Eesti Maaülikool
Estonian University of Life Sciences

CHANGES IN ACUTE-PHASE PROTEINS' CONCENTRATIONS OF NEONATAL RUMINANTS IN RELATION TO COLOSTRUM AND WEIGHT GAIN

ÄGEDA FAASI VALKUDE KONTSENTRATSIOONI MUUTUSED, NENDE SEOSED TERNESPIIMA JA MASSI-IIBEGA VASTSÜNDINUD MÄLETSEJALISTEL

KRISTEL PEETSALU

A Thesis
for applying for the degree of Doctor of Philosophy
in Veterinary Sciences

Väitekiri
filosoofiadoktori kraadi taotlemiseks loomaarstiteaduse erialal

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In memory of my father

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following four original research papers (**I–IV**), referred to in the text by their respective Roman numerals:

I Niine, T., **Peetsalu, K**., Nieminen, M., Oksanen, A., Soveri, T., Orro, T, 2017. *Giardia* and *Cryptosporidium* infections in neonatal reindeer calves: relation to the acute phase response. Comp. Immunol. Microbiol. Infect. Dis. 54, 45-50.

II **Peetsalu, K**., Tummeleht, L., Kuks, A., Orro, T., 2019. Serum amyloid A and haptoglobin concentrations in relation to growth and colostrum intake in neonatal lambs. Livest. Sci. 220, 217-220.

III Niine, T., **Peetsalu, K**., Tummeleht, L., Kuks, A., Orro, T. 2018. Acute phase response in organic lambs associated with colostrum serum amyloid A, weight gain, and *Cryptosporidium* and *Giardia* infections. Res. Vet. Sci. 121, 117-123.

IV **Peetsalu, K**., Niine, T., Loch, M., Dorbek-Kolin, E., Tummeleht, L., Orro, T., 2022. Effect of colostrum on the acute phase response in neonatal dairy calves. J. Dairy Sci. doi: 10.3168/jds.2021-21562. [published online ahead of print, 2022 May 6]

The contribution of authors to the research papers

Paper	Original idea, study design	Data collection, sample analysis	Data analysis	Manuscript preparation
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II	TO, **KP**	TO, AK, **KP**, LT	**KP**, TO	All
III	TO, TN, **KP**	TN, TO, AK, **KP**, LT	TN, TO	All
IV	TO, **KP**, TN	TO, **KP**, ED, ML, TN	**KP**, TO, ML	All

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ABBREVIATIONS

ADWG – average daily weight gain

AGP – alpha 1-acid glycoprotein

Alb – albumin

APP – acute-phase proteins

APR – acute-phase response

BRSV – bovine respiratory syncytial virus

Cr – ceruloplasmin

CRP – C reactive protein

EDTA – ethylenediaminetetraacetic acid

Fib – fibrinogen

GF – growth factor

GGT – gamma glutamyltransferase

Glob – globulins

HL – halofuginone lactate

Hp – haptoglobin

IgG – immunoglobulin G

IGF-1 – insulin-like growth factor-1

IL – interleukin

IL-1ra – interleukin 1 receptor antagonist

IFN-γ– interferon gamma

LBP – lipopolysaccharide-binding protein

LPS – lipopolysaccharide

SAA – serum amyloid A

SD – standard deviation

SEM – standard error of the mean

TNF – tumour necrosis factor

1. INTRODUCTION

The globally widespread intensive farming management can cause numerous problems for the ruminants that are bred for meat or milk production. The animals are put under pressure to grow fast and gain weight already as neonates. Physiological development and body growth of a young animal always needs a lot of resources and suitable environmental conditions. Overcrowding and poor farm hygiene can lead to substantial environmental pathogenic pressure and overall stress. For the good of young animals and farmers, the growth period must support the physiological development processes and species-specific needs as much as possible. Therefore, it is important to understand the normal development period of the neonatal animals.

