milk with no dry period in the first lactation after implementation of dry period length treatments are related with the improved EB and reduced milk yield in these cows. In the second lactation after implementation of dry period length treatments, cows with no dry period had similar NAb levels in plasma and in milk as cows with a dry period of 30-d or 60-d. Moreover, in the second lactation after implementation of dry period length treatments, cows with no dry period had less pronounced improvement of EB and had less milk yield losses compared with the first after implementation of dry period length and dietary treatments (Chen et al., 2015).

Omitting the dry period not only results in a less negative EB, but is also known to result in an increased SCC in milk in the subsequent lactation (Annen et al., 2004, Klusmeyer et al., 2009). Also in the current study, cows with a 0-d dry period had a higher SCC in milk, compared with cows with a 30-d or a 60-d dry period. In a previous study, a high SCC due to omitting the dry period was associated with a reduced milk yield, resulting in a reduced diluting effect (Steeneveld et al., 2013). Also in the current study, incorporating daily milk yield as a covariate in the statistical model reduced the effect of dry period length on the SCC (Van Knegsel et al., 2014). This means that the effect of length of the dry period on SCC is partly explained by a reduced diluting effect (Van Knegsel

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et al., 2014).

Somatic cell count has been related previously to titers of IgM binding KLH or LPS in milk (Ploegaert, 2010). In the current study, the odds ratios of NAb titers in milk for the relative odds of a high SCC were all greater than 1. This suggests that increasing titers of IgG and IgM-binding KLH or LPS in milk are associated with an increased odds of a high SCC. In contrast, increasing titers of IgM binding KLH and IgG binding LPS in plasma were associated with a decreased odds of a high SCC. A high SCC and increased levels of antibodies in milk may both rest on increased vascular permeability after intramammary inflammation (Korhonen et al., 2000). In addition, Fc receptor mediated transfer for IgG and IgM was detected for mammary gland polymorphonuclear neutrophils in dairy cows (Lascelles, 1979, Hurley and Theil, 2011). The other suggested mechanism that results in a high level of NAb in milk might be that immunoglobulins are produced locally by lymphocytes located near the glandular epithelium (Lascelles, 1979, Persson et al., 1992).

In the current study, increasing NAb levels in milk were associated with increased odds of high SCC and clinical mastitis occurrence, which is in line with earlier work (Ploegaert, 2010). Moreover, the current study shows that specific high NAb in plasma are associated with a decreased odds of clinical mastitis occurrence. A previous study reported that increasing titer of IgM binding KLH in plasma was associated with a decreased odds of clinical mastitis occurrence (unadjusted odds ratio = 0.958) (Thompson-Crispi et al., 2013). In the current study, the odds ratio for IgM binding KLH in plasma is in line with that of this earlier work (odds ratio = 0.81), but not statistically different from 1 (95% confidence interval = 0.60 to 1.02), which is possibly consequence of low statistical power. It has been suggested that the level of NAb in plasma or milk may be an additional variable to select for mastitis resistance in dairy cows (Ploegaert, 2010, Thompson-Crispi et al., 2013).

It can be questioned whether an increase in the level of NAb in plasma or milk relates only to past or present intramammary inflammation, or could also act as an indicator for future mammary infections. Therefore, the high SCC and clinical mastitis occurrence was associated with the levels of NAb in plasma or milk in the 3 weeks before the high SCC and clinical mastitis occurrence. Increasing plasma NAb titer for IgM binding KLH in the week before the occurrence of high SCC were associated with a decreased odds of high SCC occurrence. Moreover, increasing titers of IgM binding KLH or LPS in plasma in

the 3 weeks before the incidence of the disease was associated with decreased odds of clinical mastitis occurrence. Further studies are needed to confirm these relationships and study the possible value of NAb's as early indicators for reduced mammary health in dairy cows.

In the current study, although the dry period length did affect SCC in milk, the dry period length did not affect clinical mastitis occurrence. From an earlier study, it was concluded that applying a 0-d dry period may impose a greater odds of a high SCC because cows are not treated with long-acting intramammary antibiotics, although it lowered the odds of new infections during the dry period (Annen et al., 2004, Collier et al., 2012). Furthermore, other studies reported no differences in the clinical mastitis occurrence between cows with a short dry period and cows with a conventional dry period (Enevoldsen and Sørensen, 1992, Watters et al., 2008, Shoshani et al., 2014). This might imply that omitting and shortening the dry period affects SCC, but not clinical mastitis occurrence.

In contrast to earlier work, the current study monitored the cows for 2 subsequent lactations after a shortened or omitted dry period. The results for the second lactation after implementation of dry period length treatments differed from those of the first lactation after implementation of dry period length treatments for several aspects. Firstly, in contrast to the first lactation, in the second lactation after implementation of dry period length treatments dry period length had no effect on NAb titers in plasma and milk. This might be due to reduced differences in milk yield, and reduced differences in EB between dry period lengths in the second lactation (Chen et al., 2016). Secondly, the average parity of the cows in the second lactation was greater than that in the first lactation. Earlier studies showed that older cows have higher NAb titers (Van Knegsel et al., 2007), and milk yield losses due to shorter dry periods are smaller than for younger cows (Van Knegsel et al., 2014). Thirdly, after the first lactation after implementation of dry period length treatments, some cows that were planned for a 0-d dry period, dried off naturally, and were allocated to the 0→30–d dry period group. In contrast to cows with a 30-d or 60-d dry period, cows with a 0→30-d dry period were not treated with an intramammary antibiotic at the time of drying off. Although different dry-off protocols were used for the 0→30–d and the 30-d dry period, the SCC was not statistically different between cows with a 30-d and a 0→30-d dry period. Similar SCC levels in the second lactation between the groups might be due to small or zero differences in milk yield for different dry period groups.

Earlier studies showed that older cows have a higher SCC than younger cows (Annen et al., 2004, Steeneveld et al., 2013), which is in line with the current study for both lactations. However, cows with parity > 4 in the second lactation had a lower SCC when they were fed a glucogenic ration compared with a lipogenic ration. In the current study parity × ration interaction not only affected the SCC, but also NAb titers. Earlier studies showed that higher NAb titers binding KLH in plasma increased with parity (Van Knegsel et al., 2007), and are higher in adult cows than in calves (Srinivasan et al., 1999). Exogenous factors or environmental stimuli enhance NAb development (Prokešová et al., 1996), and therefore might explain an increase in NAb titers with age.

The current study implies that, compared with the first lactation after implementation of dry period length and dietary treatments, the effects on NAb titers and mammary health by shortening or omitting the dry period are different in the second lactation after implementation of dry period length and dietary treatments. As discussed above, in response to dry period length treatment in both lactations, differences in EB, milk yield, DMI level and parity may underlie differences in mammary health and NAb titers. Further studies are required to study the life-long effects on mammary health of dairy cows by shortening or omitting the dry period. Moreover, results of this study indicate that IgM-binding KLH in plasma can be an early predictor of clinical mastitis occurrence in dairy cows. At the moment, studies are ongoing aiming at identification of factors that predict mammary health after a shortened or omitted dry period in dairy cows.

4.6 Conclusions

Cows with a 0-d dry period had higher SCC, higher NAb titers for isotypes IgG and IgM in milk, and higher titer of IgG-binding LPS in plasma compared with cows with a 30-d or 60-d dry period in the first lactation after implementation of dry period length. However, after repeated shortening or omitting the dry period, the effects of omitting the dry period on NAb titers in plasma and milk disappeared. In addition, increasing titers of NAb-binding KLH or LPS in milk for both lactations were associated with increased odds of a high SCC and increased odds of clinical mastitis occurrence. In contrast, increasing titers of IgM binding KLH and IgG binding LPS in plasma for both the first and second lactations after implementation of dry period length and dietary treatments were associated with decreased odds of a high SCC and decreased odds of clinical mastitis occurrence. Titer of IgM binding KLH in plasma in a week and in all 3 weeks before the

incidence of disease were associated with high SCC and clinical mastitis occurrence, respectively. This implies that IgM binding KLH in plasma can be an early predictor of high SCC and clinical mastitis occurrence in dairy cows.

4.7 Acknowledgements

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CHAPTER 5

Effect of maternal dry period length on colostrum immunoglobulin content, and natural and specific antibody titers in calves

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5.1 Abstract

The objective was to study the effect of dry period length in dairy cows on immunoglobulin content and natural antibodies (**NAb**) titers in colostrum, growth and plasma natural and specific antibody titers in plasma of calves. Holstein-Friesian dairy cows (N = 167) were randomly assigned to 3 dry period lengths (0, 30 or 60 d). Colostrum production, concentration of colostrum IgG and IgM, and titers of NAb (isotypes IgG and IgM) binding keyhole limpet hemocyanin (**KLH**) and human serum albumin (**HuSA**) in colostrum were measured. Female calves were immunized with both KLH and HuSA in week 6 and 10 of life. Titers of NAb and specific antibody (**SpAb**) for isotypes IgG, IgM and total immunoglobulin (**IgT**) binding KLH or HuSA were determined in plasma of female calves. Primary and secondary antibody responses to KLH or HuSA from week 6 and 10 were expressed as the increase in antibody titers to week 10 and 11 of life after primary and secondary challenges, respectively. Pregnancy length for cows with a 0-d dry period was 3 d shorter, compared with cows with a 30-d or a 60-d dry period. Birth weight of calves from cows with a 0-d dry period was lower compared with calves from cows with a 30-d dry period. Growth of calves until 12 weeks of life was not affected by dry period length. Colostrum production and IgG and IgM concentration in colostrum were lower for cows with a 0-d dry period than a 60-d dry period. Natural IgG and IgM titers binding KLH or HuSA were lower in colostrum from cows with a 0-d dry period compared with cows with a 30-d or a 60-d dry period. Natural antibody titers (IgG, IgM and IgT) binding KLH or HuSA in plasma were lower during the first 2 weeks of life for calves from cows with a 0-d dry period compared with calves from cows with a 30-d or 60-d dry period. After primary and secondary immunization of calves with KLH and HuSA, SpAb titers of calves were not affected by dry period length. After secondary immunization the response of IgG and IgT binding KLH was higher in plasma of calves from cows with a 0-d dry period. The results of this study demonstrate that although omission of the dry period of dairy cows leads to lower plasma NAb titers in calves during the first 2 weeks of life, SpAb titers in calves were not affected and even the secondary antibody responses were enhanced, compared with calves from cows with a 30- or 60-d dry period.

Key words: continuous milking, colostrum, antibodies, calf.

5.2   Introduction

Omission of the dry period of cows results in better energy balance (**EB**), and metabolic health in the next lactation (Andersen et al., 2005, Rastani et al., 2005). Omission of the dry period is controversial because of a potential decrease in milk production in the subsequent lactation (Annen et al., 2004, Santschi et al., 2011a). A meta-analysis including 24 randomized controlled trials (Van Knegsel et al., 2013) reported that shortening or omitting the dry period reduced milk production and increased milk protein content. In addition, shortening the dry period tended to reduce the incidence of ketosis in the subsequent lactation. Earlier studies showed that shortening the dry period to 4 weeks did not affect colostrum immunoglobulin content (Annen et al., 2004, Klusmeyer et al., 2009, Rastani et al., 2005, Watters et al., 2008), pregnancy length (Santschi et al., 2011b) and birth weight of calves (Pezeshki et al., 2007, Rastani et al., 2005), compared with a dry period of 8 weeks. Omission of the dry period, however, reduced colostrum immunoglobulin content (Annen et al., 2004, Klusmeyer et al., 2009, Rastani et al., 2005, Verweij et al., 2014), which may affect the immune competence of the calf. Adequate and sufficient passive immune transfer of maternal antibodies via colostrum intake may improve the immune competence of calves, and would reduce the risk of diseases and infections during the pre-weaning period (Oliveira et al., 2010).

Natural antibodies (**NAb**) may play an important role in the development of immune competence. Natural antibodies are part of the innate immune system (Matter and Ochsenbein, 2008, Vollmers and Brändlein, 2009) and can be defined as antibodies present in healthy animals under the absence of antigen stimulation (Avrameas, 1991, Baumgarth et al., 2005) as opposed to specific antibodies (**SpAb**) that are raised to antigens after immunization. Natural antibodies are polyreactive with low affinity binding with various antigens (Casali and Notkins, 1989). Natural antibodies link innate and adaptive immunity, since they target antigens to the splenic marginal zone enhancing T-cell and T-cell dependent antibody responses (Matter and Ochsenbein, 2008), and activate complement activity. Zinkernagel (2012) proposed that NAb form an important protection for resistance to infection. In chickens, NAb binding *Megathura crenulata*-derived keyhole limpet hemocyanin (**KLH**) were related to a higher probability of survival during the laying period (Star et al., 2007, Sun et al., 2011). In mammals (Ochsenbein et al., 1999, Thornton et al., 1994, Tomer and Shoenfeld, 1988) and birds (Lammers et al., 2004, Parmentier et al., 2004), various specific humoral

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immune responses are enhanced by high NAb titers or are positively correlated with high NAb titers. On the other hand, high NAb levels might interfere with or prevent subsequent SpAb responses (Parmentier et al., 2008), whereas low NAb levels might facilitate or initiate higher SpAb responses (Sinyakov et al., 2002).

Natural antibodies were found in plasma of newborn calves (Srinivasan et al., 1999). In dairy cows, calves obtain maternal antibodies passively from the dam through colostrum. Natural antibodies in plasma of newborn calves were lower than in adult cows (Srinivasan et al., 1999) and NAb titers differ widely among adults (Ploegaert et al., 2011, Srinivasan et al., 1999). In addition, titers of NAb binding KLH or lipopolysaccharide (**LPS**) were related to the EB of dairy cows in early lactation (Van Knegsel et al., 2007). Knowledge on the effect of shortening or omitting the dry period of dairy cows on NAb in colostrum and the consequences for immune competence and development of calves is, however, absent.

As calves obtain passive immunity via colostrum, it was hypothesized that management or dietary strategies that affect immunoglobulin content in colostrum may affect immune competence of calves. The first objective of the present study was to evaluate the effect of dry period length in dairy cows on immunoglobulin content and NAb titers in colostrum, and growth and plasma NAb and SpAb titers of calves. The second objective was to study the responses of total immunoglobulin (**IgT**), and the isotypes IgG and IgM binding KLH and human serum albumin (**HuSA**) after primary and secondary immunization in these calves at week 6 and 10, respectively.

5.3   Materials and Methods

5.3.1 Experimental Design, Animals and Colostrum Sampling

The Institutional Animal Care and Use Committee of Wageningen University approved the experimental protocol. Holstein-Friesian dairy cows (N = 167) were selected from the Dairy Campus Research dairy herd (WUR Livestock Research, Lelystad, the Netherlands), blocked for parity, calving date, milk yield and BCS, and randomly assigned to treatments. Cows enrolled in our study were clinically healthy and had SCC in milk $< 250 \times 10^3$ cell/mL. Treatments consisted of 3 dry period lengths: 0, 30 or 60 d and 2 early lactation ration (glucogenic or lipogenic) in a 3×2 factorial design. The experiment started with 60 primiparous and 107 multiparous cows. Cows were housed in a freestall with slatted floor and cubicles and milked twice daily (0500 h and 1630 h).

5.3.2 Rations

Ration composition was described previously (Van Knegsel, et al. 2014). Prepartum, dry cows received a dry cow ration and lactating cows received a lactation ration supporting 25 kg of milk. From 10 d prepartum, cows were fed 1 kg/d of the experimental concentrate (glucogenic or lipogenic). Postpartum, all cows received 1 kg of experimental concentrate which was increased stepwise by 0.5 kg/d until concentrate supply reached 8.5 kg/d at d 17 postpartum. Experimental concentrates were provided by a computerized feeder located in the freestall. In addition, lactating cows received 1 kg/d of standard lactation concentrate in the milking parlor. Forage did not differ between diets, was supplied ad libitum and consisted of grass silage, corn silage, wheat straw and a protein source (rapeseed meal or soybean meal) in a ratio of 39:25:25:11 (DM basis). Postpartum, forage consisted of grass silage, corn silage, straw, and a protein source (rapeseed meal or soybean meal) in a ratio 51:34:2:13 (DM basis).

5.3.3 Management of Calves

Immediately after birth, calves were removed from dam. When calves were born between 2200 until 0500 h (N = 42) they were removed from the dam at 0500 h. Calves were weighed and within 24 h of life they received 4 L of colostrum in 2 portions from their mother. Secretion of the first milk was collected and weighed directly after calving, except when the cow calved between 2200 until 0500 h (N = 42). Colostrum obtained after parturition was agitated and colostrum samples (10 ml) were stored at −20°C. At 24 h after birth, calves were fed with milk replacer (CP 22% and fat 17%) (2×/d, 2 L). After 2 d, calves were moved to the calf-raising farm and were also fed milk replacer twice a day, until 60-d of life. After the first week of life, all calves were fed the same mixture of hay and grains (Agrifirm Feed, Apeldoorn, the Netherlands) based on requirements for preweaned Holstein calves. At 60-d of life, calves were weaned from milk replacer. From 60-d of life on, calves received a diet based on requirement for weaned Holstein calves. During first 2 weeks of life, calves were housed in individual hutches located approximately 60 cm apart. After 2 weeks, calves were kept in groups of 6 to 8 calves with straw-bedding. Body weight of female calves was recorded at birth and in week 2, 4, 6, 8, 10 and 12 after birth. Disease incidences were recorded.

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5.3.4 *Blood Sampling and Immunization*

Blood samples of female calves (N = 63) were taken from the jugular vein immediately after birth and every Wednesday in week 1, 2, 4, 6, 7, 8, 10, 11, and 12 of life in Vacutainer tubes (10 mL, Becton Dickinson and Co., Franklin Lakes, NJ) containing heparin to determine titers of total (IgT), IgG and IgM natural and specific antibody titers binding KLH or HuSA. Blood samples were centrifuged at 3,000 × g at 4°C, for 15 min immediately after sample collection, and plasma was stored at -20°C until analysis. Female calves were immunized twice with a combination of both KLH and HuSA. Solutions of KLH and HuSA were prepared by dissolving 2 mg of KLH (H7017, Sigma Aldrich Co., St Louis, MO) and 0.2 mg HuSA (A8763, Sigma Aldrich) in 2.5 mL PBS (0.1 *M*, pH = 7.2). Calves received a 2.0 mL i.m. injection in the neck at week 6 of life, and a second injection at week 10 of life.

5.3.5 *Analysis of Immunoglobulin Concentration in Colostrum*

Colostrum was not sampled during weekends. Total concentration of IgG and IgM in colostrum was determined by an indirect ELISA method using the Bovine IgG ELISA Quantitation Kit (Cat. No. E10-118, Bethyl Laboratories Inc., Montgomery, TX), and the Bovine IgM ELISA Quantitation Kit (Cat. No. E10-101, Bethyl Laboratories). Kit performance was optimized for the stated protocol using affinity purified sheep anti-bovine IgG-heavy chain or IgM coating antibody, bovine reference serum and horseradish peroxidase (PO) conjugated sheep anti-bovine IgG-heavy chain or IgM detection antibody. Standard dilutions from 500-7,800 ng/mL of bovine IgG and 1,000-15,625 ng/mL of bovine IgM were used according to manufacturer's procedures.

5.3.6 *Analysis of Natural and Specific Antibodies in Colostrum and Plasma*

Natural antibody titers binding KLH and HuSA in plasma of female calves were measured by indirect ELISA as outlined by Van Knegsel et al. (2007). The same ELISA procedures were also used to measure NAb titers in colostrum and SpAb titers binding KLH and HuSA in plasma of female calves. Plates were coated with 4 µg/mL of KLH or 4 µg/mL of HuSA (100 µL/well). Natural antibody of the IgG isotype in colostrum were detected either using 1:40,000 diluted sheep polyclonal anti-bovine IgG-heavy chain conjugated to horseradish PO (Cat. No. E10-118P, Bethyl Laboratories), Natural antibody of the IgM isotype in colostrum was detected using 1:20,000 diluted rabbit polyclonal anti-bovine IgM-whole

molecule conjugated to horseradish PO (Cat. No. A10-100P, Bethyl Laboratories). Serial dilutions for isotypes (IgG and IgM) in colostrum samples started at 1:10 (8 steps). Natural antibodies and SpAb isotypes IgM and IgG in plasma were detected either using 1:40,000 diluted sheep polyclonal anti-bovine IgG-heavy chain conjugated to horseradish PO (Cat. No. E10-118P, Bethyl Laboratories), or 1:20,000 diluted rabbit polyclonal anti-bovine IgM-whole molecule conjugated to horseradish PO (Cat. No. A10-100P, Bethyl Laboratories) or 1:20,000 diluted rabbit anti-bovine IgG heavy and light chain conjugated to PO (Cat. No. A10-102P, Bethyl Laboratories) for IgT. Serial dilutions for isotypes (IgG, IgM, and IgT) in plasma samples started at 1:40 (4 steps). Titers were expressed as log2 values of the dilutions that gave an extinction closest to 50% of Emax, where Emax represents the highest mean extinction of a standard positive (pooled) serum present on every microtiter plate (Ploegaert et al., 2007). Preliminary analysis showed the highest response of antigen SpAb after primary and secondary immunizations in weeks 10 and 11 of life, respectively. Therefore, response to primary antigen challenges was defined by subtracting the SpAb titers for isotype IgG, IgM and IgT binding KLH and HuSA at week 10 after primary immunization with titers at week 6. Response to secondary antigen challenges was defined by subtracting the SpAb titers for isotype IgG, IgM and IgT binding KLH and HuSA at week 11 after secondary immunization with titers at week 10.