Neonatal ruminants are particularly vulnerable to potential pathogens, as the syndesmochorial type of placentation prevents the transfer of antibodies from mother to foetus. The transition from a protected intrauterine environment to extrauterine life requires the development of the immune system as a major part of the adaptation process. It has been known for almost a century that neonatal ruminants obtain passive immunity for the first encountered pathogens from colostrum. Besides the maternal immunoglobulins attained in this way, the innate immune system of the newborn ruminant plays a major role in their protection from pathogens.

The acute phase response is a complex systemic early-defense reaction, with the goal of reestablishing organism homeostasis and promote healing (Cray *et al.,* 2009). It is a first-line defence mechanism of the organism, induced by invading pathogens or tissue injury (Baumann and Gauldie, 1994). During the APR, acute-phase proteins (APPs) are synthesised in the liver or to a smaller extent in local tissues (Gabay and Kushner, 1999; Gruys *et al*., 2005). APPs have been studied in ruminants as sensitive quantitative biomarkers for various disease conditions and stress reactions. However, during the neonatal period, age-dependent changes in the concentrations of APPs occur (Orro *et al*., 2006, 2008; Tóthová *et al*., 2016). As the neonatal period has a long-term influence on the life of animals, these APPs measured in the neonatal period might indicate how the neonatal ruminants adapts to the outside environment (Orro *et al*., 2006; Seppä-Lassila *et al.,* 2017).

The exact mechanisms of the fluctuation of APPs during the neonatal period still need to be resolved. The main focus in this current thesis was on development of the APR and APPs in neonatal ruminants. The changes in concentrations of serum amyloid A (SAA) (**I–IV**), haptoglobin (Hp) (**I–IV**), fibrinogen (Fib) (**I**), and albumin (Alb) (**I-IV**) were evaluated. The influence of colostrum intake on neonate APR has been evaluated (**II–IV**). Furthermore, to investigate possible influences of changes in APPs concentration on the neonatal animals, they were followed for a longer period and associated with further weight gain (**I–IV**).

The model species in the studies were ruminants, whose breeding and management conditions differed from one another. While reindeers are semi-domesticated animals living in a large territory, having mother–offspring bond, dairy calves are kept in an intensive management farm system separated from their mothers after birth. The lambs have mother–offspring contact, and live in a larger herd with access to outdoors beside a barn.

Sampling the neonates of three ruminant species brought up in varying breeding systems allowed the assessment of the neonatal period APR in different environmental scenarios including the specific effects of the particular year. Since there are only a few studies addressing the changes of the APPs in ruminants during the neonatal period, the studies included in the current thesis provide comparisons of normal postnatal adaptation and development in different ruminant species. To complex the approach, the colostrum effect on neonatal APR was studied. Assessed during the developmental period of the neonate ruminant, some APPs serve as markers of inflammatory processes in the organism that can lead to lower growth in long term. Understanding the natural adaptation processes, including the development of the immune system and contributing factors is necessary in order to be able to meet the needs of the animals and identifying the factors that influence the APR.

2. REVIEW OF THE LITERATURE

2.1. Immune functions in neonate ruminants

The immune system of mammals changes throughout their lives, from the foetal period until ageing. However, the most extensive immune system development and maturation occur during the neonatal period and in the youth of animals.

After birth, neonatal ruminants are exposed to different pathogens that are potentially dangerous to their health and wellbeing (Barrington and Parish, 2001). At birth, the immune system of ruminants has been developed but not fully functional, and the responses to invading pathogens are not at the levels of immunocompetent adult animals. This means that the development of the immune system up to maturity is time-dependent, and thus, neonatal animals are at a higher risk of morbidity and mortality (Svensson *et al.,* 2006; Raboisson *et al.,* 2013). The immune system of bovines is mature at approximately 6–8 months of age (Chase *et al*., 2008); in other words, the levels of immune factors reach adult values at puberty in all ruminants.

The development of white blood cell populations begins at the foetal stage while the prenatal immune system develops (Cortese, 2009). The percentage of T lymphocytes in the peripheral blood has been shown to increase constantly in the bovine foetus, from 1% at 3 months to 45% at term foetus, while that of B cells remains low throughout gestation (1%). Monocytes first appear in the peripheral blood around the fourth month of gestation in the foetuses of calves (Senogles *et al*., 1979). The immune system of the foetus can develop an immune response against foreign antigens and obtain immunocompetency to different pathogens, but it depends on the gestation time and especially on the type of infection (Barrington and Parish, 2001; Cortese, 2009). However, after birth and during the neonatal period, ruminants are highly dependent on passively transferred immunity and innate immunity.