5.3.7 Statistical Analysis

Three pairs of twins (4 calves from cows with a 60-d dry period and 2 calves from cows with a 0-d dry period) and 3 stillborn calves (2 calves from cows with a 0-d dry period and 1 calf from cow with a 60-d dry period) were excluded from the analysis. Three female calves, 1 calf from a cow with a 30-d dry period and 2 calves from cows with a 0-d dry period died in the rearing unit before week 6 and were removed from analysis. The ANOVA PROC MIXED procedure (SAS version 9.2; SAS Institute Inc., Cary, NC, Littell et al., 1996) was used to analyze colostrum immunoglobulin content, pregnancy length, birth weight, growth, and plasma antibody titers. Preliminary analysis showed no effect of dietary treatments on colostrum, plasma and growth variables, therefore ration was excluded from the analysis. Colostrum immunoglobulin content, NAb titer binding KLH or HuSA in colostrum, calf growth and the response after primary antibody challenge at 6 weeks and secondary antibody challenge at week 10 were analyzed with dry period length (0, 30 or 60 days), parity (2, 3 or >3), and their interaction included as fixed

effect (model 1). Preliminary analysis showed there was no difference between concentrations of IgG and IgM in colostrum collected directly after calving and concentrations of IgG and IgM in colostrum from cows which calved during 2200 – 0500 h (N = 42). Thus, both colostrum samples from cows which calved during night and cows which calved during the day were included in the analysis. Pregnancy length and birth weight for all calves were analyzed with dry period length (0, 30 or 60 d), parity (2, 3 or > 3), sex and interactions included as fixed effects (model 2). Natural antibody titers (week 0 until 6) and specific antibody titers after primary (week 6 until 10) and secondary immunization (week 10 until 12) in plasma were analyzed with dry period length (0, 30 or 60 d), parity (2, 3 or > 3), week (1 - 12) and interactions included as fixed effects (model 3). In model 3, calf was considered as the repeated subject. A first-order auto-regressive structure [**AR (1)**] was the best fit and was used to account for within-calf variation. For comparison of dry period length effects *P*-values are presented after a Bonferroni adjustment. Model assumption regarding normality of residuals was evaluated by examining whether skewness and kurtosis were close to 0. Values are presented as LSMEANS with their pooled SEM, unless otherwise stated. Pearson correlation coefficient (PROC CORR in SAS) were used to test the correlation between NAb titers (IgG and IgM) binding KLH or HuSA in colostrum and NAb titers (IgG and IgM) binding KLH or HuSA in plasma of calves.

5.4   Results

5.4.1 Colostrum

Cows with a 60-d dry period had greater colostrum production at first milking after calving compared with cows with a 0- or 30-d dry period (7.7 vs. 5.3 vs. 5.1 ± 0.6 kg for cows with 60- vs. 30- vs. 0-d dry; respectively, *P* < 0.01). Cows with a 0-d dry period had lower IgG and IgM concentration in colostrum (Figure 5.1a) compared with cows with a 30-d or 60-d dry period. There were no differences in IgG or IgM concentration in colostrum between cows with a 30-d and cows with a 60-d dry period. Concentration of IgG in colostrum increased with parity (20.9 vs. 27.0 vs. 30.8 ± 2.2 mg/mL for parity 2 vs. 3 vs. >3, respectively; *P* = 0.01). Titers for IgG and IgM binding KLH and HuSA in colostrum were lower for cows with a 0-d dry period compared with cows with a 30-d or 60-d dry period (Figure 5.1b). Natural antibody titers in colostrum increased with parity, except natural IgM binding HuSA.
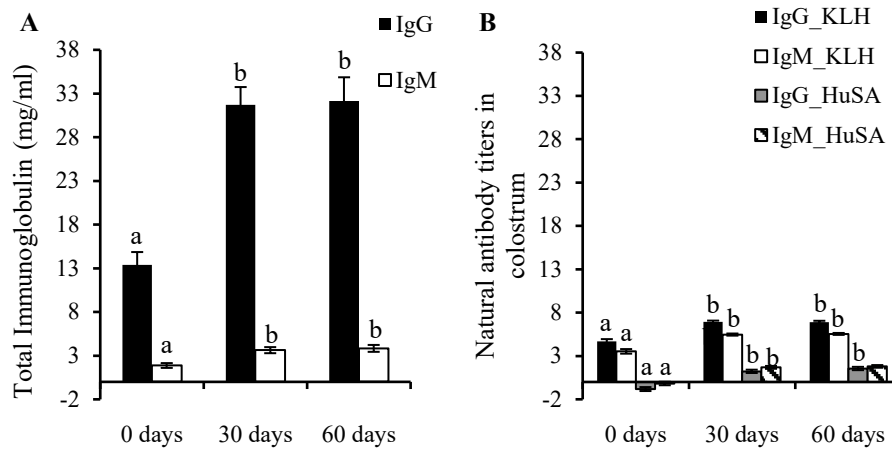
Figure 5.1 Concentration of IgG and IgM (A.) and natural antibodies (NAb) for isotype IgG and IgM titers binding keyhole limpet hemocyanin (KLH) and human serum albumin (HuSA) (B.) in colostrum from cows with 3 different dry period lengths (0, 30 or 60 d). Values are means (± SEM) per dry period length. a, b, c values between dry period length with different superscripts differ ($P < 0.05$).

5.4.2 Birth Weight and Growth of Calves

Calves from cows with a 0-d dry period were born 3 d earlier ($P = 0.01$), and had a lower birth weight ($P = 0.04$) compared with calves from cows with a 30-d or 60-d dry period (Table 5.1). Omitting the dry period resulted in lower birth weight of the male calves (43.7 vs. 46.5 vs. 46.2 ± 0.8 kg for 0-d vs. 30-d vs. 60-d dry period, respectively, $P = 0.02$) but did not affect birth weight of the female calves (42.3 ± 0.8 kg). Cows with a 0-d dry period had shorter pregnancy length and lower birth weight compared with cows with a 30-d or 60-d dry period. Pregnancy length showed positive relation with birth weight of calves from cows with 0-d dry period. Body weight of the female calves before first immunization at week 6 was not different between dry period lengths (56.9 vs. 60.6 vs. 58.9 ± 0.2 kg, for 0-d vs. 30-d vs. 60-d dry period, respectively, $P = 0.12$). Growth of female calves from day 0 until week 12 of life and live weight of female calves at week 12 (data not shown) were not different between dry period lengths of the cows ($P = 0.19$ and $P = 0.18$, respectively). Disease incidence of calves did not differ among dry period lengths.

Table 5.1 Birth weight, pregnancy length and growth of calves during the first 12 weeks of life from cows with different dry period lengths (0, 30, or 60 d) (LSM ± SEM).

Performance	Dry period lengths (d) [3]			SEM	P-values[5]			
	0	30	60		D	P	S[4]	D × P
Calves (no.)	54	54	51					
Birth weight (kg) all[1]	42.9[a]	44.7[b]	44.4[ab]	0.55	0.04	0.27	<0.01	0.23
Pregnancy length (days)	278[a]	280[b]	281[b]	0.63	0.01	0.12	0.25	0.82
Growth (kg/day)[2]	0.6	0.7	0.7	0.02	0.19	0.04	nm	0.90

[1] Birth weight of all calves (male and female) excluding twins and stillborn calves;
[2] Growth of female calves. Three calves were removed from the analysis, because they died in the rearing unit before week 6 of life;
[3] Values within dry period length in the same row with different superscripts differ ($P < 0.05$); [4] nm = not included in the model; [5] D = dry period; P = parity; S= sex.

5.4.3 Natural Antibody Titers in Plasma of Calves

Calves from cows with a 0-d dry period had lower natural IgG, IgM and IgT in plasma compared with calves from cows with 60-d dry period (Table 5.2), except for IgG binding HuSA. Natural antibody titers for calves from cows with a 0-d dry period were specifically lower immediately after calving (week 0), and in week 1 and 2 (Figure 5.2). Natural IgT binding KLH and HuSA in plasma increased with parity (2.2 vs. 2.5 vs. 3.0 ± 0.2 and 1.4 vs. 1.9 vs. 1.9 ± 0.1 for parity 2 vs. 3 vs. > 3 for IgT binding KLH and HuSA, respectively). Natural IgT binding KLH and HuSA in plasma of calves were affected by dry period × parity interaction ($P < 0.01$ and $P = 0.02$, respectively). Titers of natural IgG and IgM binding KLH and HuSA in colostrum correlated positively with titers of natural IgG and IgM binding KLH and HuSA in plasma of calves at calving ($r = 0.73$, $P < 0.01$ and $r = 0.70$, $P < 0.01$ for IgG and IgM binding KLH, respectively, and $r = 0.41$, $P < 0.01$ and $r = 0.65$, $P < 0.01$ for IgG and IgM binding HuSA, respectively). There were no correlations between NAb titers present in colostrum and NAb titers in plasma of calves after 2 weeks.

5.4.4 Primary and Secondary Antibody Responses to KLH and HuSA

Primary and secondary SpAb titers for all isotypes binding KLH and HuSA in plasma of calves did not differ between dry period lengths (Table 5.3). All SpAb titers (IgG, IgM, and IgT) binding KLH or HuSA in plasma of calves increased following primary and secondary immunization (Figure 5.2) except for IgM binding KLH or HuSA after the secondary immunization. Specific antibody titers for isotype IgG and IgT binding HuSA in calves increased with parity of the cows.

After primary immunization, calves from younger cows (parity 2) had lower specific IgG titers binding HuSA (2.0 vs. 3.0 vs. 2.8 ± 0.3 for parity 2 vs. 3 vs. > 3, respectively; $P = 0.05$) and lower IgT titers binding HuSA (2.8 vs. 4.4 vs. 4.0 ± 0.3 for parity 2 vs. 3 vs. > 3, respectively; $P < 0.01$) compared with calves from older cows. After secondary immunization, calves born from younger cows had lower specific IgG titers binding HuSA (3.7 vs. 5.2 vs. 5.0 ± 0.4 for parity 2 vs. 3 vs. > 3, respectively; $P = 0.04$) and IgT titers binding HuSA (4.9 vs. 7.0 vs. 6.4 ± 0.5 for parity 2 vs. 3 vs. >3, respectively; $P = 0.01$) compared with calves from older cows. After secondary immunization, there was a dry period length × week interaction for titers of IgG ($P < 0.01$) and IgT binding KLH ($P = 0.02$). Calves born from cows with a 0-d dry period had higher plasma titers of specific IgG binding KLH and IgT binding KLH compared with calves born from cows with a 30- or a 60-d dry period.

After primary antigen challenge, the IgG binding KLH response tended to be higher for the calves from cows with a 0-d dry period compared with calves from cows with a 30-d or 60-d dry period (Table 5.4). After primary antigen challenge, IgT binding KLH response was higher for the calves from cows with a 0-d dry period compared with calves from cows with a 30-d or 60-d dry period. Also secondary IgG and IgT responses binding KLH were enhanced in calves born from mothers with a 0-d dry period, compared with a 30-d or 60-d dry period. The responses of SpAb in plasma of calves after both primary and secondary immunization between a 30-d and a 60-d dry period were not different.

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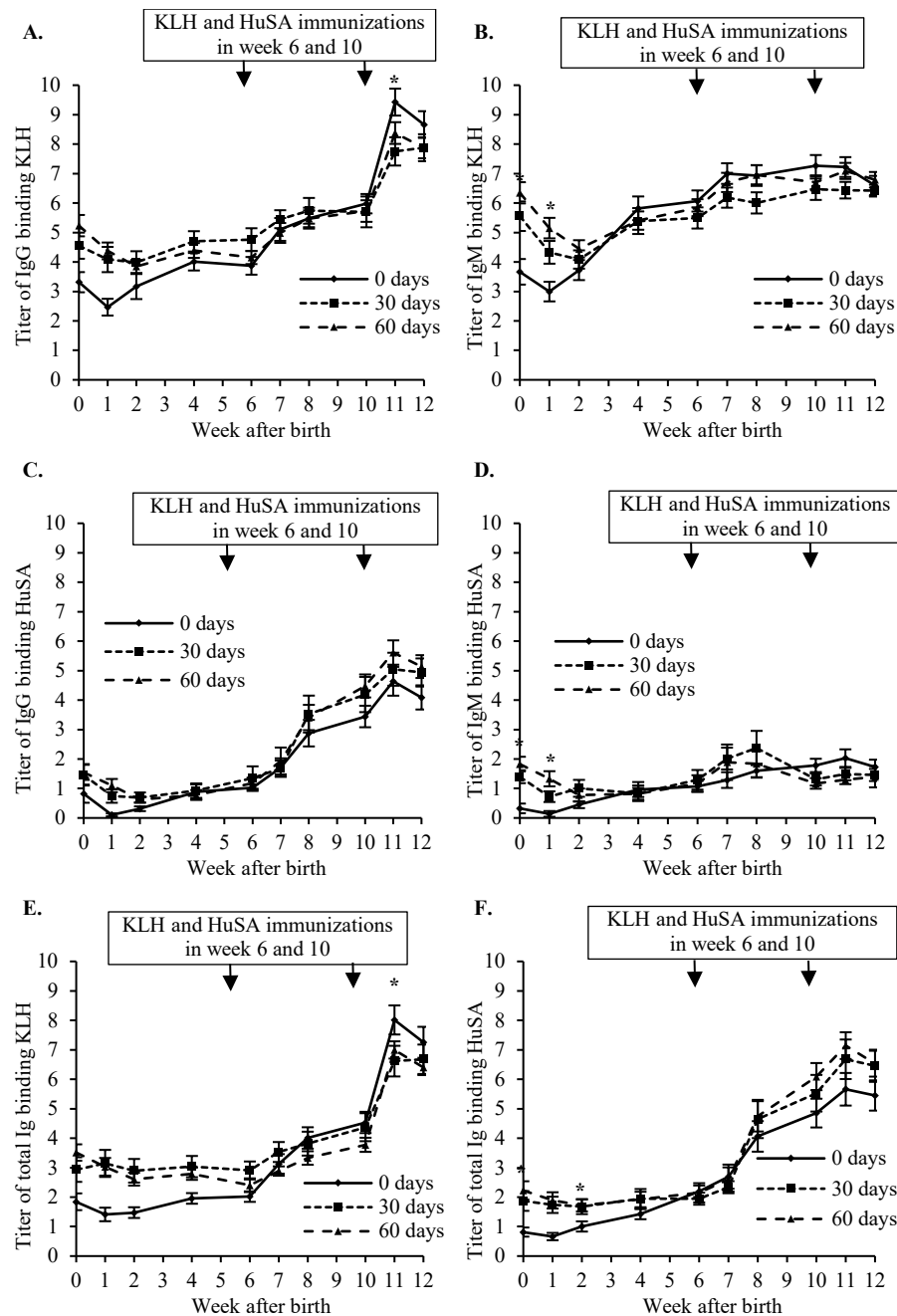**Figure 5.2** Natural antibodies (week 0 until week 6) and specific antibodies (after primary and secondary immunization) for IgG, IgM, and total immunoglobulin (IgT) titers binding keyhole limpet hemocyanin (KLH) (A, C, and E) and human serum albumin (HuSA) (B, D, and F) in plasma of female calves from dairy cows with 0, 30 or 60 days dry period. Values represent means (± SEM) per dry period length (DPL) per week (W).

Table 5.2 Natural antibody titers (IgG, IgM and and total immunoglobulin (IgT)) binding keyhole limpet hemocyanin (KLH) and human serum albumin (HuSA) from week 0 until 6 in plasma of calves from cows with different dry period length (0, 30, or 60 d) (LSM ± SEM).

Antibody[1]	Dry period length[2] (d)			SEM	P-values[3]				
	0	30	60		D	P	W	D × P	D × W
Female calves (no.)	23	19	21						
IgG-KLH	3.41[a]	4.52[b]	4.40[b]	0.22	<0.01	0.38	<0.01	0.20	0.14
IgM-KLH	4.52[a]	4.97[a,b]	5.47[b]	0.23	0.02	0.68	<0.01	0.46	<0.01
IgG-HuSA	0.66	1.14	1.06	0.14	0.08	0.33	<0.01	0.07	0.36
IgM-HuSA	0.58[a]	1.01[a,b]	1.18[b]	0.15	0.01	0.69	<0.01	0.63	0.01
IgT-HuSA	0.25[a]	1.87[b]	2.06[b]	0.16	<0.01	0.02	<0.01	<0.01	0.01
IgT-KLH	1.74[a]	3.05[b]	2.97[b]	0.21	<0.01	0.06	0.01	0.02	0.08

[1] Antibody names are combination of immunoglobulin isotype and antigen abbreviation.; [2] Values within dry period length in the same row with different superscripts differ ($P < 0.05$); [3] D = dry period; P = parity; W=week.

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Table 5.3 Specific antibody titers for isotopes IgG, IgM and Total immunoglobulin (IgT) after primary and secondary immunization to KLH (keyhole limpet hemocyanin) and HuSA (human serum albumin) in plasma of female calves (N=63) from cows with three different dry period length (0, 30 or 60 d) (LSM±SEM).

Immunization[2]	Antibody[1]	Dry period length[3] (d)			SEM	P-values[4]				
		0	30	60		D	P	W	D × P	D × W
	Female calves (no.)	23	19	21						
Primary	IgG-KLH	5.12	5.44	5.02	0.30	0.59	0.78	<0.01	0.34	0.61
Primary	IgM-KLH	6.77	6.07	6.57	0.27	0.18	0.98	<0.01	0.47	0.71
Primary	IgG-HuSA	2.35	2.68	2.82	0.30	0.52	0.05	<0.01	0.92	0.73
Primary	IgM-HuSA	1.43	1.76	1.51	0.25	0.64	0.95	<0.01	0.99	0.11
Primary	IgT-HuSA	3.56	3.60	4.05	0.33	0.50	<0.01	<0.01	0.98	0.40
Primary	IgT-KLH	3.41	3.68	3.12	0.26	0.35	0.48	<0.01	0.42	0.13
Secondary	IgG-KLH	8.01	7.15	7.21	0.38	0.19	0.43	<0.01	0.34	<0.01
Secondary	IgM-KLH	6.99	6.48	6.84	0.24	0.33	0.26	0.17	0.18	0.41
Secondary	IgG-HuSA	4.15	4.72	5.15	0.41	0.22	0.04	0.01	0.93	0.68
Secondary	IgM-HuSA	1.84	1.44	1.23	0.21	0.11	0.53	0.28	0.46	0.68
Secondary	IgT-HuSA	5.42	6.21	6.77	0.48	0.13	0.01	<0.01	1.00	0.62
Secondary	IgT-KLH	6.58	5.94	5.69	0.37	0.21	0.73	<0.01	0.19	0.02

[1] Antibody names are combination of antigen abbreviation and immunoglobulin isotype; [2] Values on primary and secondary immunization are the number after 1st immunization and the number after 2nd immunization, respectively.[3] Values within dry period length in the same row with different superscripts differ ($P < 0.05$); [4] D = dry period; P = parity; W = week.

Table 5.4 The response of specific antibody for isotopes IgG, IgM and Total immunoglobulin (IgT) to KLH (keyhole limpet hemocyanin) and HuSA (human serum albumin) in plasma of female calves from cows with three different dry period length (0, 30 or 60 d) (LSM ± SEM).

Increase (response)[3]	Antibody[1]	Dry period length[2] (d)			SEM	P-values	
		0	30	60		Dry period	Parity
	Female caves (no.)	23	19	21			
First	IgG-KLH	2.13	0.96	1.56	0.34	0.06	0.37
	IgM-KLH	1.15	0.98	0.81	0.36	0.79	0.68
	IgG-HuSA	2.53	2.84	3.42	0.46	0.37	0.04
	IgM-HuSA	0.72	0.01	-0.002	0.28	0.12	0.95
	IgT –HuSA	2.78	3.59	4.06	0.52	0.19	0.04
	IgT-KLH	2.53[a]	1.49[ab]	1.35[b]	0.35	0.04	0.47
Second	IgG-KLH	3.37[a]	2.03[b]	2.59[ab]	0.35	0.03	0.06
	IgM-KLH	-0.07	-0.05	0.38	0.30	0.49	0.45
	IgG-HuSA	1.17	0.84	1.14	0.29	0.68	0.19
	IgM-HuSA	0.23	0.17	0.13	0.21	0.94	0.29
	IgT-HuSA	0.79	1.14	1.05	0.27	0.62	0.42
	IgT –KLH	3.39	2.22	3.16	0.34	0.05	0.04

[1] Antibody names are combination of antigen abbreviation and immunoglobulin isotype; [2] Values on primary and secondary immunization are the number after 1st immunization and the number after 2nd immunization, respectively. [3] First increase (response) is titers at week 10 minus titers week 6 and second increase is titers at week 11 minus week 10.

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5.5 Discussion

The present study shows that a 0-d dry period for dairy cows resulted in lower NAb titers binding KLH or HuSA in calves in the first 2 weeks of life, compared with calves from cows with a 30-d or 60-d dry period. Specific antibody titers binding KLH and HuSA after primary immunization in week 6 or secondary immunization in week 10 of life did not differ between calves born from mothers with different dry period lengths. After secondary immunization, however, calves from cows with a 0-d dry period showed higher IgG and IgT binding KLH titers compared with calves from cows with a 30-d or 60-d dry period. This suggests that specific humoral immune competence of calves from cows with a 0-d dry period length was not negatively affected.

Natural antibodies detected in plasma of calves immediately after birth are likely derived from colostrum since calves are born without antibodies in blood (Srinivasan et al., 1999). In our study, NAb titers in colostrum were positively correlated with NAb titers in plasma of calves during the first 2 weeks of life. In addition, natural IgG and IgM binding to KLH and HuSA in plasma of calves was correlated positively with natural IgG and IgM binding to KLH and HuSA in colostrum. In the current study, concentrations of IgG and IgM and natural IgG and IgM binding KLH or HuSA in colostrum were reduced when cows had a 0-d dry period compared with cows with a 30-d or 60-d dry period. This study concurs with results of earlier studies (Annen et al., 2004, Klusmeyer et al., 2009, Rastani et al., 2005, Verweij et al., 2014), which detected a reduction in IgG concentration in colostrum after omission of the dry period compared with a conventional dry period. Concentration of IgG was lower in present study compared with earlier studies (Annen et al., 2004, Klusmeyer et al., 2009, Rastani et al., 2005, Watters et al., 2008). In these earlier studies, single radial immunodiffusion assays (RID) was used to assess antibody level in colostrum (Annen et al., 2004, Klusmeyer et al., 2009, Rastani et al., 2005, Watters et al., 2008). In the present study, immunoglobulin concentration in colostrum was assessed by an ELISA and results were comparable with previous studies using ELISA [for IgG concentrations; 32.62 mg/mL (Horn et al., 2010) or 41.5 mg/mL (Verweij et al., 2014)].

In the present study, cows with a 0-d dry period, but not cows with a 30-d dry period, had lower colostrum production and lower colostrum immunoglobulin content at first milking compared with cows with a 60-d dry period, which is in line with previous studies (Rastani et al., 2005, Klusmeyer et al., 2009, Verweij et al., 2014). Moreover, it seems that although IgG mass produced at day of calving was

lower for cows with a 0-d dry period, the total IgG mass produced during colostrogenesis is not different from cows with a 60-d dry period (Baumrucker et al., 2014), which was explained by additional IgG production during the prepartum period for cows with a 0-d dry period, compared with cows with a 60-d dry period. This implies that colostrum production for cows with a 0-d dry period is not compromised, but secretion is distributed over more days, resulting in a lower colostrum secretion at the day of calving (Baumrucker et al., 2014).

Variation in immunoglobulin content in colostrum and milk has been explained by stage of lactation of the cows, milking processes or milking frequency (Stelwagen et al., 2009). In addition, the current study shows variation between parities. Young cows had lower NAb titers binding HuSA in colostrum than older cows, which is consistent with previous studies (Kruse, 1970, Muller and Ellinger, 1981, Pritchett et al., 1991). Moreover, in the current study natural IgT binding KLH and HuSA were lower in plasma of calves born from young cows than in calves from older cows. The same results were found for SpAb, the titers of IgT binding HuSA and specific IgG binding HuSA were lower after primary and secondary immunization in plasma of calves from young cows than in calves from older cows. Our study is in line with Conneely et al. (2013) which suggested that older cows are more likely to be exposed to a greater number of antigens in their life (Larson et al., 1980), resulting in more transfer of the antibodies from blood to colostrum and greater antibody titers in plasma of calves. In the present study all calves received 4 L colostrum; hence, intake of NAb by calves may directly reflect the concentration of (natural) IgG, and IgM present in the consumed colostrum. The current results show that albeit calves from cows with a 0-d dry period had lower titers of NAb binding KLH or HuSA in the first 2 weeks of life, calves from cows with a 0-d dry period eventually had similar titers of SpAb binding KLH and HuSA both after primary and secondary immunization. This suggests that calves with lower titers of NAb in the first 2 weeks of life mounted a higher SpAb response after primary and secondary immunization than calves with higher titers of NAb in the first 2 weeks of life. The titers of NAb may facilitate subsequent specific antibody responses (Lammers et al., 2004). The low level of NAb and high subsequent antibody response in our study were in line with earlier studies which reported that low NAb titers are related to high levels of SpAb following vaccination or immunization (Sinyakov and Avtalion, 2009), and high NAb titers that are related to low specific antibody responses (Parmentier et al., 2008). As expected, an IgG response was found after secondary challenge. Secondary IgG

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response of calves born from cows with a 0-d dry period was enhanced after secondary immunization.

Higher NAb titers in plasma have been related with higher survival rates and less disease incidence in birds (Star et al., 2007, Sun et al., 2011), fish (Kacharnakova et al., 2006, Sinyakov et al., 2002), reptiles (Ujvari and Madsen, 2011) and mammals (Ochsenbein et al., 1999, Thornton et al., 1994). It remains to be studied whether temporarily lowered NAb titers as found in calves from cows with no dry period in the present study may have consequences for disease incidence and immune competence of the calves later on. Calves from cows with the 0-d dry period received 47 percent less immunoglobulin via colostrum, whereas the calves from cows with a 30- or 60-d dry period received 25 percent more immunoglobulin than recommended [100 g of IgG by NRC (2001)]. Calves from cows with a 0-d dry period received less NAb in the colostrum but seem to be able to come to the same level of NAb during the first 6 weeks of life; thus, apparently their intrinsic capacity to produce NAb themselves may be greater than calves from cows with a 30-d or 60-d dry period. The data even indicated that calves with low NAb titers mount higher SpAb responses.

In the current study, omission of the dry period was also accompanied by a shorter pregnancy length and lower birth weight of the calves compared with calves from cows with a 30-d or 60-d dry period. In agreement with previous studies, in the current study no difference between the 30-d and the 60-d dry period was noted with respect to pregnancy length (Santschi et al., 2011b) and birth weight of calves (Pezeshki et al., 2007, Rastani et al., 2005). In the present study, no differences in growth and disease incidence of the calves were observed during the observation period of 12 weeks of life. The hygiene conditions in our study can be expected to be high. Calves from study were all raised at a specific calf raising farm. This farm is located approximately 15 km from the main research farm and raises only dairy calves for this specific research farm. The physical environmental conditions, such as housing conditions and the level of hygiene, will influence mortality and morbidity rates, effectiveness of good nutrition, and management programs (Davis and Drackley, 1998). In the present study, no intentional infection was performed; therefore no information on disease resistance was obtained. Apparently, in the current study, omitting the dry period did result in lower immunoglobulin contents and lower NAb titers in colostrum and as a consequence low NAb titers during the first 2 weeks of life in plasma of the calves, without negative consequences for growth and disease incidence during the first 12 weeks

of life. It can be hypothesized that calves that do not have the ability to recover their NAb levels, have more health problems and compromised performance in later life. During the limited observation period of 12 weeks, this was not the case. More long-term studies are needed that monitor health of calves from cows that received different dry period lengths under conditions with for example a lower hygiene status.

5.6 Conclusions

Omitting the dry period of dairy cows resulted in lower plasma NAb titers in calves in the first 2 weeks of life compared with calves from cows with a dry period of 30-d or 60-d. Lower NAb in plasma of calves was likely caused by lower immunoglobulin content in colostrum compared with calves from cows with a dry period of 30-d or 60-d. Lower NAb in plasma did not affect calf growth and SpAb titers. Calves from cows with a 0-d dry period showed a greater capacity to produce NAb themselves in the first 6 weeks and showed an increased capacity to mount specific antibody responses as exemplified during the secondary response. This suggested that calves from cows with a 0-d dry period had earlier immune maturation and had faster response to challenges than other calves.

5.7 Acknowledgements

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CHAPTER 6

Natural autoantibodies in *Bos taurus* calves during the first twelve weeks of life

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6.1   Abstract

Natural autoantibodies (**NAAb**) have a role in maintaining physiological homeostasis and prevention of infections, and have been found in mammalian species tested so far. Albeit NAAb levels rise with age, little is known about the origin, function, regulation and initiation of NAAb in young animals. The present study addressed the presence of IgM and IgG NAAb binding glutamate dehydrogenase (**GD**), carbonic anhydrase (**CA**), myosin (**MYO**) and transferrin (**TRANS**) from before drinking colostrum until the first 12 weeks of life in plasma of female calves. In addition, NAAb to these 4 self-antigens were also measured in colostrum and in plasma of their mothers during 3 weeks before calving. Titers of NAAb binding GD, CA, MYO and TRANS were detected in plasma of cows before calving, in colostrum, and in plasma of calves before and after drinking of colostrum. Levels of NAAb in colostrum were positively related with levels of NAAb in plasma of cows. Before colostrum intake, levels of NAAb in plasma of calves were not related with levels of NAAb in plasma of their mother but were influenced by parity of their mother. After colostrum intake, levels of NAAb in plasma of calves in the first week of life were positively related with levels of NAAb in colostrum. Low NAAb levels in colostrum were related with low NAAb in plasma of calves in the first week of life, but after 2 weeks of life the relation between colostrum and plasma of calves was absent. In conclusion, NAAb are already present in the unborn calf, and levels of neonatal NAAb during the early weeks of life are affected by levels of maternal NAAb obtained via colostrum.

Key words: autoantibodies, calf, colostrum, maternal antibody transfer, dry period.

6.2   Introduction

Natural antibodies (**NAb**) are a humoral part of innate immunity (Matter and Ochsenbein, 2008, Vollmers and Brändlein, 2009), and have been proposed to be involved in preventing infection and maintenance of physiological homeostasis (Ehrenstein and Notley, 2010, Lutz, 2012). Natural antibodies are polyreactive with low affinity binding with various antigens (Casali and Notkins, 1989), and are present in healthy animals in the absence of antigen stimulation (Avrameas, 1991, Baumgarth et al., 2005). Natural antibodies have been divided into 2 classes: overt and cryptic NAb. Overt NAb bind antigens that the individual has never encountered before, such as keyhole limpet hemocyanin (**KLH**). Cryptic NAb or so-called natural autoantibodies **(NAAb)** are antibodies that bind to self-antigens or slightly changed self-antigens (neo-epitopes). Natural autoantibodies are thought to be involved in inactivation of cytokines, prevention of inflammation, clearance of metabolic waste, perform various homeostatic roles within the immune response (Cojocura et al., 2009), and have a role in the prevention of autoimmunity (Nguyen et al., 2015). Antibodies binding a variety of self-antigens such as myosin, thyroglobulin (Lutz et al., 2009), heat shock proteins (Cohen, 2013), solubilized extracts of histologically normal organs were found in humans (Stahl et al., 2000) and in healthy calves (Khobondo et al., 2015). In dairy cows, NAb binding exo-antigens (Ploegaert et al., 2011, Thompson-Crispi et al., 2013, Mayasari et al., 2015) as well as self-antigens (Van Knegsel et al., 2012) were described earlier. Little is known of the routes that lead to the production of NAAb in healthy young individuals. It was proposed that NAAb may arise by cross reactivity with the intestinal microflora (Kamada et al., 2013), are initiated by dietary compounds, directed to neo-epitopes (Lutz et al., 2009, 2012), rest on random VDJ recombination in B-cells maintained by self-antigens (Quintana and Cohen., 2004), or reflect levels of maternal antibodies after consumption of colostrum or milk. In dairy cows, maternal antibodies are not actively transferred to the fetus before birth, but calves obtain maternal antibodies passively from the dam through colostrum during the first 24 hours of life. Thus, after first colostrum intake antibody titers in calves likely reflect maternal antibody titers in colostrum during the first weeks of life until neonatal antibody production starts. Until start of neonatal antibody production, adequate and sufficient passive immune transfer of maternal antibodies via colostrum intake provide immune competence of calves, which is a prerequisite to lower the risk of diseases and infections during the pre-weaning period (Oliveira et al., 2010).

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To our knowledge, little information is present about the presence of NAAb in colostrum and naïve calves and the relation between NAAb levels in cows and their calves during the early weeks of life. Earlier, we studied health and energy metabolism in cows with different dry period lengths. Omission of the dry period in dairy cows reduced NAb titers in colostrum compared with cows with a short (30-d) or conventional dry period (60-d) (Mayasari et al., 2015). Moreover, lower NAb titers in colostrum were reflected by lower titers of NAb binding keyhole limpet hemocyanin (**KLH**) and human serum albumin (**HuSA**) in plasma of calves in the first 2 weeks of life. No IgM and IgG NAb binding KLH or HuSA were detected in plasma of calves before colostrum intake. In the present study, we evaluated the level of NAAb to 4 self-antigens: glutamate dehydrogenase (**GD**), carbonic anhydrase (**CA**), myosin (**MYO**) and transferrin (**TRANS**) in plasma of calves before and after colostrum intake, and in plasma and in colostrum of their mother. Antibodies to those 4 self-antigens were found in plasma of dairy cows. Previous studies reported that self-antibodies to GD (Schulz et al., 2014) and CA (Puscas et al., 2001) are related with metabolic and health disorders in mammals. Cows with subclinical ketosis had higher non esterified fatty acid concentration and GD activation after calving (Schulz et al., 2014). Carbonic anhydrase also functions in regulation of pH and fluid balance (Badger and Price, 1994) as observed during inflammation (Mihaylova et al., 2008). Another study reported that NAAb binding MYO and TRANS in milk of cows were associated with sensitivity for mastitis (Van Knegsel et al., 2012). Myosin is a fibrous protein which has a role in muscle contraction (Schmitt., 1968). Transferrin is a serum globulin which works in the complexing and transport of iron (Crichton and Charloteauxwauters., 1987).

In the current study, it was hypothesized that NAAb binding GD, CA, MYO and TRANS are present in plasma of cows and in colostrum, but absent in plasma of calves before colostrum intake. Moreover, if omission of the dry period in cows reduced NAb titers in colostrum, NAAb titers in colostrum and in plasma of their calves may be reduced as well. Moreover, the NAAb titers in plasma of calves after colostrum intake are expected to reflect the NAAb titers of their mother (in plasma and colostrum). The present study aimed to evaluate NAAb to GD, CA, MYO and TRANS in mother and calves before and after colostrum intake until 12 weeks of age and to study relationships between these titers in mother, colostrum and the calves.

6.3   Materials and Methods

6.3.1 *Experimental Design, Animals and Rations*

The Institutional Animal Care and Use Committee of Wageningen University approved the experimental protocol. The registration number of the experimental protocol is 2010026. Blood and colostrum samples originated from an experiment that was designed to evaluate the effect of dry period length and ration composition on health of cows and calves. The experimental design, treatments of dry period lengths and ration composition in cows were described earlier by Van Knegsel et al. (2014). In short, Holstein-Friesian dairy cows (N = 167) were selected from the Dairy Campus research herd (WUR Livestock Research, Lelystad, the Netherlands) blocked according to parity, calving date, milk yield in previous lactation and body condition score (**BCS**), and randomly assigned to treatments. Treatment consisted of 3 dry period lengths: 0, 30 or 60 d and 2 lactation rations (glucogenic or lipogenic ration). For the current study, female calves born to these cows (N = 63) were monitored.

Management of Cows

Cows were housed in a freestall with slatted floor and cubicles and milked twice daily (at 0500 and 1630 h). Prepartum, dry cows received a dry cow ration and lactating cows received a lactating cow ration supporting 25 kg of milk per day. Forage was supplied ad libitum and consisted prepartum of grass silage, corn silage, wheat straw and a protein source (rapeseed meal or soybean meal) in a ratio of 39:25:25:11 (DM basis). Postpartum, forage consisted of grass silage, corn silage, straw, and a protein source (rapeseed meal or soybean meal) in a ratio 51:34:2:13 (DM basis). From d 10 before expected calving onwards, cows of all treatments were fed 1 kg/d glucogenic or lipogenic concentrate and increased postcalving stepwise with 0.5 kg/d until the concentration supply reached 8.5 kg/d. Main ingredient for glucogenic concentrate was corn and main ingredients for lipogenic concentrate were sugar beet pulp, palm kernel, and rumen protected palm oil. Concentrate and forage were supplied separately.

Colostrum Sampling and Management of Calves

Colostrum sampling and management of the calves were described earlier (Mayasari et al., 2015). Distribution of cows, colostrum sampling, plasma samples of cows, plasma samples of female calves before and after colostrum intake across dry period length treatments group are shown in Table 6.1. Colostrum was

collected and weighed immediately after calving from each quarter of mammary gland and pooled in 1 sample per cow. Calves received the colostrum from their mother via an artificial teat. Colostrum samples (N = 138; 10 mL) obtained after parturition were agitated and stored at −20°C, until analysis.

Immediately after birth, calves were removed from the dam. When calves were born between 2200 and 0500 h (N = 42), calves were removed from the dam at 0500 h. Calves were weighed and within 24 h of life, they received 4 L of colostrum in 2 portions from their mother. After 24 h after birth, female calves were fed with milk replacer (crude protein 22% and fat 17%) (2 x/ d 2 L). After 2 days, calves were moved to the calf raising farm and were also fed milk replacer twice a day, until 60-d of life. After the first week of life, all calves were fed the same mixture of hay and grains (Agrifirm Feed, the Netherlands) based on requirements for preweaned Holstein calves. At 60-d of life, calves were weaned from milk replacer. From 60-d of life calves received a diet based on requirement for weaned Holstein calves. During first 2 weeks of life, calves were housed in individual hutches located approximately 60 cm apart. After 2 weeks, calves were kept in groups of 6 to 8 calves with straw bedding. Body weight of female calves was recorded at birth and in week 2, 4, 6, 8, 10 and 12 after birth and reported earlier (Mayasari et al., 2015). Disease incidences and treatments of the female calves were recorded during the first 12 weeks of life.

In an earlier study, we evaluated the effect of maternal dry period on specific antibody responses (**SpAb**) to keyhole limpet hemocyanin (**KLH**) and human albumin serum (**HuSA**) in plasma of calves (Mayasari, et al 2015). Therefore, calves received primary and secondary immunizations with KLH and HuSA in week 6 and 10, respectively. Briefly, after 6 weeks of life, primary SpAb titers binding KLH and HuSA did not differ between calves from cows with different dry period lengths. However, secondary antibody responses were enhanced in calves from cows with a 0-d dry period (Mayasari et al., 2015).

6.3.2 Blood Sampling

For cows, blood samples were taken from the tail vain for NAAb measurement (N = 92 cows), weekly from week -3 till -1 relative to calving. For calves, blood samples were taken from jugular vein for NAAb measurement at several time points : before colostrum intake (N = 52), after colostrum intake at day 2 (after 24 h) (N = 59) and every Wednesday in weeks 1, 2, 4, 6, 7, 8, 10, 11, and 12 of life (N = 63). Blood samples were taken in Vacutainer tubes (10 mL; Greiner

Bio-One GmbH, Kremsmunster, Austria) containing heparin as anticoagulant. Blood samples were centrifuged at 3000 × *g* at 4°C, for 15 min within 2 h after collection. Plasma was transferred and stored at -20 °C until analysis.

6.3.3 *Analysis of NAAb in Plasma of Cows, Colostrum, and Plasma of Calves*

Titers of NAAb binding the 4 self-antigens GD, CA, MYO and TRANS in plasma of calves and cows and in colostrum were measured by an indirect enzyme-linked immunosorbent assay (ELISA) technique as outlined by Van Knegsel et al. (2012). In brief, plates were coated with 4 μg/mL of GD (G7882, Sigma), CA (C3934, Sigma), MYO (M0531, Sigma) or TRANS (T1408, Sigma), respectively, in 100 μl/well. Natural autoantibodies of the IgG isotype were detected either using 1:20,000 (cow) or 1:40.000 (calf, colostrum) diluted sheep polyclonal anti-bovine IgG-heavy chain conjugated to horseradish peroxidase (**PO**) (Cat. No. E10-118P, Bethyl, Laboratories). Natural autoantibodies of the IgM isotype in plasma of cows and calves and in colostrum were detected using 1:20,000 diluted rabbit polyclonal anti-bovine IgM-whole molecule conjugated to horseradish PO (Cat. No. A10-100P, Bethyl, Laboratories). Four step serial dilutions for both IgG and IgM in colostrum and in plasma samples of cows and calves started at 1:40. After washing, a substrate containing tetra methyl benzidine (Sigma Aldrich Chemie, Steinheim, Germany) and 0.05 percent hydrogen peroxide was added, and incubated for 10 min at room temperature. The reaction was stopped by adding 1.25 M sulfuric acid. Extinctions were measured with a Multiskan reader (Lab Systems, Helsinki, Finland) at a wavelength of 450 nm. Titers were expressed as log2 values of the dilutions that gave an extinction closest to 50 percent of Emax, where Emax represents the highest mean extinction of a standard positive (pooled) serum present on every microtiter plate (Ploegaert et al., 2007).

6.3.4 *Statistical Analysis*

All data of NAAb titers in cows (plasma and colostrum) and in plasma of calves approximated normality of residuals by examining whether skewness and kurtosis were in a range of -2 until 2. Moreover, the data was analysed using PROC MIXED in SAS 9.2 (SAS Inst. Inc. Cary, NC). The NAAb titers for IgG and IgM binding GD, CA, MYO and TRANS in plasma of cows before calving were analysed with dry period length (0, 30 or 60 d), parity (1, 2 or > 2), week (week -3 till -1 relative to calving), and their 2 way-interactions included as fixed effect. Cow was considered as the repeated subject. A first order auto-regressive [**AR (1)**] variance-

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covariance structure was the best fit according to Akaike's corrected information criterion (Model 1). The NAAb titers for IgG and IgM binding GD, CA, MYO and TRANS in colostrum and in plasma of calves before colostrum intake were analysed with dry period length (0, 30 or 60 d), parity of the cow (2, 3 or > 3) and their 2-way interaction included as fixed effect (Model 2).

The NAAb titers for IgG and IgM binding GD, CA, MYO and TRANS after colostrum intake (week 0 until 6) and specific antibody titers after primary (week 6 until 10) and secondary immunization (week 10 until 12) in plasma were analysed with dry period length (0, 30 or 60 d), parity (2, 3 or >3), week (1, 2,... or 12) and their 2-way interactions included as fixed effects. Calf was considered as the repeated subject. A first order auto-regressive variance-covariance structure was determined to be the best fit according to Akaike's corrected information criterion (Model 3). To compare dry period length effects, P-values are presented after a Bonferroni adjustment. Values are presented as least squares means (**LSM**) with their pooled standard errors of the mean (**SEM**), unless otherwise stated. Preliminary result showed primary and secondary immunization (in week 6 and 10, respectively) of KLH and HUSA did not affect the titers for IgG and IgM binding GD, CA, MYO and TRANS.

To assess the relationship between NAAb titers in plasma of cows with NAAb titers in colostrum or with NAAb titers in plasma of female calves (before and after colostrum intake), the average NAAb of the cows was included as continuous variable in model 2 with all 2-way interactions (Model 4). Model 4 was applied for 4 different time periods and the availability of plasma in cows and their female calves; (1) titers before colostrum intake (naïve calves) (N = 27), (2) titers after colostrum intake at day 2 (after 24 h) (N = 32), (3) the average titers in weeks 1 and 2 (N = 34), and (4) the average titers in weeks 4 till 12 of life (N = 34). Preliminary analysis showed there was no interaction between NAAb titers in cows and dry period length, therefore this interaction was excluded from the model. To assess the relationship between NAAb titers in colostrum with NAAb titers in plasma of calves after colostrum intake, the NAAb titers in colostrum was included as continuous variable in model 3 with all 2-way interactions (Model 5). The regression coefficients (β) from the statistical model and the P-value corresponding to the β are displayed.

6.4 Results

Natural autoantibody (NAAb) titers for isotypes IgG and IgM binding glutamate dehydrogenase (GD), carbonic anhydrase (CA), myosin (MYO), and

transferrin (TRANS) were detected in plasma of cows before calving (Table 6.2), in colostrum (Figure 6.1) and in plasma of calves before and after colostrum intake (Figure 6.2 and Table 6.3).