The innate immune system components (including complement, collectins and ficolins, lysozymes, antimicrobial peptides, APPs, etc.) and cells (monocyte and macrophage lineage and granulocytes) provide the first protection against infectious agents or invading pathogens (Firth *et al.,* 2005). At the time of birth, most of the immune system

components are present in the newborn organism, although the soluble mediators might be in suboptimal concentrations, and cellular components of the adaptive immune system are naïve or in quiescent states (Firth *et al.*, 2005). The neutrophil functions are partially immature after birth (Hauser *et al.*, 1986), but from the first week of life, neutrophilic granulocytes are functional for effective response (Kampen *et al.*, 2006). The number of circulating natural killer (NK) cells is higher in calves (Kulberg *et al.*, 2004). Colostrum ingestion has influence on increase in the percentage of phagocytising polymorphonuclear leukocytes and monocytes (Menge *et al.*, 1998) indicating its influence on innate immunity.

The cornerstones of the adaptive immune system are T and B lymphocytes. The levels of T cell subpopulations in calves are comparable with those in adult cows; and B cell population increase with age (Kampen *et al.*, 2006). In calves, the mean percentage of CD4+ lymphocytes increases to some degree during the first 6-8 weeks and in the first week of life the blood lymphocyte population consists of 25% of γδ T cells (Kampen *et al.*, 2006). While γδ T cells have many properties similar to those of the innate immune system and serve as a link between innate and acquired immunity, higher levels of these cells can compensate for the lower responses of the immature adaptive system during the neonatal period (Bonneville *et al.*, 2010; Heinrichs, 2017). The newborn adaptive immune response is biased toward the Th2 cell lineage, and cell-mediated and memory responses are suppressed (Chase *et al.*, 2008). During the gestation period, the polarisation towards Th2 is important for foetus maintenance; however, after birth, the neonate needs to obtain a balance between Th1 and Th2 abundance (Morein *et al.*, 2002; Adkins *et al.*, 2004). The cell-mediated immune responses reach adult levels at approximately 2 weeks of life in calves (Barrington and Parish, 2001). The number of B lymphocytes reaches adult levels in calves after 2–3 weeks of life, but the lymphocytes still have a lower antibody-producing activity during the first month after birth (Senogles *et al.*, 1979; Nagahata *et al.*, 1991; Kampen *et al.*, 2006).

The colostrum is the first feed for neonatal ruminants and is thus an essential source of nutrients. In addition, colostrum provides immunological protection to offspring, serving as a unique body fluid with a dual role in neonates (Blum and Hammon, 2000; Vorbach *et al.*, 2006; Stelwagen *et al.*, 2009). Colostrum provides both immediate

specific and non-specific immune protection. The ruminant placenta is of the synepitheliochorial type (Wooding, 1992), which prevents immunoglobulin transfer from mother to foetus, and thus, neonate ruminants are agammaglobulinemic after birth and highly dependent on colostrum antibodies for proper immune protection. The consumption of good-quality colostrum is critical for neonatal ruminants, providing immunological protection (Chase *et al.*, 2008), and failed transfer of passive immunity increases the risk of infections considerably.

Furthermore, colostrum contains different hormones, growth factors (GF), and a variety of components associated with innate immunity, such as different peptides, small proteins, and enzymes (Vorbach *et al.*, 2006). During the last decade, the importance of specific colostrum proteins has been investigated (Hernández-Castellano *et al.*, 2015). The exact bioactive roles of different colostrum proteins in neonates still need clarification.

2.2. Acute-phase response

All living organisms must maintain homeostasis to ensure their survival and reproduction. Throughout evolution, unique protection mechanisms have coalesced to the immune system of animals, which is activated in case of disturbances and contributes to maintaining homeostasis in the organism through several physiological systems.