6.4.1 Natural Autoantibodies in Plasma of Cows before Calving (Model 1)

From week 3 till 1 before calving, cows with a 30-d dry period had higher plasma titers of IgG binding GD and IgM binding CA compared with cows with a 60-d dry period (Table 6.2). There was a dry period length ×week interaction for titers of IgG binding GD and CA ($P < 0.05$). Cows with a 60-d dry period had lower IgG binding GD or CA a week before calving compared with week 2 or 3 before calving (GD; 8.2 vs. 8.0 vs. 7.3 ± 0.03, CA; 6.3 vs. 6.0 vs. 5.5 ± 0.2 for weeks -3 vs. -2 vs. -1). Cows with a 0-d dry period or cows with a 30-d dry period had no difference of IgG binding GD or CA titers between different weeks prepartum. Titers of IgM binding CA and IgM binding TRANS decreased with parity (CA; 6.5 vs. 6.3 vs. 5.8 ± 0.1, TRANS 6.9 vs. 6.5 vs. 5.4 ± 0.1 for parity 1 vs. 2 vs. > 2, respectively).
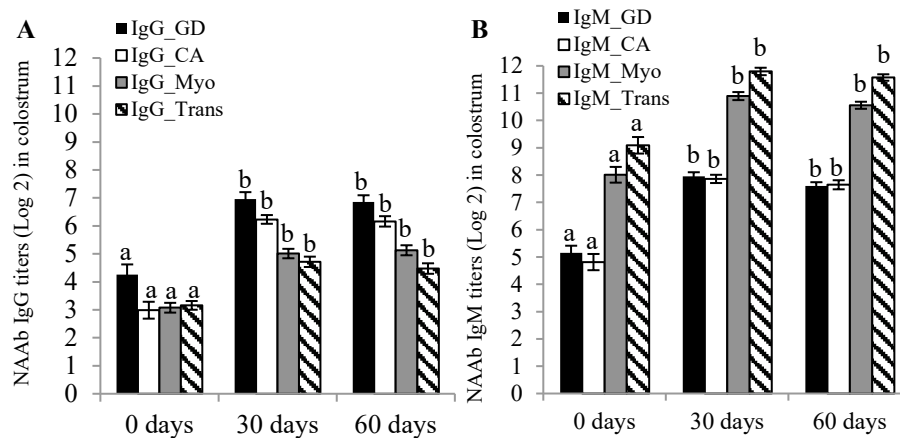
Figure 6.1 Natural autoantibodies titers (Log 2) for isotype IgG (A) and IgM (B) titers binding glutamate dehydrogenase (GD), carbonic anhydrase (CA), myosin (MYO) and transferrin (TRANS) in colostrum from dairy cows with different dry period lengths (0, 30 or 60 d) (Means ± SEM). a, b, c values between dry period length with different superscripts differ ($P < 0.05$).

6.4.2 Natural Autoantibodies in Colostrum (Model 2)

Cows with a 0-d dry period had lower titers for both IgM and IgG NAAb binding GD, CA, MYO and TRANS in colostrum compared with cows with a 30-d

or 60-d dry period (Figure 6.1). A dry period length × parity interaction was present for both titers of IgM binding GD and CA ($P < 0.05$). Older cows (parity ≥ 3) had higher titers of IgM binding GD and CA when cows had 0-d dry period compared with younger cows (GD; 4.4 vs. 5.4 vs. 5.8 ± 0.4 CA; 4.0 vs. 5.3 vs. 5.3 ± 0.5 for parity 2 vs. 3 vs. > 3, respectively). Contrary, older cows had lower titers of IgM binding GD and CA when cows had a 60-d dry period compared with younger cows (GD; 7.9 vs. 7.5 vs. 7.3 ± 0.3 CA; 8.1 vs. 7.4 vs. 7.4 ± 0.3 for parity 2 vs. 3 vs. > 3, respectively).

Table 6.1 Distribution of cows, colostrum samples, plasma in cows and plasma of female calves (before and after colostrum intake) across the dry period length.

	N	Dry period length		
		0 d	30 d	60 d
Cows in the experiments	167	56	55	56
Colostrum	138	44	45	49
Plasma of cows (before calving)	92	30	32	30
Plasma of female calves before colostrum intake	52	19	16	17
Plasma of female calves after colostrum intake	63	23	19	21

6.4.3 Natural Autoantibodies in Plasma of Calves before Colostrum Intake (Model 2)

Natural autoantibodies for both isotypes (IgG and IgM) binding GD, CA, MYO and TRANS were detected in plasma of calves at birth before colostrum intake (Figure 6.2 and Table 6.2). Dry period length of the mother did not affect NAAb titers in calves before colostrum intake. Before colostrum intake, calves from younger cows had higher titers of IgM binding GD and MYO in plasma compared with calves from older cows (GD; 2.1 vs. 1.1 vs. 1.3 ± 0.1, MYO; 4.2 vs. 2.7 vs. 2.4 ± 0.1 for parity 1 vs. 2 vs. > 2, respectively, $P < 0.05$).

6.4.4 Natural Autoantibodies in Plasma of Calves after Colostrum Intake (Model 3)

After colostrum intake (week 1) until immunizations of KLH and HuSA at week 6 of life, NAAb for isotypes IgG and IgM binding GD, CA, MYO and TRANS in plasma of calves were affected by dry period treatment of the mother especially in the first 2 weeks of life (Figure 6.2). Primary and secondary immunizations of KLH and HuSA in week 6 and 10 of life did not affect NAAb titers for IgG and IgM binding GD, CA, MYO or TRANS in plasma of calves. After colostrum intake (week 1) until 6 weeks of life, calves from cows with a 0-d

dry period had lower NAAb IgG and IgM binding GD, CA, MYO and TRANS in plasma compared with calves from cows with a 60-d (Table 6.2). Calves from cows with a 30-d dry period had no difference in IgM and IgG NAAb titers binding GD, CA, MYO or TRANS in plasma compared with calves from cows with a 60-d dry period, except for IgG binding MYO. From week 1 until 6 of life, NAAb for isotypes IgG and IgM binding GD, CA, MYO and TRANS in plasma of calves were affected by dry period and week interaction.

6.4.5 Relationship between NAAb Titers in Plasma of Cows and NAAb Titers in Colostrum and in Plasma of Calves before Colostrum Intake (Model 4)

NAAb titers (IgG and IgM) binding GD, CA, MYO and TRANS in plasma of cows before calving were all positively related with NAAb titers (IgG and IgM) binding GD, CA, MYO and TRANS in colostrum (Table 6.4). There were no relationships between NAAb titers to 4 self-antigens in plasma of cows before calving with NAAb titers to 4 self-antigens in plasma of their calves before colostrum intake.

6.4.6 Relationship between NAAb Titers in Plasma of Cows and NAAb Titers in Plasma of Calves after Colostrum Intake (Model 4)

Titer of IgG binding CA in plasma of calves after colostrum intake at day 2 was positively related with titer of IgG binding CA in plasma of cows (Table 6.4). The other titers were not correlated. In weeks 1 and 2 after birth, titers of IgG binding GD, CA and TRANS in plasma of calves were positively related with titers of IgG binding GD, CA and TRANS in plasma of cows. However, NAAb titers in plasma of calves from week 4 until 12 of life were not related with NAAb titers in plasma of cows.

6.4.7 Relationship between NAAb titers in Colostrum and in Plasma of Calves after Colostrum Intake (Model 5)

Titers of NAAb (IgG and IgM) binding GD, CA, MYO and TRANS in colostrum correlated positively with titers of NAAb (IgG and IgM) binding GD, CA, MYO and TRANS in plasma of calves after colostrum intake (at d 2) (Table 6.5). No relations were found between NAAb titers present in colostrum and NAAb titers in plasma of calves after 2 weeks of life.

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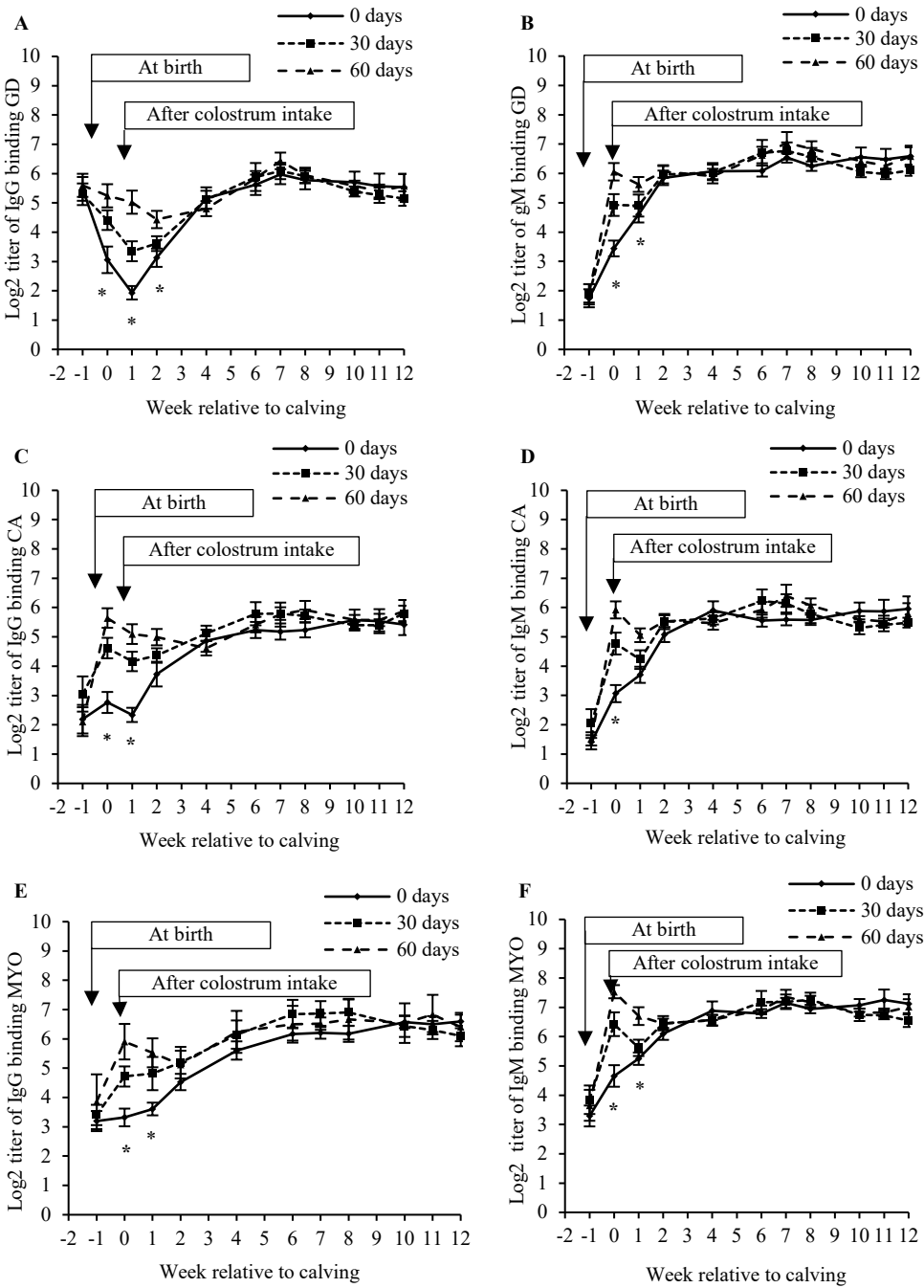
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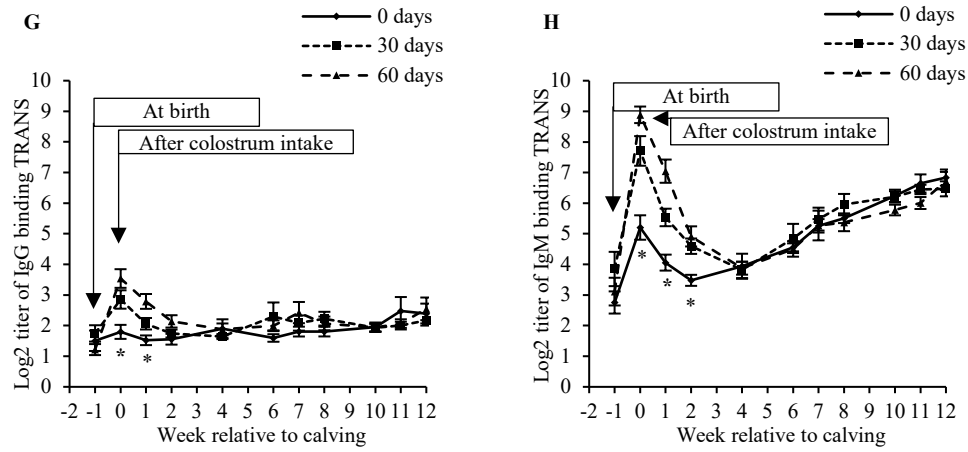**Figure 6.2** Plasma natural autoantibodies (IgG and IgM) titers binding glutamate dehydrogenase (GD; A and B), carbonic anhydrase (CA; C and D), myosin (MYO; E and F) and transferrin (TRANS; G and H) of calves at birth (before colostrum intake), after drinking colostrum from cows with 3 different dry period lengths (0, 30, or 60 d). Values are means (± SEM) per dry period length (DP) per week (W). * *P*-value < 0.05.

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Table 6.2 Plasma natural autoantibody titers (IgG and IgM) binding glutamate dehydrogenase (GD), carbonic anhydrase (CA), myosin (MYO), and transferrin (TRANS) in plasma of dairy cows with different dry period lengths (0, 30 or 60 d) during the precalving period[1] (LSM ± pooled SEM).

Item	Dry period lengths (d)			SEM	P-values[2]				
	0	30	60		Dry period	Parity	Week[1]	D × P	D × W
Cows (N = 92)	30	32	30						
NAAb IgG-GD[3]	8.09ab	8.67b	7.81a	0.23	0.03	0.39	0.05	0.88	0.01
NAAb IgM-GD[3]	6.33	6.67	6.35	0.15	0.18	0.41	0.75	0.61	0.30
NAAb IgG-CA[3]	6.14	6.45	5.94	0.18	0.12	0.71	<0.01	0.93	0.03
NAAb IgM-CA[3]	6.02a	6.49b	6.01a	0.15	0.03	0.01	0.35	0.11	0.58
NAAb IgG-MYO[3]	6.20	5.88	5.71	0.24	0.34	0.96	0.90	0.91	0.08
NAAb IgM- MYO[3]	6.20	6.57	6.30	0.20	0.38	0.63	0.38	0.31	0.11
NAAb IgG-TRANS[3]	4.82	4.89	4.78	0.19	0.93	0.94	<0.01	0.08	0.30
NAAb IgM- TRANS[3]	6.51	6.72	6.64	0.15	0.64	0.05	<0.01	0.27	0.21

[a,b] Values within dry period length in the same row with different superscripts differ ($P < 0.05$); [1] Week -3, -2, and -1 relative to calving; [2] D = dry period; P = parity (1, 2 and > 2); W=week (-3 till -1 relative to calving); [3] Antibody names are combination of natural autoantibody (NAAb), antigen abbreviation (GD, CA, MYO or TRANS) and immunoglobulin isotype (IgG or IgM).

Table 6.3 Plasma natural autoantibody titers (IgG and IgM) binding glutamate dehydrogenase (GD), carbonic anhydrase (CA), myosin (MYO), and transferrin (TRANS) in plasma of female calves from cows with different dry period lengths (0, 30 or 60 days) before colostrum intake and after colostrum intake till week 6 of life (LSM ± pooled SEM).

Item	Dry period length (d)			SEM	P-values[1]				
	0	30	60		Dry period	Parity	Week	D × P	D × W
Calves before colostrum intake (N = 52)	19	16	17						
NAAb IgG-GD[2]	6.09	5.66	5.85	0.39	0.73	0.08	NA	0.99	NA
NAAb IgM-GD[2]	1.33	1.43	1.71	0.29	0.62	0.04	NA	0.79	NA
NAAb IgG-CA[2]	2.44	2.15	2.21	0.56	0.93	0.38	NA	0.13	NA
NAAb IgM-CA[2]	1.02	1.52	1.41	0.34	0.54	0.37	NA	0.53	NA
NAAb IgG-MYO[2]	2.91	3.26	3.91	0.65	0.55	0.35	NA	0.22	NA
NAAb IgM- MYO[2]	2.89	3.16	3.30	0.43	0.78	0.01	NA	0.46	NA
NAAb IgG-TRANS[2]	1.56	1.59	1.17	0.34	0.64	0.57	NA	0.76	NA
NAAb IgM- TRANS[2]	2.30	3.32	2.93	0.46	0.28	0.19	NA	0.72	NA
Calves after colostrum intake (N = 63)	23	19	21						
NAAb IgG-GD[2]	3.86[a]	4.47[ab]	5.06[b]	0.25	<0.01	0.16	<0.01	0.80	<0.01
NAAb IgM-GD[2]	5.24[a]	5.74[b]	6.06[b]	0.17	<0.01	0.20	<0.01	0.25	<0.01
NAAb IgG-CA[2]	3.82[a]	4.81[b]	5.18[b]	0.21	<0.01	0.71	<0.01	0.21	<0.01
NAAb IgM-CA[2]	4.68[a]	5.29[b]	5.36[b]	0.17	0.01	0.10	<0.01	0.17	0.01
NAAb IgG-MYO[2]	4.67[a]	5.53[a]	5.93[b]	0.39	0.06	0.54	<0.01	0.41	0.02
NAAb IgM- MYO[2]	5.96[a]	6.46[b]	6.84[b]	0.16	<0.01	0.36	<0.01	0.45	<0.01
NAAb IgG-TRANS[2]	1.65[a]	2.13[b]	2.44[b]	0.16	<0.01	0.80	<0.01	0.72	<0.01
NAAb IgM- TRANS[2]	4.25[a]	5.30[b]	5.86[b]	0.21	<0.01	0.80	<0.01	0.44	<0.01

[a,b] Values within dry period length in the same row with different superscripts differ (*P*<0.05); [1] D = dry period, P = parity (2, 3 and >3), W=week (1, 2…6 weeks of life), NA= not applicable.; [2] Antibody names are combination of natural autoantibody (NAAb), antigen abbreviation (GD, CA, MYO or TRANS) and immunoglobulin isotype (IgG or IgM)

Table 6.4 Relationship between the average titers of natural auto antibodies (NAAb) binding glutamate dehydrogenase (GD), carbonic anhydrase (CA), myosin (MYO), and transferrin (TRANS) in plasma of cows before calving[1] with NAAb in colostrum, NAAb in plasma of calves before colostrum intake and after colostrum intake[2]. Values represent regression coefficients (β) and *P*-value.

	n[4]	Cows (week -3 until -1 relative to calving)[1,3]			
		IgG_GD	IgM_GD	IgG_CA	IgM_CA
Colostrum	74	0.454 (0.01)	0.364 (0.02)	0.388 (0.02)	0.570 (<0.01)
Calves (before colostrum intake)	26	0.541 (0.09)	-0.403 (0.26)	-0.805 (0.13)	-0.376 (0.20)
Calves (after colostrum intake at d 2)	32	0.220 (0.42)	0.336 (0.31)	0.690 (0.05)	0.531 (0.09)
Calves (week 1- 2 weeks of life)	34	0.360 (0.05)	0.168 (0.41)	0.482 (0.04)	0.012 (0.96)
Calves (4-12 weeks of life)	34	-0.134 (0.46)	0.126 (0.54)	0.192 (0.35)	-0.077 (0.69)

Table 6.4 Continued

	n[4]	Cows (week -3 until -1 relative to calving)[1,3]			
		IgG_MYO	IgM_MYO	IgG_TRANS	IgM_TRANS
Colostrum	74	0.846 (<0.01)	0.482 (<0.01)	0.533 (<0.01)	0.467 (0.01)
Calves (before colostrum intake)	26	-0.189 (0.50)	0.131 (0.71)	-0.466 (0.07)	0.297 (0.42)
Calves (after colostrum intake at day 2)	32	0.393 (0.11)	0.325 (0.13)	0.364 (0.07)	0.702 (0.09)
Calves (week 1- 2 weeks of life)	34	0.193 (0.24)	0.029 (0.82)	0.243 (0.02)	0.363 (0.11)
Calves (4-12 weeks of life)	34	-0.090 (0.51)	0.099 (0.32)	0.128 (0.30)	-0.074 (0.68)

[1] NAAb titers in plasma of cows are the average titer in weeks −3 till −1 relative to calving. [2] Calves were classified based on weeks to NAAb (titer before colostrum intake, titer after colostrum intake at day 2 (after 24 h), the average titer in the first 2 weeks (week1 and 2) and the average titer in weeks 4 till 12 of life). [3] Antibody names are combination of natural autoantibody (NAAb), antigen abbreviation (GD, CA, MYO, or TRANS) and immunoglobulin isotype (IgG or IgM). [4] Samples available in plasma of cows and colostrum (n = 74, 0-d = 21; 30-d = 25 and 60-d = 28) and in calves (in several time points: plasma of calves before colostrum intake (n = 26, 0-d = 8; 30-d = 10 and 60-d = 8), plasma of calves after colostrum intake (at day 2) (n = 32, 0-d = 11; 30-d = 10 and 60-d = 11), plasma of calves after colostrum in week 1 and 2, plasma of calves after colostrum intake from week 4 till 12 (n = 34, 0-d = 12; 30-d = 11 and 60-d = 11).

Table 6.5 Relationship between the average of natural auto antibodies (NAAb) binding glutamate dehydrogenase (GD), carbonic anhydrase (CA), myosin (MYO), and transferrin (TRANS) in colostrum and in plasma of calves after drinking colostrum. Values represent regression coefficient (β) and *P*-value.

| | n | Colostrum | | | | | | | |
		IgG_GD[1]	IgM_GD[1]	IgG_CA[1]	IgM_CA[1]	IgG_MYO[1]	IgM_MYO[1]	IgG_TRANS[1]	IgM_TRANS[1]
Day 2	50	0.658(<0.01)	0.600(<0.01)	0.570(<0.01)	0.519(<0.01)	0.270(<0.01)	0.557(0.02)	0.506(<0.01)	0.717(<0.01)
Week 1	49	0.568(<0.01)	0.082(0.51)	0.430(<0.01)	0.104(0.32)	0.477(0.04)	0.176(0.13)	0.309(<0.01)	0.250(0.09)
Week 2	51	0.481(<0.01)	0.223(0.03)	0.224(0.06)	0.030(0.75)	0.281(0.21)	0.140(0.08)	0.261(<0.01)	0.312(0.02)
Week 4	50	0.205(0.14)	-0.009(0.94)	0.116(0.32)	0.032(0.75)	0.029(0.92)	-0.084 (0.40)	0.105(0.36)	-0.062(0.68)
Week 6	51	0.180(0.29)	0.286(0.05)	0.229(0.05)	0.209(0.08)	0.110(0.67)	0.142 (0.17)	0.146(0.27)	0.080(0.60)
Week 7	49	0.126(0.35)	0.245(0.07)	0.152(0.19)	0.178(0.14)	0.117(0.45)	0.098 (0.28)	0.218(0.10)	-0.017(0.92)
Week 8	51	0.130(0.30)	0.114(0.27)	0.138(0.17)	0.106(0.23)	0.133(0.62)	0.115(0.17)	0.086(0.39)	0.006(0.96)
Week 10	51	0.071(0.55)	-0.063(0.58)	0.037(0.71)	-0.038(0.66)	0.035(0.89)	0.009(0.91)	0.046(0.49)	-0.051(0.55)
Week 11	51	0.065(0.61)	-0.020(0.82)	0.142(0.12)	0.068(0.40)	-0.045(0.85)	0.020(0.79)	0.041(0.46)	-0.030(0.71)
Week 12	50	0.230(0.20)	0.034(0.76)	0.249(0.04)	0.089(0.48)	-0.002(0.99)	-0.110(0.27)	0.323(0.01)	-0.100(0.37)

[1] Antibody names are combination of natural autoantibody (NAAb), antigen abbreviation (GD, CA, MYO, or TRANS) and immunoglobulin isotype (IgG or IgM).

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6.5 Discussion

In the present study IgM and IgG antibodies binding 4 self-antigens: GD, CA, MYO and TRANS, were detected in plasma of cows before calving, in colostrum, and in plasma of their calves both before and after colostrum intake. Previous studies showed that antibodies reacting to self-antigens can be found in plasma and milk from healthy non-immunized dairy cows (Van Knegsel et al., 2012) and in plasma from calves of 20-26 of weeks old (Khobondo et al., 2015). Together, these findings show that NAAb are present in young and old dairy cows.

The present study showed that NAAb of both antibody isotypes (IgG and IgM) binding GD, CA, MYO and TRANS were present in plasma of calves before colostrum intake. Earlier, NAb binding the exo-antigens KLH and HuSA were found in plasma from calves after colostrum intake but not before colostrum intake (Mayasari et al., 2015). The study of Mayasari et al. (2015) suggested that NAb binding exo-antigens during the first weeks of life depend on maternal transfer via colostrum and subsequent sensitization to environmental antigens, whereas the current study suggest that NAAb may be already initiated by self-antigens during the fetal phase. Knowledge of origin, induction and transfer of NAAb is scarce. The presence of NAAb may reflect cross reactivity to microbiota (Kamada et al., 2013), or represent idiotypes either initiated or maintained by self-antigens, or alternatively initiated and maintained by maternal antigens transferred from the mother to the fetus. The presence of NAAb in antigen-free mice (Haury et al., 1997), in human fetal cord blood (Lacroix-Desmazes et al., 1998) and in plasma from calves before colostrum intake (this study) suggest that NAAb do reflect auto-responsiveness. In fetal calf's, lymph nodes IgM+ cells are present at 60 d in gestation, and from day 130 plasma immunoglobulins are found (Schultz et al., 1973). It is tempting to speculate that auto-antigens in the fetus are involved in the production of auto-antibodies. VDJ recombinations and somatic mutations provide a large degree of antigen receptor diversification that is required to combat infections, but as a consequence high levels of autoimmune B cells are found in healthy humans (Wardemann and Nussenzweig, 2007) indicating that yet to be identified mechanisms prevent auto-immune diseases. In addition, repeated immunizations with exo-antigens in this study did not affect NAAb levels in the calves. This study confirms previous studies in man that NAAb binding auto-antigens like DNA, hormones, histones, enzymes, receptors, major histocompatibility antigens, and intercellular matrix components are present in healthy individuals (Poletaev and Osipenko, 2003) even without any challenges

and even without transfer of passive maternal antibodies via colostrum or intrauterine IgG transfer (Madi et al., 2009).

In the current study, titers of NAAb binding GD, CA, MYO and TRANS increased in plasma of calves after colostrum intake. A week after colostrum intake the levels of NAAb in plasma decreased in all calves, but especially in calves that received colostrum with lower levels of IgG binding GD and CA, and IgM binding TRANS, i.e. from cows with a 0-d dry period. Natural autoantibody titers for calves from cows with a 0-d dry period were specifically lower immediately after colostrum intake and in week one, but after 4 weeks the differences in titers between the 3 groups of calves were absent, suggesting an increased production of NAAb to GD, CA, MYO and TRANS in calves from cows with a 0-d dry period. Earlier we suggested that the lower NAb titers in plasma in the first 2 weeks of life of calves, which were born from cows with a 0-d dry period, were likely caused by lower immunoglobulin content in colostrum (Mayasari et al., 2015). Moreover, in this earlier study NAb titers in plasma of calves from cows with a 0-d dry period increased more between weeks 2 and 6 of age, compared with calves from cows with a 30-d or 60-d dry period. In the current study, there was no difference in the level of antibodies against self-antigens for calves from cows with a 30-d dry period, compared with calves born to cows a 60-d dry period. This is in line with the earlier study that calves born to cows with a 30-d dry period had no differences in antibodies against exo-antigens in plasma compared with calves born to cows with a 60-d dry period (Mayasari et al., 2015). Together, these findings suggest that the dynamics of NAAb binding GD, CA, MYO and TRANS in plasma of calves after colostrum intake were similar with the dynamics of NAb binding exo-antigens in calves after colostrum intake. Calves from cows with a 0-d dry period had lower NAb or NAAb in the first 2 weeks of life but eventually they had similar titers of NAb or NAAb from 6 weeks of life onwards.

In the current study, NAAb for isotypes IgG and IgM binding 4 autoantigens in plasma of calves increased after colostrum intake, except for IgG binding GD. We have no explanation for different behavior of IgG binding GD. Our observation suggested that calves already have NAAb before colostrum intake, which is markedly affected by colostral NAAb, but the level of IgG binding GD titers decreased very fast within a week after calves produced NAAb themselves. Since after a few weeks there were no differences anymore between the calves, it is questioned, whether NAAb have a specific function in colostrum and are selectively transfer to plasma. In addition, it is also questioned whether NAAb are

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waste products in the colostrum since calves seem to be able to make NAAb by themselves. A sudden increase in maternal antibodies may result in a response of the calf to get rid of IgG binding GD but this is all very speculative. A previous study showed that the mechanisms of low levels of antibodies in plasma after colostrum intake were either antigen specific or non-antigen specific (Banks, 1982). Another study showed that the concentration of colostral antibodies absorbed, the half-life of each class (isotypes) and the catabolism rate of each isotype determined the level of antibodies in plasma of calves (Murphy et al., 2014).

The low NAAb titers and subsequent higher increase in NAAb levels in calves from the 0-d dry period treated cows are in line with earlier studies which reported a negative relation between NAb and antibody responses following vaccination or immunization (Sinyakov and Avtalion., 2009). In man, colostrum of healthy mothers contains a broad spectrum of IgA (Pribylova et al., 2012, Quan et al.,1997) and IgM (Vassilev and Veleva., 1996) binding various autoantigens, which may modulate or supplement neonatal innate immunity (Pribylova et al., 2012). On the other hand, maternal antibodies, apart from protection of neonates to infection, also perform long term self-perpetuating imprinting of the neonatal B-cell repertoire by preventing development of activated self–reactive B cells by masking cross-reactive self during infections (Fink et al., 2008).

In our study, age (parity) of the mother affected the level of NAAb in plasma of calves before colostrum intake. Older cows with no dry period compared with young cows had lower IgM NAAb titers in plasma and they had higher IgM NAAb in colostrum. A previous study reported that old and young cows had no differences in NAAb in plasma or milk (Van Knegsel et al., 2012). Other studies reported that older cows had higher NAb titers binding exo-antigens like KLH and HuSA in colostrum than young cows (Muller and Ellinger, 1981, Pritchett et al., 1991, Mayasari et al., 2015). The present study is not in line with Conneely et al. (2013) who suggested that older cows have higher antibody levels because they are likely to be exposed more to a greater number of antigens in their life (Larson et al., 1980). Low NAAb in plasma of cows may result from transfer of antibodies from blood to colostrum. Furthermore, IgM NAAb in plasma of calves before colostrum intake were lower in plasma of calves from older cows. In man, several genes (e.g. V_H genes) encoding some NAAb in the mother were shown to predominate in the newborn (Lacroix-Desmazes et al., 1998). In mice, the level of IgM binding self-antigens was high in plasma during fetal development and in

newborns (Nobrega et al., 1996). Yet, we have no explanation how parity of the cows may affect the level of NAAb in plasma of their calves, because cows do not transfer antibodies to the fetus. Therefore, it is tempting to speculate that the level of NAAb in plasma of naïve calves may represent a health status biomarker of themselves or their mother.

To our best knowledge, this is the first study presenting the relationship between cows (plasma and colostrum) and their calves during the first weeks of life with respect to NAAb. In the current study, omitting the dry period resulted in lower levels of IgM binding CA in plasma of cows 3 weeks before calving and lower levels of IgG and IgM binding GD, CA, MYO or TRANS in colostrum compared with cows with a 30-d or a 60-d dry period. Earlier studies (Rastani et al., 2005, Verweij et al., 2014, Mayasari et al., 2015) reported that omission of the dry period reduced colostrum immunoglobulin contents and reduced NAb levels in colostrum at day of calving (Mayasari et., al 2015). Although titers of NAAb were detected in plasma of calves immediately after birth, the titers of NAAb in plasma of the calves before colostrum intake had no correlations with the averages of NAAb in plasma of their mother. The NAAb titers in plasma of calves increased directly after colostrum intake indicating that the high plasma NAAb titers in calves immediately after colostrum intake are likely derived from colostrum (Srinivasan et al., 1999). The levels of NAAb in plasma of calves during the first week of life were positively correlated with the level of NAAb in colostrum. This suggests that the level of antibodies in plasma of calves during the first weeks of life reflect the level of antibodies in colostrum and plasma of their mothers. It is suggested that Fc receptor mediated transfer for IgG and IgM (Lascelles, 1979, Hurley and Theil, 2011) has a role in transferring antibodies from plasma to colostrum and milk in dairy cows (Kacskovics et al., 2000). The level of NAAb in colostrum had no relation with the levels of NAAb in plasma of calves after 2 weeks of life. Low NAAb levels after colostrum intake coincided with a faster increase of NAAb levels within 3 till 4 weeks after birth. Thus, dry period treatment of cows had temporary transgenerational effects on calves. Similar with the increase of NAb to exo-antigens (Mayasari et al., 2015), calves may build up their own immune repertoire after the first 2 weeks of life regardless of their mother. In humans, high titers of NAAb binding self-antigens in plasma of the mother might reduce the incidence of diseases of their offspring (Tzioufas and Routsias, 2010). Our data indicate that the 2 weeks period in which calves received

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less colostrum from (0-d dry period) mothers did not result in enhanced disease sensitivity during the 12 weeks observation period.

6.6   Conclusions

In conclusion, IgG and IgM antibodies binding GD, CA, MYO and TRANS were detected in plasma of cows before calving, in colostrum and in plasma of calves before and after colostrum intake. The level of NAAb in plasma of calves before colostrum intake was not affected by the level of NAAb of their mother but were affected by parity of their mothers. After colostrum intake, calves from cows with no dry period (i.e. receiving the lower amount of maternal NAAb after drinking) had a rapid increase in NAAb to similar levels as the other calves. Our data indicate that; (1) low levels of maternal NAAb in colostrum affect production of neonatal NAAb positively and (2) independent of dry period length, healthy calves reach a specific level of NAAb from 6 weeks of age. Future studies should reveal the consequences of low or high levels of (other) NAAb from either neonatal or maternal of origin on disease sensitivity of calves later in life.

6.7   Acknowledgements

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CHAPTER 7

General discussion

7.1 Introduction

In early lactation, dairy cows experience a negative energy balance (**NEB**) caused by a sudden increase of energy requirement for milk production while the dietary energy intake is limited. The NEB has been related to an increased occurrence of metabolic disorders (Grummer, 1993), infectious diseases (Collard et al., 2000), reduction in fertility (Butler, 2003), inflammation (Bionaz et al., 2007, Trevisi et al., 2012), immunosuppression (Mallard et al., 1998) and oxidative stress (Sordillo et al., 2009). Cows with NEB and elevated plasma free fatty acid (**FFA**) and β-hydroxybutyrate (**BHB**) concentrations have a higher occurrence of clinical problems (Ospina et al., 2010b, Chapinal et al., 2011, Seifi et al., 2011) and reduced fertility (Walsh et al., 2007, Ospina et al., 2010a). Extensive research efforts have been made to find ways to reduce the health problems in early lactation by improving the energy balance (**EB**) in dairy cows (as reviewed by Ingvartsen and Moyes, 2013, Van Knegsel et al., 2014a).

Recently, there has been an interest in shortening the dry period as a strategy to improve EB in early lactation of high yielding dairy cows. Shortening or omitting the dry period shifts milk yield partially from postcalving to precalving period and, herewith, improves EB (Rastani et al., 2005, Van Knegsel et al., 2014b) and metabolic status in early lactation (Andersen et al., 2005, de Feu et al., 2009). In addition, shortening or omitting the dry period also improved fertility by reducing the interval from calving to first ovulation postpartum (Gümen et al., 2005, de Feu et al., 2009, Chen et al., 2015b). Meta-analysis showed that a short dry period tended to reduce the risk of ketosis (odd ratio = 0.75; P = 0.09) but did not affect occurrence of clinical mastitis, retained placenta or displaced abomasum compared with a conventional dry period (Van Knegsel et al., 2013). Studies on consequences for disease incidence of omitting the dry period are rare and often limited on animal numbers, but studies of Rastani et al. (2005) and Schlamberger et al. (2010) showed no effect of omitting the dry period on diseases incidence (clinical mastitis, retained placenta, ketosis and lameness) compared with a conventional dry period. It can be hypothesized that if shortening or omitting the dry period improves the EB of cows in early lactation, this will also improve health and fertility of dairy cows with a short or no dry period, compared with cows with a conventional dry period. The main objective of this thesis was to investigate effects of dry period length and dietary energy source on health status related variables in dairy cows and their calves.

7.2 Biomarkers for Health in Dairy Cows

Animal health is an important factor for productive life time in dairy cows (Booth et al., 2004, Carlén et al., 2004). A clinical health problem can be determined by clinical examinations combined with various diagnostic tests (Petersen et al., 2004). Measuring health status through various diagnostic tests is essential to generate an important research tool when studying pathogenesis and inflammatory processes. In the present study, we refer to health problems as clinical health problems related to inflammation, such as clinical mastitis, metritis, retained placenta and fever. Subclinical disease as a response of inflammatory processes but impact on animal health may remain undetected and may be indirectly reflected by impaired growth and fertility (Petersen et al., 2004). Identification of biomarkers for various clinical and subclinical diseases would be useful to assess early diagnosis, treatment, and prevention of important diseases (Petersen et al., 2004). A biomarker is defined as a biological characteristic that can be objectively measured as an indicator of normal biological processes or a response to environmental intervention (Van Altena, 2016). In this thesis, potential biomarkers are associated with health status in dairy cows such as acute phase proteins (**APP**), liver functionality variables (Bionaz et al., 2007, Bertoni et al., 2008), oxidative stress (Bernabucci et al., 2005) and natural antibodies (**NAb**) (Van Knegsel et al., 2007a, Ploegaert et al., 2010) are used. In this thesis, we refer to those potential biomarkers as health variables.

In human and veterinary medicine, levels of APP in plasma have been used as a biomarker for infection and inflammation. As quantitative biomarkers for infection, APP can be used in diagnosis, prognosis and in monitoring response to general health screening in dairy cows (Petersen et al., 2004, Gruys et al., 2005, Eckersall and Bell, 2010). During NEB and inflammation, pro-inflammatory cytokines stimulated hepatic synthesis of positive APP, such as haptoglobin and ceruloplasmin, and impaired hepatic synthesis of negative APP such as albumin and cholesterol (Bionaz et al., 2007, LeBlanc, 2012). Bilirubin and paraoxonase are commonly used as biomarkers for liver status around calving (Bionaz et al., 2007, Bertoni et al., 2008). High bilirubin and low paraoxonase levels have been associated with liver damage (Bionaz et al., 2007, Bertoni et al., 2008). Reactive metabolites oxygen (**ROM**) and ferric reducing antioxidant power (**FRAP**) are known as oxidative stress variables. Ferric reducing antioxidant power is an indicator of antioxidant status (Jacometo et al., 2015, Konvičná et al., 2015), and acts to neutralize the production of reactive intermediates (e.g. ROM) caused by oxidative stress in early lactation (Esposito et al., 2014). Creatinine is a marker for

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mobilization of body protein. Serum NAb levels have been suggested as biomarkers for general disease resistance in dairy cows (Van Altena, 2016). Natural antibodies have an important function to provide not only the first line of defence but also to maintain homeostasis, to promote phagocytes of dead cells, and to prevent infectious and autoimmune diseases (Cojocaru et al., 2009). The level of antibodies in plasma is highly related to disease resistance with respect to infections and metabolic disorders (Baumgarth et al., 2005).

7.3   Effect of Shortening or Omitting the Dry Period on Health Variables

This paragraph discusses the effects of dry period length on inflammatory biomarkers (positive and negative APP), liver functionality (paraoxonase and bilirubin), oxidative stress (ROM and FRAP), NAb, NAAb and creatinine over 2 subsequent lactations.

7.3.1 Effect of Dry Period Length on Inflammatory Biomarkers

Omitting the dry period increased positive APP levels (ceruloplasmin and haptoglobin) and negative APP (cholesterol) levels in plasma in the first subsequent lactation after implementation of dry period length treatments **(Chapter 2)**. In the second subsequent lactation after implementation of dry period length treatments, omitting the dry period resulted in higher ceruloplasmin levels in plasma but did not affect plasma cholesterol levels compared with a dry period of 30-d or 60-d. In the second subsequent lactation after implementation of dry period length, cows which were planned for 0-d but dried themselves off (0→30-d) had a lower plasma ceruloplasmin levels compared with cows with a 0-d dry period (**Chapter 3**). This finding is partly unexpected. It was hypothesized that if EB and metabolic status are improved, due to omitting or shortening of the dry period, this will reduce the inflammatory status (i.e. higher levels of negative APP, and lower levels of positive APP) during the transition period. Previous studies showed that in early lactation cows with a better EB and improved metabolic status had increased negative APP in plasma like cholesterol (Kaneene et al., 1997, Trevisi et al., 2009). It can be suggested that the increased cholesterol levels in plasma in the first subsequent lactation after implementation of dry period length in the present study is not only related to better EB in cows with a 0-d dry period but also because of the differences in energy intake and ration composition. Earlier, ceruloplasmin and haptoglobin were negatively related to EB (Bionaz et al., 2007). In the current study, however, the improved EB in cows with 0-d dry period did not result in lower ceruloplasmin or haptoglobin levels in plasma, compared with cows

with a 60-d dry period. In addition, ceruloplasmin and haptoglobin levels in plasma were positively related to occurrence of clinical health problems. Also in earlier studies, the increased levels of ceruloplasmin and haptoglobin were associated with health problems of cows in early lactation (Conner et al., 1988, Chassagne et al., 1998, Sheldon et al., 2001). It appears that, besides dry period length and EB, also the occurrence of clinical health problems might cause increased levels of haptoglobin and ceruloplasmin in plasma of dairy cows in early lactation.

7.3.2 *Effect of Dry Period Length on Liver Functionality*

Chapter 2 described that in the first subsequent lactation after implementation of dry period length treatments, omitting the dry period improved the EB and reduced bilirubin levels in plasma which was in line with expectations. The reduced bilirubin levels in plasma in cows with a 0-d dry period means that these cows have better liver function compared with cows with a 60-d dry period, which is related to the better EB and reduced milk yield in these cows. It was hypothesized that if EB is improved, due to omitting the dry period, bilirubin levels in plasma will reduce as well. Plasma bilirubin levels were positively correlated with the degree of fat infiltration of the liver which is caused by the accumulation of triacylglycerol (**TAG**) in the liver (West, 1990). Also in the current experiment, cows with a 0-d dry period had a lower plasma FFA and liver TAG concentration, compared with cows with a 30-d or 60-d dry period (Chen et al., 2015a). The lower liver TAG concentration was in line with reduced plasma bilirubin concentration in cows with a 0–d dry period. In the second lactation after implementation of dry period length treatments, plasma bilirubin levels were not different among cows with different dry period lengths (**Chapter 3**). However, cows which were planned for a 0-d dry period but dried themselves off (0→30-d) had a higher plasma bilirubin levels compared with cows with a 0-d, 30-d or 60-d dry period. The increased bilirubin in cows with 0→30-d dry period suggests that these cows have worse liver function compared with cows with a 0-d, 30-d, or 60-d dry period, which was explained by the more severe NEB in these cows. Reduced bilirubin levels indicated better clearance of secretory enzymes in the liver as response to liver cell damage and have been associated with a better EB (Assenat et al., 2004, Bertoni et al., 2008). Chen et al. (2016) reported for this experiment that in the second subsequent lactation, cows which were planned for 0-d but dried themselves off (0→30-d) had severe NEB compared with cows with a 0-d or 30-d dry period. Moreover, in the second subsequent lactation, omitting the dry period

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resulted in a less pronounced improvement of EB and metabolic status in early lactation compared with the first subsequent lactation after implementation of dry period length treatments (Chen et al., 2016).

It can be hypothesized that if EB improves and bilirubin in plasma reduces by omitting the dry period, the levels of paraoxonase in plasma will increase in cows with a 0-d dry period. Paraoxonase is known as a liver protein with hydrolase activity and may serve as an index of liver function because it is drastically reduced in chronic liver damage (Bionaz et al., 2007) or during lipid metabolic disorders (Ferré et al., 2002, Aviram and Rosenblat, 2004, Turk et al., 2004). As mentioned before, cows with a 0-d dry period had better liver functionality which was indicated by reduced TAG in liver and plasma bilirubin levels, however, but not with respect to paraoxonase. In **Chapter 2**, paraoxonase was negatively associated with haptoglobin and ROM which could be related to a more severe inflammatory condition or due to oxidative stress. Paraxonase is also known as an anti-inflammatory variable (James and Deakin, 2004) and as an important anti-oxidant variable (Bell et al., 2010). Thus, we suggest in that case the reduced paraoxonase levels in plasma could not be explained by liver status but might be explained by inflammation or oxidative stress.

7.3.3 *Effect of Dry Period Length on Oxidative Stress*

Omitting the dry period resulted in increased oxidative stress (increased ROM levels in plasma) over 2 subsequent lactations which was not related to the better EB and improved metabolic status in these cows (**Chapter 2 and 3**). High ROM levels in plasma were earlier associated with severe NEB indicated by increased BHB and FFA concentrations (Bernabucci et al., 2005, Pedernera et al., 2010). In early lactation, cows with a 0-d dry period produced less milk yield, resulting in lower metabolic demand and lower oxygen requirement compared with cows with a 60-d dry period. Accumulation of reactive oxygen species can be prevented by reduced oxygen requirement for metabolic processes (Spears and Weiss, 2008) and increased the availability of antioxidant defence systems such as FRAP (Abuelo et al., 2015). In the current study, there was no effect of dry period length on FRAP levels in plasma. Together with low paraoxonase levels in plasma in cows with a 0-d dry period, availability of antioxidant FRAP and paraoxonase levels in plasma is not sufficient to prevent oxidative stress in these cows. It is suggested that high ROM levels in plasma in cows with a 0-d dry period could be

explained by the occurrence of health problems related to inflammation, rather than directly explained by the (better) EB in these cows.

7.3.4 *Effect of Dry Period Length on Natural Antibodies*

Increased incidences of infectious diseases in cows with NEB have been attributed to suboptimal immune function in early lactation (Mallard et al., 1998). In the present study (**Chapter 4**), cows with a 0-d dry period not only had improved EB in early lactation but also higher somatic cell count (**SCC**), higher NAb titers for isotypes IgG and IgM binding keyhole limpet hemocyanin (**KLH**) and lipopolysaccharides (**LPS**) in milk, and higher titer of IgG binding LPS in plasma compared with cows with a 30-d or 60-d dry period in the first lactation after implementation of dry period length treatments. In addition, cows with 0-d dry period had higher IgG natural autoantibodies (**NAAb**) binding myosin (**MYO**) in plasma compared with cows with a 60-d dry period (Table 7.1). In the second lactation after implementation of a short or no dry period, the effects of omitting the dry period on NAb titers binding KLH and LPS in plasma and milk and NAAb binding MYO in plasma disappeared. Natural antibodies act as a first line of defence against infection (Ochsenbein et al., 1999, Zinkernagel, 2012) but also to maintain homeostasis, to promote phagocytes of dead cells, and to prevent infectious and autoimmune diseases (Cojocaru et al., 2009). In an earlier study, high NAAb binding MYO in milk of cows were associated with severe NEB (β = -0.001, P < 0.10), low dry matter intake (**DMI**) (β = -0.049, P < 0.05) and occurrence of clinical mastitis (Van Knegsel et al., 2012). In the study of Van Knegsel et al. (2012), NAAb binding MYO was measured in milk, but not in plasma. Measuring antibodies in milk compared with plasma is easier but may give different results due to low phenotypic correlation between NAbs in blood and NAbs in milk.

In the second lactation after implementation dry period length treatments, cows with a 0-d dry period compared with cows with a 30-d dry period have a lower NAAb IgM binding carbonic anhydrase (**CA**; enzyme that involve in the regulation of pH during inflammation), but not compared with cows with a 60-d dry period (Table 7.1). This is may be related to the fact that in the second subsequent lactation, omitting the dry period resulted in a less pronounced improvement of EB and metabolic status in early lactation compared with the first subsequent lactation after implementation of dry period length treatments (Chen et al., 2016). Carbonic anhydrase have functions in regulation of pH and fluid balance (Badger and Price, 1994) as observed during inflammation (Mihaylova et al.,

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2008). However, NAAb binding CA in plasma is associated with SCC (Figure 7.2). It is suggested that the higher NAb titers in plasma and in milk for cows with a 0-d dry period in first and second lactations after implementation of dry period length treatments are related with the improved EB and reduced milk yield in these cows (**Chapter 4**). It might be speculated that NAAb binding CA in plasma reflects a subclinical udder infection in cows with a 0-d dry period.

7.3.5 *Effect of Dry Period Length on Creatinine*

In the first lactation after implementation dry period length treatments, dry period length affected creatinine levels dependent on parity. Cows with parity ≥ 3 had higher levels of creatinine in plasma when had a 0-d dry period compared with cows with parity 2 (**Chapter 2**). In the second subsequent lactation after implementation dry period length treatments, creatinine did not different among dry period length treatments. However, cows which were planned for a 0-d dry period but dried themselves off (0→30-d) had higher plasma creatinine levels compared with cows with a 60-d dry period (**Chapter 3**), which was possibly related to the lower EB in these cows. In the first subsequent lactation of the current experiment, during the first 2 weeks after calving cows with parity ≥ 3 cows showed less reduction in milk yield when the dry period was omitted compared with cows with parity 2. Creatinine typically decreases around calving due to milk production (Kokkonen et al., 2005). Previous studies from Annen et al. (2004) and Santschi et al. (2011) showed a reduction in milk yield after shortening or omitting the dry period for cows with parity 2, but not for cows with parity > 3. Thus, it suggests that the increased creatinine levels in plasma of cows with a 0-d dry period in the first subsequent lactation could be explained by parity. In the current experiment, cows which were planned for a 0-d dry period but dried themselves off (0→30-d) not only had severe NEB but also had high precalving BCS (Chen et al., 2016). Cows with severe NEB, also have the more severe mobilization of muscle (Graugnard et al., 2012). Previous study from Pires et al. (2013) showed cows with high BCS group had higher plasma creatinine and severe NEB compared with cows with low BCS. Thus, this study suggested that the increased plasma creatinine levels in cows which were planned for a 0-d dry period but dried themselves off (0→30-d) was related to high precalving BCS and a more severe NEB in these cows.

Table 7.1 Plasma natural autoantibody titers (IgG and IgM) binding glutamate dehydrogenase (GD), carbonic anhydrase (CA), myosin (MYO), and transferrin (TRANS) of dairy cows with varying dry period lengths (0, 30, 60, or 0→30[l] days) and fed either a glucogenic (G) or a lipogenic (L) ration in first and second lactations after implementation of dry period length treatments (LSM ± pooled SEM).

Item	Dry period length (d)					Ration			P-values[2]							
	0	30	60	0→30[l]	SEM	G	L	SEM	D	R	P	W[3]	D×R	D×P	D×W	P×R
1st lactation[1]																
Cows (n=92)	30	32	30	NA	46	46	46									
NAAb IgG-GD[4]	8.28	8.33	7.87	NA	0.17	8.00	8.32	0.14	0.11	0.09	0.41	0.26	0.04	0.56	0.02	0.46
NAAb IgM-GD[4]	6.19	6.21	6.00	NA	0.10	6.02	6.25	0.08	0.25	0.04	0.07	<0.01	0.16	0.46	0.86	<0.01
NAAb IgG-CA[4]	6.10	6.28	5.84	NA	0.13	6.09	6.06	0.11	0.06	0.86	0.22	<0.01	0.29	0.21	0.38	0.97
NAAb IgM-CA[4]	5.86[ab]	6.02[a]	5.61[b]	NA	0.11	5.82	5.84	0.09	0.02	0.86	<0.01	<0.01	0.19	0.13	0.64	0.24
NAAb IgG-MYO[4]	6.01[a]	5.60[ab]	5.42[b]	NA	0.17	5.56	5.80	0.14	0.05	0.21	0.86	0.01	0.17	0.63	0.44	0.81
NAAb IgM-MYO[4]	5.87	6.08	5.77	NA	0.11	5.79	6.04	0.09	0.11	0.04	0.85	<0.01	0.03	0.06	0.88	<0.01
NAAb IgG-TRANS[4]	4.60	4.52	4.37	NA	0.13	4.38	4.61	0.11	0.49	0.13	0.94	<0.01	0.34	0.01	0.84	0.16
NAAb IgM-TRANS[4]	6.20	6.15	6.09	NA	0.09	6.12	6.16	0.07	0.68	0.69	0.01	<0.01	0.11	0.03	0.60	<0.01
2nd lactation[1]																
Cows (n=114)	17	34	44	19		58	56									
NAAb IgG-GD[4]	8.58	8.47	8.26	8.37	0.17	8.35	8.50	0.12	0.50	0.37	0.53	0.03	0.73	0.53	0.75	0.05
NAAb IgM-GD[4]	7.78	8.00	7.86	7.83	0.10	7.78	7.95	0.07	0.37	0.08	<0.01	<0.01	0.19	0.32	0.26	0.02
NAAb IgG-CA[4]	6.65	6.66	6.67	6.62	0.15	6.61	6.70	0.11	1.00	0.55	0.48	<0.01	0.50	0.40	0.11	0.08
NAAb IgM-CA[4]	7.12[a]	7.54[b]	7.37[ab]	7.18[ab]	0.10	7.27	7.34	0.07	0.02	0.49	<0.01	<0.01	0.11	0.09	0.27	<0.01
NAAb IgG-MYO[4]	5.45	5.31	5.60	5.70	0.17	5.42	5.61	0.12	0.32	0.25	0.07	0.03	0.55	0.18	0.49	0.07
NAAb IgM-MYO[4]	4.69	4.91	4.83	4.99	0.12	4.86	4.86	0.09	0.48	0.97	<0.01	<0.01	0.52	0.07	0.37	<0.01
NAAb IgG-TRANS[4]	4.25	4.19	4.17	4.42	0.18	4.14	4.38	0.13	0.76	0.17	0.49	<0.01	0.09	0.87	0.89	<0.01
NAAb IgM-TRANS[4]	4.41	4.45	4.45	4.25	0.11	4.36	4.42	0.08	0.57	0.58	0.08	<0.01	0.02	0.52	0.33	<0.01

Continued on the next page

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Table 7.1 continued

7.4 Effect of Dietary Energy Source on Health Variables in Dairy Cows

Feeding a glucogenic or lipogenic ration did not affect occurrence of health problems in dairy cows in early lactation in both lactations in the present experiment. In the first lactation after implementation of dry period and dietary treatments, feeding a glucogenic or lipogenic ration affect negative APP and NAb, dependent on parity. In the first lactation after implementation of dry period and dietary treatments, cows with parity 2 and fed a glucogenic ration had higher NAb titer in plasma and milk (**Chapter 4**) compared with cows with parity 2 and fed a lipogenic ration. Moreover, cows with parity > 3 and fed a glucogenic ration had higher cholesterol levels (**Chapter 2**) compared with cows with parity > 3 and fed a lipogenic ration. In an earlier study, a glucogenic ration reduced milk energy output and herewith improved the EB of dairy cows in early lactation, compared with a lipogenic ration (Van Knegsel et al., 2007b), which was related to higher titers of NAb binding KLH in plasma. Also in the current experiment, feeding a glucogenic ration in early lactation improved EB by a reduction in milk energy output, realised by a reduction in milk fat content, compared with a lipogenic ration (Van Knegsel et al., 2014b). Moreover, this improvement in EB was also associated with an improved metabolic status (e.g. reduced plasma FFA and BHB concentrations), independent of dry period length (Chen et al., 2015a). Severe NEB has been associated with inflammation as indicated by increased haptoglobin and reduced cholesterol (Bionaz et al., 2007, Trevisi et al., 2012). Thus, it could be expected that when feeding a glucogenic ration improves the EB in early lactation it also reduces inflammation and increases NAb titers in plasma. In the second lactation after implementation of dry period length and dietary treatments, ration had more effect on health variables, compared with the first lactation. In the second lactation after implementation of dry period length treatments, cows fed a lipogenic ration had higher plasma cholesterol level in plasma compared with cows fed a glucogenic ration, independent of dry period length (**Chapter 3**). In addition, cows

with a 0-d dry period and fed a lipogenic ration had higher FRAP and creatinine levels in plasma compared with cows with a 0-d dry period and fed a glucogenic ration (**Chapter 3**). In the second lactation after implementation of the dry period length treatments, Chen et al. (2016) reported for the current experiment that a glucogenic ration compared with a lipogenic ration resulted in lower plasma FFA and BHB concentrations. Reduced plasma FFA and BHB concentrations have been associated with increased cholesterol (Bionaz et al., 2007, Trevisi et al., 2012) but not related to plasma FRAP (Wullepit et al., 2012) or creatinine (Bionaz et al., 2007). However, no ration effect on FRAP in the study of Wullepit et al. (2012) and no dietary treatment in the study of Bionaz et al. (2007) and Trevisi et al. (2012). Earlier, cholesterol levels were influenced by fat supplementation (Carroll et al., 1990). Thus, it might be speculated that the high cholesterol levels in the second lactation is related to the high fat content in lipogenic ration, compared with a more glucogenic ration.
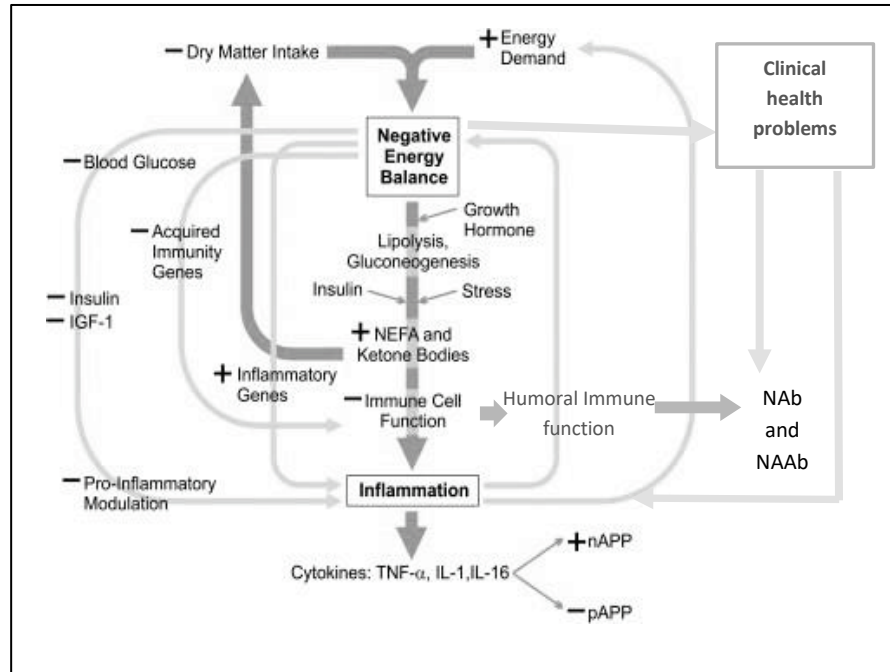
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Figure 7.1 Interaction among immunity, endocrinology, metabolism, and clinical health problems in dairy cows during the transition period (*adapted from Esposito et al., 2014*).

7.5 Interaction among Immunity, Endocrinology, Metabolism and Clinical Health Problems in Dairy Cows.

Inflammation and dysregulated immune function have been associated with NEB and metabolic disorders in dairy cows in early lactation (Esposito et al., 2014). Figure 7.1 illustrates that NEB in dairy cows in early lactation is not only related to metabolic disorders and inflammation but also to immunosuppression and occurrence of clinical health problems. During NEB, many metabolic hormones such as insulin, growth hormone and their receptor changes as a response to physiological needs in this period (Goff and Horst, 1997). During NEB, the growth hormone-IGF axis uncouples due to a down-regulation of liver growth hormone receptor which is associated with a reduction of insulin concentration (Butler, 2003). Low insulin concentration also increase mobilization of triacylglycerol (**TAG**) deposits as FFA and ketone bodies (Grummer, 1993). High FFA and ketone

bodies in plasma during severe NEB increase the risk for fatty liver and ketosis (Grummer, 1993), dysfunctional immune cells (Mallard et al., 1998, Lacetera et al., 2005) and increase pro-inflammatory cytokines (Bionaz et al., 2007, Trevisi et al., 2012). Pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 regulate APP concentration in plasma (Baumann and Gauldie, 1994). The liver plays a central role in these changes, as the hepatic synthesis of many proteins increase (positive APP) whiles the synthesis of other proteins decrease (negative APP). Cows with severe NEB as indicated by high FFA and ketone bodies concentrations in plasma have been related to the occurrence of health problems related to inflammation such as clinical mastitis (Mallard et al., 1998, Sordillo et al., 2009), metritis and retained placenta (Enevoldsen and Sørensen, 1992, Butler, 2003, Bertoni et al., 2009). Negative energy balance has been associated with level of humoral innate immunity such as low NAb in plasma (Van Knegsel et al., 2007a) (**Chapter 4**) and low IgM antibodies (Lacetera et al., 2005). Cows with NEB and uterine infection had higher haptoglobin, lower albumin and lower paraoxonase in plasma (Schneider et al., 2013) (**Chapter 3**). Cows with clinical mastitis had higher levels of haptoglobin in plasma (Pyörälä, 2003) and lower titers of NAb binding KLH in plasma (Ploegaert et al., 2010, Thompson-Crispi et al., 2013) (**Chapter 4**), compared with healthy cows. Clinical health problems related to inflammation might be reflected by the changes in inflammatory biomarkers (**Chapter 2 and 3**) and titers of NAb (**Chapter 4**).

7.6 What Explains (Lack of) Effects of Dry Period Length and Dietary Treatments on Health in Dairy Cows

The current study shows that omitting the dry period not only improves EB and metabolic status but also influences health variables such as increased levels of positive APP, increased levels of negative APP, reduced levels of bilirubin, increased oxidative stress, increased NAb levels in plasma and milk. It was hypothesized that an improvement of the EB due to shortening or omitting the dry period would be beneficial for health of the dairy cows, the changes of health variables due to dry period length treatments could not be completely explained by the effects of dry period on the EB. It can be questioned whether the changes of health variables in the current experiment was directly due to dry period length effects, ration effects or indirectly through effect of dry period length on differences in EB, mammary health, clinical health problems, body condition, or uterine health of cows.

7.6.1 Effect of Energy Balance on Health Variables in Dairy Cows

As mentioned previously, omitting the dry period improved EB (Rastani et al., 2005, Van Knegsel et al., 2014a) and metabolic status in early lactation (Andersen et al., 2005, de Feu et al., 2009, Chen et al., 2015a) compared with a conventional dry period. A better EB is only related to low plasma bilirubin levels but is not related to APP, oxidative stress, creatinine and NAb (**Chapter 2 and 3**). Milk yield is positively related to the levels of bilirubin. Dry matter intake is negatively related to levels of bilirubin. Since the combination of both milk yield and DMI results in EB, it can be suggested that the effect of dry period length on bilirubin levels was associated with alterations in EB and metabolic status. In the present experiment, cows with NEB had increased plasma concentrations of FFA and bilirubin. This finding is expected because the FFA concentration was positively related to the levels of bilirubin in plasma (Qualmann et al., 1997). Variations of FFA and bilirubin concentrations over time were the most pronounced in the first 2 weeks after calving, compared with the period of week 1 to 9 postcalving. In the present experiment, FFA and bilirubin concentrations in plasma were reduced after the first 2 weeks after calving which may indicate better metabolic status and liver functionality after week 2 in lactation. Thus, EB explains effect of dry period length on bilirubin.

7.6.2 Effect of Mammary Health on Health Variables on Dairy Cows

In the present experiment, effect of dry period length on inflammatory biomarkers and oxidative stress variables in plasma could partly be explained by clinical mastitis. In addition, effect of dry period length on NAb titers in plasma and milk could partly be explained by both the high SCC ($\geq$ 250,000 cell/mL) and clinical mastitis. In the current experiment, omitting the dry period increased SCC in milk in the subsequent lactation, but did not affect the occurrence of clinical mastitis compared with shortening or conventional dry period both in the first and second lactations (**Chapter 4**). The increased SCC in milk was related to decreased titer of IgG binding LPS in plasma (**Chapter 4**). In the current study, SCC did not relate to inflammatory biomarkers and oxidative stress (**Chapter 2 and 3**). Clinical mastitis was associated with decreased IgG binding LPS titers in plasma (**Chapter 4**), increased ceruloplasmin and ROM levels in plasma, but not related to other health variables (Table 7.2). Earlier, clinical mastitis has been associated with decreased IgM binding KLH titers in plasma (Thompson-Crispi et al., 2013), increased plasma haptoglobin levels (Pyörälä, 2003), increased bilirubin levels

(Minuti et al., 2015) and decreased plasma paraoxonase levels (Turk et al., 2012). It was suggested that the level of NAb in plasma or milk may be an additional variable to select for clinical mastitis resistance in dairy cows (Ploegaert et al., 2010, Thompson-Crispi et al., 2013). The increased NAb levels in milk were associated with an increased SCC and clinical mastitis occurrence, which is in line with earlier work (Ploegaert et al., 2010). However, the mechanism underlying the relationship between high NAb in plasma with a decreased clinical mastitis occurrence remains to be elucidated. Results of the current experiment indicate that IgM-binding KLH in plasma can be an early predictor of clinical mastitis occurrence in dairy cows. Further studies are required to evaluate factors that predict mammary health after a shortened or omitted dry period in dairy cows.

7.6.3 *Effect of Clinical Health Problems on Health Variables in Dairy Cows*

In **Chapter 2, 3 and 4**, we reported that higher NAb titers, higher ceruloplasmin, higher haptoglobin, lower albumin and higher ROM in plasma were related to the occurrence of clinical health problems. It is known that the occurrence of clinical health problems is associated with severe or prolonged inflammation (Trevisi et al., 2011) and oxidative stress (Trevisi et al., 2010) around calving. Changes in inflammatory biomarkers and oxidative stress after calving of cows with different dry period lengths were related to the occurrence of health problems in the present experiment, but maybe many of these initiate around calving and several remained subclinical (Petersen et al., 2004, Trevisi et al., 2011). Clinical health problems is a very general term, and specific diseases (metabolic or infectious diseases) might have different effects on variables relation to inflammation or oxidative stress (Bertoni et al., 2009, Sordillo and Aitken, 2009). From this perspective, we suggest that the dry period length may not directly be a causative factor for inflammation and oxidative stress around calving. Moreover, severe NEB could indirectly be a risk factor for inflammation diseases (e.g. clinical mastitis and endometritis).

7.6.4 *Effect of Body Condition on Health Variables in Dairy Cows*

In the present study, higher precalving BCS was related to higher creatinine in plasma of dairy cows from week 3 before calving until 4 weeks after calving. No relationship between precalving BCS with plasma APP, liver functionality variables, oxidative stress and NAb levels in plasma in dairy cows was present in the current study. Our finding indicated that precalving BCS in the second lactation

after repeated implementation of dry period length treatments may be a risk factor for severe NEB and metabolic disorders which is in line with earlier study (Bernabucci et al., 2005) but BCS was not related to inflammatory or oxidative stress variables.

7.6.5 *Effect of Uterine Health on Health Variables in Dairy Cows*

A severe NEB in early lactation has been related with a greater risk for metritis or endometritis (Butler, 2003). Cows with endometritis have an increased culling rate and impaired fertility among others by reduced pregnancy rates (LeBlanc et al., 2002). Uterus palpation and monitoring of vaginal discharge (**VD**) has been widely used to diagnose clinical endometritis in dairy cows (LeBlanc et al., 2002, Gilbert et al., 2005, Williams et al., 2005, Prunner et al., 2014). Vaginal discharge has been related to high levels of haptoglobin in plasma (Huzzey et al., 2009, Dubuc et al., 2010, Ametaj et al., 2014). In the second lactation, uterine health status (**UHS**) was monitored by scoring VD based on a 4-point scoring system (0, 1, 2 or 3) in week 2 and 3 after calving. Cows were classified as having a healthy uterine environment (**HU**, VDS = 0 or 1 in both week 2 and 3), non-recovering uterine environment (**NRU**, VDS = 2 or 3 in week 3) or a recovering uterine environment (**RU**, VDS = 2 or 3 in week 2 and VDS = 0 or 1 in week 3). In **Chapter 3**, we reported that cows with **NRU** had higher levels of haptogloblin and lower paraoxonase in plasma compared with cows with **HU**. Cows with a NRU had lower levels of albumin compared with cows with **RU**. Schneider et al. (2013) reported that cows with infection in the uterus had lower levels of albumin, lower paraoxonase and higher haptoglobin in plasma compared with cows with a healthy uterus. In the present experiment, uterine health status of the cows was not related to NAb, NAAb, cholesterol, albumin, bilirubin, FRAP and creatinine in plasma. These results indicate that the increase in levels of +APP and oxidative stress variables in cows with a 0-d dry period in the first and second lactation after implementation of dry period length treatments might be explained by uterine health status as one of clinical health problems related to inflammation but not EB.

7.7 The Relationships between Health Variables and DPL, EB, SCC, Clinical Mastitis, Clinical Health Problem, BCS, or UHS

Table 7.2 shows an overview about the relationships between health variables (NAb, inflammatory biomarkers and oxidative stress) and DPL, EB, SCC, clinical mastitis (CM), clinical health problems (CHP), precalving BCS, or UHS in

the first 2 weeks after calving in the first and second lactation after implementation dry period treatments. Dry period length influenced levels of IgG binding LPS or MYO, IgM binding CA, ceruloplasmin, cholesterol, bilirubin, paraoxonase, ROM and creatinine. When including EB, SCC, clinical mastitis, CHP, BCS, or UHS as covariate in the model for each analysis, some health variables were associated with the covariate in the first 2 weeks after calving.

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Table 7.2 Relationship between health variables (natural (auto) antibodies, inflammatory biomarkers and oxidative stress) and dry period length (DPL), energy balance (EB), somatic cell count (SCC), clinical mastitis (CM), clinical health problem (CHP), body condition score (BCS), or uterine health status (UHS) in the first 2 weeks after calving.

Variables	Values (1, 2) indicate an effect in lactation 1, lactation 2 or both[a, b]						
	DPL	EB	SCC	CM	CHP	BCS	UHS
Natural antibodies[c]							
IgG binding KLH	-	-	2	-	-	-	-
IgM binding KLH	-	-	-	-	-	-	-
IgG binding LPS	1	2	-	-	1	-	-
IgM binding LPS	-	-	2	-	2	-	-
IgG binding GD	-	2	-	-	-	-	-
IgM binding GD	-	-	-	-	-	-	-
IgG binding CA	-	-	-	-	-	-	-
IgM binding CA	1, 2	2	2	-	-	-	-
IgG binding MYO	1	-	2	-	-	-	-
IgM binding MYO	-	-	2	2	2	-	-
IgG binding TRANS	-	2	2	-	-	-	-
IgM binding TRANS	-	-	2	-	-	-	-
Acute phase protein							
Haptoglobin (g/L)	-	-	-	-	2	-	2
Ceruloplasmin (µmol/L)	1, 2	-	-	1	1	-	-
Albumin (g/L)	-	-	-	-	1	-	2
Cholesterol (mmol/L)	1	-	-	-	-	-	-
Liver function							
Bilirubin (µmol/L)	1, 2	1, 2	-	-	1	-	-
Paraoxonase, U/mL	1	-	-	-	2	-	2
Oxidative stress							
ROM[d] (mg H_2O_2/100mL)	1, 2	-	-	1	1	-	-
FRAP[d] (µmol/L)	-	-	-	-	-	-	-
Creatinine (µmol/L)	2	-	-	-	-	2	2

[a] EB = Energy balance; SCC = Somatic cell count; CM = Clinical mastitis; CHP = Clinical health problem; BCS = Body condition score; UHS = Uterine health status; [b] EB, SCC, CHP (clinical mastitis, metritis, retained placenta and fever), and CM are in the first 2 weeks after calving in the first and second lactations; BCS (<3.75 vs. ≥3.75) is in the last month before calving in the second lactation; UHS in the second lactation categorized as 1) Healthy uterine environment (HU): vaginal discharge score (VDS) = 0 or 1 in both week 2 and 3; Recovering uterine environment (RU): VDS = 2 or 3 in week 2 and VDS = 0 or 1 in week 3; non recovering uterine environment (NRU): VDS = 2 or 3 in week 3; [c] Keyhole limpet hemocyanin (KLH), lipopolysaccharide (LPS), glutamate dehydrogenase (GD), carbonic anhydrase (CA), myosin (MYO) and Transferrin (TRANS); [d] ROM = Reactive oxygen metabolites; FRAP = Ferric-reducing antioxidant power.

7.8 Consequences of Maternal Dry Period Length on Colostrum Immunoglobulin Content and Immune Competence of Calves

7.8.1 Colostrum Immunoglobulin Content

Cows with a 0-d dry period, but not cows with a 30-d dry period, had lower colostrum production, lower colostrum immunoglobulin (IgG and IgM) content and reduced NAb and NAAb titers in colostrum at first milking compared with cows with a 60-d dry period in both the first (**Chapter 5**) and second lactation (Figure 7.2). In addition, colostrum immunoglobulin content did not differ between cows with a 30-d dry period and cows with a 60-d dry period. Furthermore, there was no difference in colostrum NAb (**Chapter 5**) and NAAb titers (**Chapter 6**) between cows with 30-d and 60-d dry period in the first lactation and in the second lactation after implementation dry period length (Figure 7.3). Although IgG mass produced at day of calving was lower for cows with a 0-d dry period over 2 subsequent lactations, the total IgG mass produced during colostrogenesis was not different between cows with a 0-d and a 60-d dry period (Baumrucker et al., 2014).

Colostrum immunoglobulins in the mammary gland originate from systemic and local sources. Colostrum immunoglobulin content is influenced by many factors such as time of milking after calving (Pritchett et al., 1994), age of the cows (Meganck et al., 2012), nutrition of the cows before calving (Quigley Iii and Drewry, 1998), season and heat stress (Nardone et al., 1997, Morin et al., 2010). Results of the present study indicate that that colostrum production for cows with a 0-d dry period is not compromised, but secretion is distributed over more days, resulting in a lower colostrum secretion at the day of calving (Baumrucker et al., 2014). It was suggested in earlier studies that providing a day or 2 non-milking prior to parturition would allow for the IgG to accumulate, thus the production and concentration of IgG would be higher in the following parturition (Baumrucker et al., 2014, Gross et al., 2014). Nevertheless, these results suggest that dry period length affect immunoglobulin content in colostrum thus also may affect immune competence of calves, since calves obtain passive immunity via colostrum.
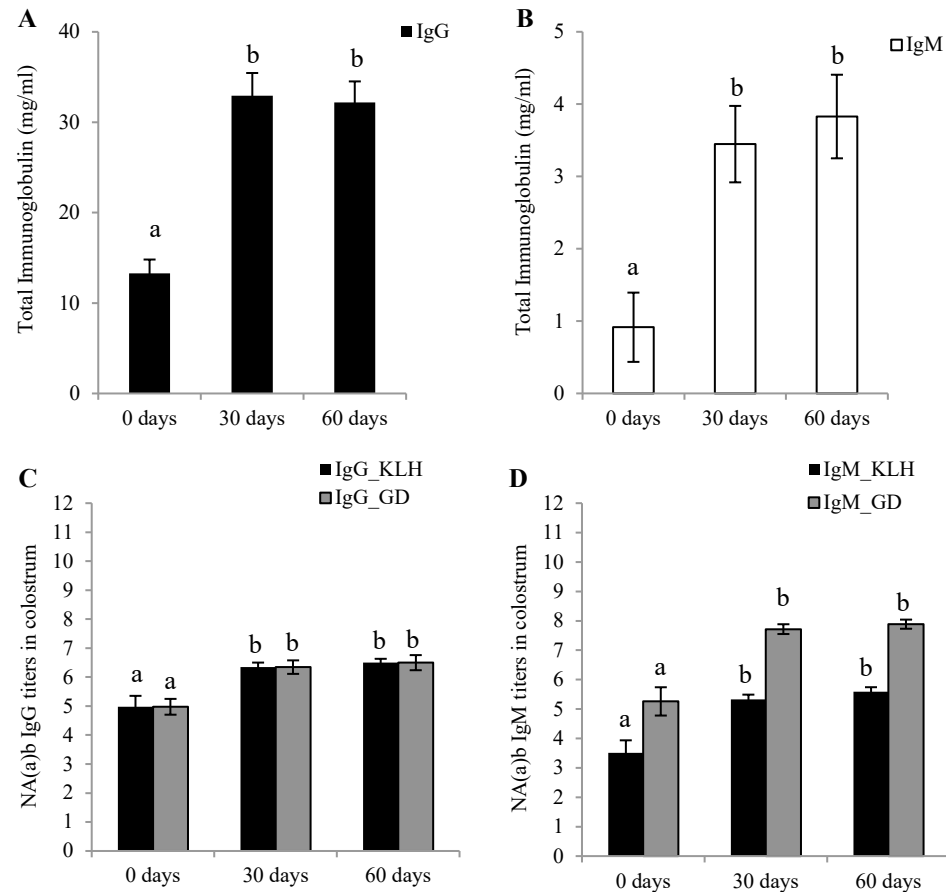
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Figure 7.2 Concentration of IgG (A) and IgM (B), natural (auto) antibodies (NAAb) binding keyhole limpet hemocyanin (KLH) and glutamate dehydrogenase (GD) for isotype IgG (C) and IgM (D) in colostrum from cows with 3 different dry period lengths (0, 30 or 60 d) in the second lactation after implementation of dry period length treatments. Values are means (± SEM) per dry period length. Bars within immunoglobulin type with different letters differ ($P < 0.05$).

7.8.2 Natural (Auto) Antibody and Specific Antibody Titers in Plasma of Calves

In the present study, NAAb (**Chapter 6**), but not NAb (**Chapter 5**) are already present in plasma of calves before colostrum intake after implementation of dry period length treatments. In the first lactation, omitting the dry period decreased NAb (**Chapter 5**) and NAAb titers (**Chapter 6**) in plasma of calves in the first week of life compared with shortening or a conventional dry period. Low NAb levels in plasma of calves are a reflection of low intake of NAb levels from

colostrum. Colostrum NAb and NAAb have a positive correlation with NAb (**Chapter 5**) and NAAb (**Chapter 6**) in plasma of the calves in the first week of life. Albeit calves from cows with a 0-d dry period had lower titers of NAb and NAAb in the first week of life, calves from cows with a 0-d dry period eventually have similar titers of SpAb binding KLH and HuSA after primary immunization at week 6 after calving. In addition, SpAb in calves were not affected by the maternal dry period length. Even the secondary antibody response to KLH in calves from cows with a 0-d dry period were enhanced compared with calves from cows with a 30-d or 60-d dry period (**Chapter 5**). Uptake of sufficient colostrum is essential for calf health, growth and performance as a dairy cow (Morin et al., 2010, Meganck et al., 2012). However, it appears that temporarily lowered NAb and NAAb titers as found in calves from cows with no dry period in the present study had no consequences for disease incidence and immune competence of the calves during the first 3 months of life.

In the present study, there were no differences in growth and disease incidence of the calves during the first 12 weeks of life in both the first (**Chapter 5**) and second lactation after implementation of maternal dry period length treatments. In the second lactation, the 0-d dry period treatment is not included in the data because in this group 6 calves were twins (5 females and 1 male) and 19 cows were naturally dried off which were therefore included in the group of a 30-d dry period. In the second lactation, the body weight of female calves prior to first immunization at week 6 was not different between dry period lengths (13.6 kg vs. 13.5 kg ±1, a 30-d vs. 60-d dry period, $P = 0.16$).The hygiene conditions in this study can be expected to be high. Calves from the present study were all raised at a specific calf raising farm from day 2 of life onwards. Davis and Drackley (1998) reported that the physical environmental conditions like housing and the level of hygiene will influence mortality and morbidity rates, effectiveness of good nutrition and management. In the present study no intentional infection was performed, therefore no information on disease resistance was obtained. In the second lactation, in total 9 calves out of 115 were infected by IBR (Infectious Bovine Rhinotracheitis) but this was not attributed to dry period length treatments (5 calves from cows with 60-d dry period and 4 calves from cows with 30-d dry period). No differences in antibody titers for KLH and HuSA were found in these calves. Further long-term studies are needed which monitor health of calves from cows with different dry period lengths under different conditions with larger number of animals.
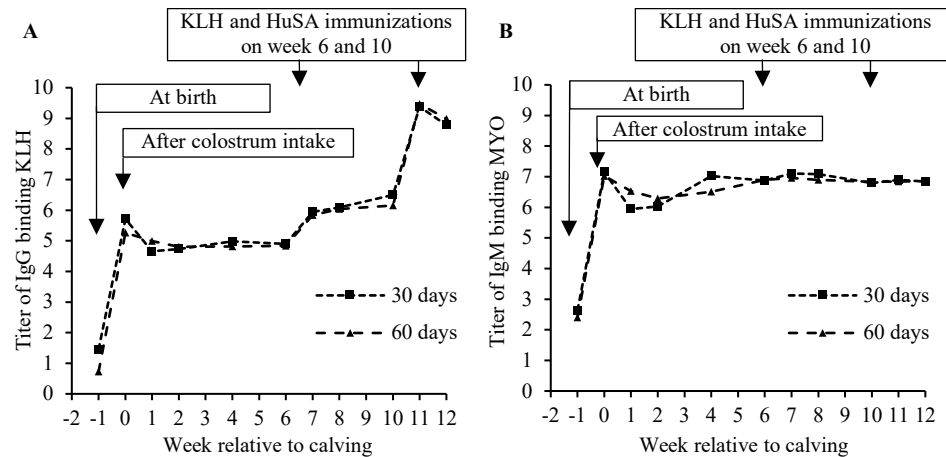
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Figure 7.3 Natural (auto) antibodies (week 0 until week 6) and specific antibodies (after primary and secondary immunization of keyhole limpet hemocyanin (KLH) and human albumin serum (HuSA)) for immunoglobulin (Ig) G titer binding KLH (A), and for IgM binding myosin (MYO) (B) in plasma of female calves from dairy cows with a 30-d or 60-d dry period in the second lactation after implementation of dry period length treatments. Values represent means per dry period length per week.

7.9 The Possible Application of Shortening or Omitting the Dry Period in Practice to Improve Animal Health and Welfare

The feasibility of shortening or omitting the dry period to improve EB and animal health not only could be experimentally shown but was implemented in practice. In practice, some dairy farmers shifted their dry period management strategy from a conventional dry period to a management strategy with a shortened or omitted dry period. An earlier study involving 13 commercial Canadian dairy herds showed that some farmers are satisfied with the strategy of shortening the dry period (35-d) because of lower incidence of mild ketosis without any negative effects on other health problems (incidence of severe ketosis, displaced abomasum, milk fever, and mastitis) and reproduction (metritis) (Santschi et al., 2011). This study did not include a 0-d dry period length treatments. A study involving 11 commercial Dutch dairy herds showed that some farmers are satisfied with the no dry period strategy because of healthier cows, decreased work load due to less dietary and group transitions, and improved fertility, whereas two farmers stopped using it because of the lower milk yield (Steeneveld et al., 2013). A follow up study involving 16 commercial Dutch dairy herds showed that some farmers are able to apply a short or no dry period on their farm over 2 consecutive lactations without a change in quantity of milk losses (Kok et al., 2017). In this study, cows

with a 30-d dry period had similar reduction in milk yield (1 kg/d) compared with cows with a 60-d dry period over 2 consecutive lactations. Cows with a 0-d dry period had reduced the additional milk yield before calving, but had increased the milk yield after calving, compared with the first omission of the dry period, which results in the same total quantity of milk losses (2 kg/d) (Kok et al., 2017). Earlier, it was suggested that farmers may use characteristics of individual cows, like parity or milk production level, to predict milk yield for individual cows in response to dry period length (Kok et al., 2017). However, it is still questioned whether omitting the dry period is beneficial for dairy cows welfare (Zobel et al., 2015). Planned stop milking before calving was shown to increase udder pressure and stress (as measured by faecal glucocorticoid metabolites) at the start of the dry period (Tucker et al.,2009, Bertulat et al., 2013) which can be expected to affect animal welfare. Cows with a 0-d dry period can be kept in the herd without regrouping and dietary changes. From this perspective, we assume that cows with 0-d dry period have less stress and therefore an improved welfare. Further study is needed to confirm whether shortening the dry period affects animal welfare.

From the economic point of view, cows with a omitted dry period had lower cost for antibiotic use and reduced management costs for regrouping the cows and dietary changes (Steeneveld et al., 2013). Earlier study involving 13 commercial Canadian dairy herds showed that shortening the dry period (35-d) would be more beneficial compared with a conventional dry period (60-d) due to additional milk yield in the precalving period, lower replacement rate, and a shorter calving interval (Santschi et al., 2011). However, knowledge about the economic impact of omitting the dry period is scarce. A field study with a large number of animals is needed to evaluate the economic impact of omitting the dry period based on health and fertility.

7.10 Conclusion

Omitting the dry period improved EB, metabolic status and fertility. The current study shows that omitting the dry period reduced plasma bilirubin level but does not have clear consistent effects on inflammation or oxidative stress compared with a short or a conventional dry period in the first and second lactation after implementation of dry period length treatments. Better liver functionality in cows with a 0-d dry period was associated with improved EB in these cows. Omitting the dry period increased both positive and negative APP, oxidative stress, and resulted in higher NAb and NAAb in plasma compared with a short or a conventional dry

period. Effects of dry period length on health variables such as APP, liver functionality markers, oxidative stress markers and NAb depend on many factors, like EB, mammary health, clinical health problems related to inflammation, BCS, and uterine health status. Changes in inflammatory variables (increased ceruloplasmin), oxidative stress marker (increased ROM) and higher NAb IgG binding LPS in plasma were a reflection of the occurrence of health problems related to inflammation. In particular clinical mastitis and reduced uterine health had consequences for the monitored variables related to inflammation, oxidative stress and humoral immune response of cows in early lactation, independent of dry period length. In the first lactation after implementation dry period length and dietary treatments, feeding a glucogenic ration in early lactation increased IgM NAb titers binding LPS in milk compared with a lipogenic ration, which could be explained partly by better EB. In the second lactation after implementation dry period length and dietary treatments, feeding a lipogenic ration in early lactation increased cholesterol levels in plasma compared with a glucogenic ration, which could be explained by high fat content in this ration. Albeit omitting the dry period in cows resulted in reduced immunoglobulin content in colostrum and NAb and NAAb levels in plasma of their calves in the first week of life, this did not affect specific immune response and growth of the calves at least in the first 12 weeks of life. The titers of NAAb in plasma of the calves were not related to NAAB in plasma of their mother before colostrum intake, but in the first week of life there was a relation with NAAB in plasma of the mother, which disappeared again thereafter. In addition, NAb and NAAb in plasma are influenced by the age of the mother. Maternal dry period length had temporary transgenerational effects on calves.

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Summary

During early lactation, modern high-producing dairy cows typically experience a period with a negative energy balance (**NEB**). The NEB results from a sudden increase in milk production after calving while feed intake capacity is limited in this period. The severe NEB results in extensive mobilization of body reserves in early lactation in dairy cows. A high mobilization of free fatty acid (**FFA**) is related to an increased occurrence of metabolic disorders like liver fattening and ketosis, higher incidence of infectious diseases, immunosuppression, inflammation and oxidative stress. Moreover, cows with NEB had a compromised liver functions and increased oxidative stress. Extensive research efforts have been made to improve the energy balance (**EB**) in early lactation in dairy cows. Shortening or omitting the dry period shifts milk yield partially from the critical period after calving to the period before calving. The reduction in milk yield in early lactation results in an improvement of EB, metabolic status, and fertility of dairy cows in early lactation. It can be hypothesized that improvement of the EB due to a short or no dry period improves health and reduces the occurrence of clinical health problems in early lactation. Studies on the effects of dry period length on health are limited and mostly limited in animal numbers. The first objective of this thesis was to study the effects of shortening the dry period on health related variables (inflammatory biomarkers, markers for liver functionality, oxidative stress and natural antibodies (**NAb**)) in dairy cows for multiple lactations. The second objective was to study the effects of dry period length on colostrum immunoglobulin content, natural (auto) and specific antibodies in plasma of calves for multiple lactations. Earlier, it was reported that feeding a glucogenic ration compared with a lipogenic ration in early lactation improved EB and metabolic status by reducing milk energy output. Shortening or omitting the dry period might reduce the requirement for glucogenic nutrients in early lactation because of the improved EB and metabolic status. Thus, it can be hypothesized that feeding a glucogenic ration may have less impact on EB but also may reduce inflammation, immunosuppression and oxidative stress in early lactation in dairy cows if cows have a better EB due to the shortened or omitted dry period.

To address these hypotheses, an experiment was performed where Holstein-Friesian dairy cows (N = 167) were assigned randomly to 3 dry period lengths (0, 30, or 60 d) and 2 early lactation rations (glucogenic or lipogenic ration). Cows were fed a glucogenic or lipogenic ration from 10 d before the expected calving date. Cows were planned to have the same dry period length and dietary treatments for 2 subsequent lactations. Before calving of the second subsequent lactation, 19

out of 39 cows in the 0-d dry period group were attributed to 0→30-d dry period group (actual days dry: 67 ± 8 d) because these cows had a milk yield of < 4 kg/d at least 30-d before expected calving date and were allowed to go dry.

In the first subsequent lactation after implementation of dry period length treatments, cows with a 0-d dry period had higher levels of cholesterol (negative APP), ceruloplasmin (positive APP), reactive metabolite oxygen (**ROM)** and tended to have higher haptoglobin levels compared with cows with a 30-d or a 60-d dry period (**Chapter 2**). Cows with a 0-d dry period had a lower plasma paraoxonase and bilirubin levels in the first 2 weeks after calving and a lower liver functionality index (**LFI)** compared with cows with a 60-d dry period. Cows with a 30-d dry period did not differ on bilirubin levels compared with cows with a 0-d or 60-d dry period. It was questioned if dry period length effects on inflammatory and oxidative stress variables could be explained by dry period length effects on EB, mammary health or occurrence of clinical health problems. High levels of ceruloplasmin and ROM were explained by the occurrence of health problems related to inflammation. Low bilirubin levels were associated with better EB. Feeding a glucogenic ration did not affect levels of positive APP, bilirubin, and oxidative stress, but improved cholesterol levels in plasma compared with a more lipogenic ration. In conclusion, cows with a 0-d dry period not only had a better EB, but also had higher levels of positive APP (ceruloplasmin) and oxidative stress compared with cows with a 30-d or 60-d dry period. No consistent effects of dry period length on inflammatory variables were observed. Moreover, occurrence of health problems did not differ between cows with different dry period lengths.

In the second lactation after implementation of dry period length and dietary treatments, the relationship between uterine health with inflammatory biomarkers and oxidative stress of dairy cows in early lactation was assessed (**Chapter 3**). Uterine health status (**UHS)** was monitored by scoring vaginal discharge (**VD)** based on a 4-point scoring system (0, 1, 2 or 3) in week 2 and 3 after calving. Cows were classified as having a healthy uterine environment (**HU**, VDS = 0 or 1 in both week 2 and 3), non- recovering uterine environment (**NRU**, VDS = 2 or 3 in week 3) or a recovering uterine environment (**RU**, VDS = 2 or 3 in week 2 and VDS = 0 or 1 in week 3). Cows with HU had lower levels of haptogloblin and higher paraoxonase in plasma compared with cows with NRU. Cows with RU had higher levels of albumin and creatinine plasma and a higher LFI compared with cows with NRU. Independent of UHS, cows in the 0→30-d dry period group had higher bilirubin and creatinine levels in plasma, which was related to more severe NEB in

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these cows. This study suggests that the also uterine health status explains peripartum changes in inflammatory biomarkers.

Mammary health and NAb in plasma and milk of cows with different dry period length were determined in both first and second lactation after implementation of dry period length and dietary treatments (**Chapter 4**). In the first lactation after implementation of dry period length treatments, cows with a 0-d dry period had higher somatic cell count (**SCC**), NAb (IgG and IgM) titers in milk and IgG binding lipopolysaccharide (**LPS**) in plasma compared with cows with a 30-d or 60-d dry period. In the second lactation after implementation of dry period length and dietary treatments, cows with a 0-d and 60-d dry period had a lower SCC compared with cows with a 30-d dry period and cows which were planned for 0-d but they dried themselves off (0→30-d) dry periods. In the second lactation after implementation of dry period length treatments, dry period length did not affect NAb titers in plasma or milk. In both lactations, ration did not affect NAb titers in plasma. The clinical mastitis occurrence was 17 percent in lactation 1 of the experiment and 25 percent in lactation 2, and did not differ according to dry period lengths or rations. For both lactations, an increasing titer of IgG binding LPS in plasma was associated with decreased odds of a high SCC and decreased odds of clinical mastitis occurrence. Also up to 3 weeks before the clinical mastitis occurrence, increasing titers of IgM binding KLH and LPS in plasma was associated with decreased odds of clinical mastitis occurrence. In conclusion, omitting the dry period increased SCC, NAb titers in milk and IgG binding LPS in plasma compared with a short (30-d) or conventional (60-d) dry period. The effects on NAb titers, however, were only present in the first year after omitting the dry period and disappeared after repeated omitting the dry period. Moreover, an increasing titer of IgG binding LPS in plasma was associated with a decreased odds of high SCC and clinical mastitis occurrence.

The effect of maternal dry period length on colostrum immunoglobulin content and immune competence of their calves was determined in the first year after implementation dry period length treatments (**Chapter 5**). Birth weight of calves from cows with a 0-d dry period was lower compared with calves from cows with a 30-d dry period, but not compared with calves from cows with a 60-d dry period. Growth of calves until 12 weeks of life was not affected by dry period length. Cows with a 0-d or 30-d dry period had lower colostrum production at first milking after calving compared with cows with a 60-d dry period. Total IgG and IgM concentration in colostrum, NAb IgG and IgM titers binding KLH or HuSA

were lower in colostrum from cows with a 0-d dry period compared with cows with a 30-d or 60-d dry period, which may be explained because Ig production during the prepartum period for cows with a 0-d dry period was already lost in milk before parturition, compared with cows with a dry period. Natural antibody titers (IgG, IgM and IgT) binding KLH or HuSA in plasma were lower during the first 2 weeks of life for calves from cows with a 0-d dry period compared with calves from cows with a 30-d or 60-d dry period. Female calves were immunized with both KLH and HuSA in week 6 and 10 of life. Specific antibodies binding KLH in calves were not affected by the maternal dry period length and the secondary antibody responses to KLH in calves from cows with a 0-d dry period were even enhanced compared with calves from cows with a 30-d or 60-d dry period, which suggested that calves from cows with a 0-d dry period had earlier immune maturation and had a faster response to immune challenges than other calves. This study indicated that although omission of the dry period of dairy cows leads to lower IgG in colostrum and lower plasma NAb titers in calves during the first 2 weeks of life, SpAb titers in calves were not affected and even the secondary antibody responses were enhanced, compared with calves from cows with a 30-d or 60-d dry period.

The presence of IgM and IgG NAAb binding glutamate dehydrogenase (**GD**), carbonic anhydrase (**CA**), myosin (**MYO**) and transferrin (**TRANS**) as representative for antibodies binding autoantigens from calves before drinking colostrum until the first 12 weeks of life in plasma was evaluated (**Chapter 6**). In addition, NAAb to these four self-antigens were also determined in colostrum and in plasma of their mothers during last 3 weeks before calving. Titers of NAAb to these four self-antigens were already present in the unborn calves, which led to the speculation that NAAb may be already initiated by self-antigens during the fetal phase. Levels of neonatal NAAb during the early weeks of life were positively related with levels of maternal NAAb in colostrum, but the relation was absent after 2 weeks of life. In addition, repeated immunizations with exo-antigens (KLH and HuSA) did not affect NAAb levels in the calves. Before colostrum intake, levels of NAAb in plasma of calves were not related with levels of NAAb in plasma of their mother but were influenced by parity of their mother. Titers of IgM NAAb binding GD and MYO in plasma of calves before colostrum intake were lower in plasma of calves from older cows than younger cows. This study indicated that NAAb are already present in the unborn calf, and levels of neonatal NAAb during the early weeks of life are related with levels of maternal NAAb obtained via colostrum.

Biological mechanisms behind the effects of shortening or omitting dry period on inflammatory biomarkers, oxidative stress, NAb and NAAb were discussed in **Chapter 7**. Omitting the dry period affects health variables such as increased levels of positive APP, increased levels of negative APP, reduced levels of bilirubin, increased oxidative stress, increased NAb and NAAb titers in plasma and increased NAb levels in milk. The changes of health variables between cows with different dry period length could not be completely explained by the effects of dry period length on the EB, but also were explained partly by the occurrence of clinical health problems in dairy cows, especially clinical mastitis and uterine health status. Additionally, the consequences of maternal dry period length on immunoglobulin content in colostrum, NAb and SpAb in plasma of calves in the first 12 weeks of life over 2 subsequent lactations were described. Omitting the dry period reduced immunoglobulin content in colostrum and also reduced NAb and NAAb titers in plasma of calves in the first week of life in both lactations. In both lactations, NAAb but not NAb were already present in the unborn calf. The levels of NAb and NAAb during the early weeks of life were positively related with maternal NAb and NAAb in colostrum, but the relation was absent after 2 weeks after calving. Maternal dry period length did not affect SpAb in calves after immunization.

Omitting the dry period improved EB, metabolic status and fertility. The conclusion of the current study was that omitting the dry period reduced plasma bilirubin level but does not have clear consistent effects on inflammation or oxidative stress compared with a short or a conventional dry period in the first and second lactation after implementation of dry period length treatments. Better liver functionality in cows with a 0-d dry period was associated with improved EB in these cows. Omitting the dry period increased both positive and negative APP, oxidative stress, and resulted in higher NAb in milk compared with a short or a conventional dry period. Effects of dry period length on health variables such as APP, liver functionality markers, oxidative stress markers and NAb depend on many factors, like EB, mammary health, clinical health problems related to inflammation, BCS, and uterine health status. Changes in inflammation variables (increased ceruloplasmin), oxidative stress marker (increased ROM) and higher NAb in milk were a reflection of the occurrence of health problems related to inflammation. In particular clinical mastitis and reduced uterine health had consequences for the monitored variables related to inflammation, oxidative stress and humoral immune response of cows in early lactation, independent of dry

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period length. Omitting the dry period increased SCC, NAb titers in milk and titers of IgG binding LPS and MYO in plasma compared with conventional dry period. The effects on NAb titers, however, were only present in the first lactation after omitting the dry period and disappeared after repeated omitting the dry period. An increasing titers of NAb in milk for both lactations were associated with increased odds of a high SCC and increased odds of clinical mastitis occurrence. Moreover, an increasing titer of IgG binding LPS in plasma was associated with decreased odds of high SCC and clinical mastitis occurrence. In the first lactation after implementation dry period length and dietary treatments, feeding a glucogenic ration in early lactation increased NAb titers compared with lipogenic diet, which could be partly explained by better EB. In the second lactation after implementation dry period length and dietary treatments, feeding a lipogenic ration in early lactation increased cholesterol levels in plasma compared with a glucogenic ration, which could be explained by high fat content in this ration. Furthermore, albeit omitting the dry period compared with shortening or conventional dry period cows resulted in reduced immunoglobulin content in colostrum and NAb and NAAb levels in plasma of their calves in the first week of life, this did not affect specific immune response and diseases incidence of the calves at least in the first 12 weeks of life. The titers of NAAb in plasma of the calves were not related to NAAb in plasma of their mother before colostrum intake, but in the first week of life there was a relation with NAAb in plasma of the mother, which disappeared again thereafter. This study implies that shortening or omitting the dry period did not negatively affect immune competence of the calves.

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List of abbreviation

APP	: Acute phase protein
AR (1)	: First-order autoregressive covariance structure
BCRs	: B-cell receptors
BCS	: Body condition score
BHB	: β-hydroxybutyrate
CA	: Carbonic anhydrase
CHP	: Clinical health problem
CM	: Clinical mastitis
CVB	: Centraal veevoederbureau
DM	: Dry matter
DMI	: Dry matter intake
DP	: Dry period
DPL	: Dry period length
DVE/ OEB	: Intestinal digestible protein and degraded protein balance
EB	: Energy balance
ELISA	: Enzyme-linked immunosorbent assay
FFA	: Free fatty acid
FRAP	: Ferric-reducing antioxidant power
GD	: Glutamate dehydrogenase
HU	: Healthy uterine environment
HuSA	: Human serum albumin
IBR	: Infectious bovine rhinotracheitis
IgA	: Immunoglobulin A
IGF-I	: Insulin-like growth factor-1
IgG	: Immunoglobulin G
IgM	: Immunoglobulin M
IgT	: Immunoglobulin Total
IL-1	: Interleukin 1
IN-LO	: Intermediate low liver functionality score
IN-UP	: Intermediate-high liver functionality score
ISO	: International standard organization
KLH	: Keyhole limpet hemocyanin
LFI	: Liver functionality index
LO	: Low liver functionality score
LPS	: Lipopolysaccharide
LSM	: Least square means

MYO	: Myosin
NAAb	: Natural auto antibodies
NAb	: Natural antibodies
NEB	: Negative energy balance
NLR	: NOD-like receptor
NRU	: Non-recovering uterine environment
OR	: Odds ratio
PMN	: Polymononuclear
PO	: Peroxidase
PRRs	: Pathogen-recognition receptors
Rho	: Spearman's rank correlation coefficient
RIA	: Radioimmunoassay
RID	: Radial immunodiffusion assay
ROM	: Reactive oxygen metabolites
ROS	: Reactive oxygen species
RU	: Recovering uterine environment
SCC	: Somatic cell count
SEM	: Standard error of the mean
SpAb	: Specific antibodies
TAG	: Tri-acyl-glycerides
TCRs	: T-cell receptors
TLR	: Toll-like receptors
TNF-α	: Tumor necrosis factor alpha
TRANS	: Transferrin
UHS	: Uterine health status
UP	: High liver functionality score
VDS	: Vaginal Discharge Score
VEM	: Dutch net energy evaluation system for dairy cattle
VLDL	: Very low density lipoprotein

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About the Author

Curriculum Vitae

Novi Mayasari (or Novi) was born as the oldest of three in Bandung, West Java-Indonesia, on the 8th of November 1982. In 2004 she completed her bachelor study in Universitas Padjadjaran, Bandung, Indonesia. At this university she focussed on animal feed and nutrition. She has done her experiments for her thesis in both Center for Life Sciences Institute Technology Bandung and Faculty of Animal Husbandry, Universitas Padjadjaran, where she studied fermentation of coffee pulp with *Pleoratus ostreatus* as feed supplementation to increase production of volatile fatty acid in cattle. Since 2005, she started to work as a research assistant at Laboratory of Animal Physiology and Biochemistry in Universitas Padjadjaran, Bandung. She focused during her research on physiological nutrition in both ruminants and non-ruminants. In 2008, she started her Master at the Department of Animal Sciences of Wageningen University with specialisation in Animal Health and Behaviour. For her MSc study she got a scholarship from Directorate of Higher Education Indonesia. She did two major theses in Adaptation Physiology Group and Animal Nutrition Group. Both theses focussed on physiological nutrition and behaviour in broiler chickens. The first thesis was about personality and feed intake in fast and slow broiler chickens. The second thesis was about effect of wet feeding and a high energy to protein ratio on gastrointestinal tract development, performance, and behaviour of broiler chicken under normal and high ambient temperature regimes. After graduated in 2010, she returned to Indonesia and continue working as a researcher and lecturer in Padjadjaran University. In 2012, she got a grant from Directorate of Higher Education Indonesia to start her PhD. In December 2012, she started her PhD at Adaptation Physiology Group in Wageningen University, The Netherlands. Her project is part of the WHYDRY project at Adaptation Physiology Group and within this project she focused on effects of dry period length and dietary energy source on physiological health variables in dairy cows and calves. In 2016, she received a WIAS PhD fellowship to travel to the Università Cattolica del Sacro Cuore, Piacenza, Italy to perform research resulting in two scientific papers which are included in this thesis. In Italy she worked at Faculty of Agriculture, Food and Environmental Sciences under the supervision of professor Erminio Trevisi. Final results of the total PhD project are included in this thesis.

Overview of scientific publications

Peer reviewed scientific publications

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

Mayasari, N., W. Rijks, G. de Vries Reilingh, G. J. Remmelink, B. Ducro, B. Kemp, H. K. Parmentier, and A. T. M. Van Knegsel. 2016. The effects of dry period length and dietary energy source on natural antibody titers and mammary health in dairy cows. Preventive Veterinary Medicine 127:1-9.

Mayasari, N., A. Van Knegsel, G. de Vries Reilingh, B. Kemp, and H. Parmentier. 2016. Natural autoantibodies in Bos taurus calves during the first twelve weeks of life. Veterinary Immunology and Immunopathology 178:70-78.

Mayasari, N., J. Chen, A. Ferrari, R. M. Bruckmaier, B. Kemp, H. K. Parmentier, A.T.M. van Knegsel, E. Trevisi. 2017. Effects of dry period length and dietary energy source on inflammatory biomarkers and oxidative stress in dairy cows. Journal of Dairy Science. *In press*

Mayasari, N., E. Trevisi, A. Ferrari, B. Kemp, B H. K. Parmentier, and A.T.M. van Knegsel. Relationships between inflammatory biomarkers and oxidative stress with uterine health in dairy cows with different dry period lengths. *To be submitted*

Conference and symposia proceedings

Mayasari, N., G. de Vries Reilingh, G. J. Remmelink, H. K. Parmentier, B. Kemp, and A. T. M. Van Knegsel. 2014. Effect of maternal dry period and nutrition on colostrum quality, natural and specific humoral immunity, and development of calves. In Book of abstract of WIAS Science Day 2014, 30 April 2014, Wageningen, the Netherlands.

Mayasari, N., G. de Vries Reilingh, G. J. Remmelink, H. K. Parmentier, B. Kemp, and A. T. M. Van Knegsel. 2015. Long-term effect of shortening dry period of dairy cows on immunoglobulin content in colostrum, natural and specific antibodies and development of calves. In Book of abstract of WIAS Science Day 2015, 5 February 2015, Wageningen, the Netherlands.

Mayasari, N., W. Rijks, G. de Vries Reilingh, G. J. Remmelink, B. Ducro, B. Kemp, H. K. Parmentier, A. T. M. Van Knegsel. 2015. Repeated shortening the dry period, but not the diet, affected natural antibody levels and somatic cell counts in milk of dairy cows. In Proceedings of the 40th Animal Nutrition Forum, 22 May 2015, Merelbeke, Belgium.

Mayasari, N., W. Rijks, G. de Vries Reilingh, G. J. Remmelink, B. Ducro, B. Kemp, H. K. Parmentier, and A. T. M. Van Knegsel. 2015. Effect of dry period length and dietary energy source in dairy cows on natural antibody titers and somatic cell count in milk. In Proceedings of the Joint Annual Meeting 2015 ADSA-ASAS, 12-16 July 2015, Orlando, Florida, USA.

Mayasari, N., W. Rijks, G. de Vries Reilingh, G. J. Remmelink, B. Kemp, H. K. Parmentier, A. T. M. Van Knegsel. 2015. Natural auto antibody in colostrum and calves with a different maternal dry period length. In Proceedings of the 5th European Veterinary Immunology Workshop, 2-4 September 2015, Vienna, Austria.

Mayasari, N., J. Chen, G. De Vries Reilingh, J. J. Gross, R. M. Bruckmaier, B. Kemp, H. K. Parmentier, A.T.M. van Knegsel. 2016. Relationships between metabolism and metabolism in dairy cows with different dry period lengths. In Proceedings of the 15th International Conference on Production Diseases in Farm Animals, 20-23 June 2016, Wageningen, the Netherlands.

Mayasari, N., J. Chen, G. De Vries Reilingh, J. J. Gross, R. M. Bruckmaier, B. Kemp, H. K. Parmentier, A.T.M. van Knegsel. 2017. Relationships between inflammatory biomarkers and oxidative stress with uterine health in dairy cows with different dry period lengths. In Book of abstract of WIAS Science Day 2017, 6 February 2017, Wageningen, the Netherlands.

Training and Supervision Plan[1]

The Basic Package (3 ECTS[2])

Ethics and Philosophy in Life Sciences	2013
WIAS Introduction Course	2013

International Conferences (4 ECTS)

ADSA-ASAS Joint Annual Meetings, Orlando, USA	2015
16th International Conference on Production Diseases in farm animals (ICPD), The Netherlands	2016
The 5th European Veterinary Immunology Workshop (EVIW) n Vienna, Austria	2015

Seminars and Workshops (4 ECTS)

2nd Workshop ; Health and Nutrition in High -Yielding Dairy Cows, Piacenza, Italy	2013
3rd Workshop ; Health and Nutrition in High -Yielding Dairy Cows, Ludwigshafen, Germany	2014
5th Workshop ; Health and Nutrition in High -Yielding Dairy Cows, Wageningen, The Netherlands	2016
WIAS Seminar ; Development in Ruminant Nutrition; Wageningen University, The Netherlands	2013
International Symposium on Dairy Cattle Nutrition 2013 "Feed efficiency in dairy cattle" Wageningen University, The Netherlands	2013
International Symposium on Dairy Cattle Nutrition 2014 "Strategies Towards a Quota-Free Dairy Production " Wageningen University, The Netherlands	2014
International Symposium on Dairy Cattle Nutrition 2015 "Dairy cows nutrition and animal health" Wageningen University, The Netherlands	2015
WIAS Science Day, Wageningen University, The Netherlands *3	2014/15/17
The 40th Animal Nutrition Research Forum, Merelbeke, Belgium	2015
The 41st Animal Nutrition Research Forum, Wageningen, The Netherlands	2016
Wageningen Indonesia Scientific Expose symposium (WISE) 2016, Wageningen, The Netherlands	2016
Dairy Symposium : Postpartum challenges for the modern high yielding dairy cows, Gent, Belgium.	2016

[1] Completed in the fulfilment of the requirements for the education certificate of the Graduate School WIAS (Wageningen Institute of Animal Science)

[2] One ECTS equals a study load of 28 hours

Presentations (9 ECTS)

BASF Workshop : 2nd Health and Nutrition in High-Yielding Dairy Cows, Oral Presentation, Ludwigshafen, Germany	2013
WIAS Science Day, 2 Poster Presentations, Wageningen, The Netherlands	2014/15
The 40th Animal nutrition research forum, Oral Presentation, Ghent, Belgium	2015
ADSA-ASAS Joint Annual Meetings, Oral Presentation, Orlando, USA	2015
EVIW, Poster Presentation, Wageningen, The Netherlands	2015
BASF Workshop : 5th Health and Nutrition in High-Yielding Dairy Cows, Oral Presentation, Wageningen, The Netherlands	2016
The 16th ICPD, Oral Presentation, Wageningen, The Netherlands	2016
Seminar of Metabolism in periparturient dairy cows, Oral Presentation, Wageningen, The Netherlands	2016

Disciplinary and Interdisciplinary Courses (7 ECTS)

Tropical Farming Systems with Livestock, Wageningen, The Netherlands	2013
Fish Immunology Workshop, Wageningen, The Netherlands	2013
Epigenesis and Epigenetics, Wageningen, The Netherlands	2014
Adaptive Animals and Livestock Farming Systems to Meet Global Changes, Rennes, France	2014
Advanced Course Immunology, Utrecht, The Netherlands	2015

Advanced Statistics Courses (3 ECTS)

Advanced Statistics: Design of Experiments	2013
Statistics for the Life Sciences	2015

PhD students' discussion groups (2 ECTS)

Animal health and immunology discussion group	2014-15

Professional Skills Support Courses (5 ECTS)

Information Literacy PhD including Endnote Introduction	2013
Scientific Publishing	2013
Course Techniques for Scientific Writing	2014
Project and Time Management	2014
PhD Competence Assessment or Job Assessment	2014
Course Supervising MSc Thesis Work	2015
WIAS course the Final Touch: Writing the General Introduction and Discussion	2016

Research Skills Training (8 ECTS)

Preparing own PhD Research Proposal	2012
External Training Period, Italy, 2 months	2016

Didactic Skills Training (6 ECTS)

Lecture in BSc course Adaptation Physiology	2015
Lecture in BSc course Infections and Disorders	2015-16
Supervision of 3 MSc Thesis Students	2014-15

Management Skills Training (6.0 ECTS)

Chair WIAS Science Day	2015
Education Committee Member	2016

TOTAL : 57 ECTS

COLOPHON