During gestation, foetuses are protected from environmental stressors. Immediately after birth, newborns are exposed to environmental agents, and their survival is highly dependent on the first-line non-specific defence mechanism of the innate immune system. The core part of the first-line non-specific defence mechanism is the APR, an evolutionarily well-conserved process that appears in all mammals, as well as in fish and invertebrates (Bayne and Gerwick, 2001; Cray, 2012).

The APR can be defined as a set of early and immediate local and complex systemic physiological reactions that are induced during the inflammation process (Baumann and Gauldie, 1994). The APR is a predetermined and well-orchestrated sequence of processes in which different cell types and protein networks are involved in the inflammatory response (Baumann and Gauldie, 1994; Ceciliani *et al.*, 2012). The specific aims of the APR are to isolate and destroy invading

pathogens and parsites, to prevent ongoing tissue damage, and to promote healing by restoring the homeostasis of the body (Baumann and Gauldie, 1994; Uhlar and Whitehead, 1999). The APR can be initiated by infection, tissue injury, trauma, stress, immunological disorders or neoplasia (Baumann and Gauldie, 1994; Gruys *et al.*, 2005). In particular, the APR is triggered by signal factor molecules, pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs) (Ceciliani *et al.*, 2012; Schrödl *et al.*, 2016). Inflammatory cells, such as granulocytes and phagocytes, express pattern recognition receptors (PRR), respond to PAMPs or DAMPs, and trigger intracellular signalling pathways, which finally results in the expression of pro-inflammatory molecules (Mogensen, 2009; Newton and Dixit, 2012; Yun *et al.*, 2014). At the site of the inflammatory stimulus, monocytes and macrophages are the predominant cells that elicit the APR by releasing pro-inflammatory cytokines, of which tumour necrosis factors (TNFs) interleukin (IL) 1 are the predominant ones (Baumann and Gauldie, 1994). Hence, inflammatory cells react locally and respond to inflammatory stimuli and cellular debris from tissue damage.

After the initiation of inflammation and the first release of alarm pro-inflammatory cytokines, the second wave of cytokines and chemokines, primary IL-6, Il-8 and monocyte chemoattractant protein, from local stromal cells, initiates a cellular and cytokine cascade (Baumann and Gauldie, 1994, Uhlar and Whitehead, 1999). Leukocytes are attracted to inflammatory sites by chemokines and in turn release further pro-inflammatory cytokines (Lloyd and Oppenheim, 1992; Uhlar and Whitehead, 1999). Locally caused inflammation is classically characterised by clinical signs such as heat, pain, swelling, loss of function, and redness (Gauldie, 1985).

Besides cytokines and chemokines, other inflammatory mediators are involved in the process of the APR (Uhlar and Whitehead, 1999). For example, transforming growth factor β (TGF-β), released during mast cell degranulation or aggregation-induced platelet activation, can also be a chemoattractant for monocytes and macrophages during the initiating event (Baumann and Gauldie, 1994).

Glucocorticoids can directly stimulate the expression of some APPs; however, they usually enhance the effect of pro-inflammatory cytokines during the transcriptional induction of APPs (Uhlar and Whitehead,

1999). Both glucocorticoids and GF modulate cytokine activity, and cytokines mainly stimulate APP gene expression (Baumann and Gauldie, 1994; Ceciliani *et al.*, 2002).

Besides local reactions at the inflammation site, cytokines also initiate systemic reactions in the organism, involving endocrine and metabolic alterations (Koj, 1996). In particular, pro-inflammatory (pyrogenic) cytokines are cofactors that cause a febrile response (Dinarello, 1999). The activation of the clotting system, kinin-forming pathways and complement occur during the systemic APR (Koj, 1996). Behavioural changes in animals, such as lethargy, anorexia, and somnolence are symptoms of systemic reactions during the APR. Nutritional changes and metabolic modifications, such as protein catabolism, increased lipolysis, and decreased gluconeogenesis, occur (Baumann and Gauldie, 1994; Gabay and Kushner, 1999; Gruys *et al.*, 2005; Borghetti *et al.*, 2009). General leukocytosis and thrombocytosis occur in the peripheral blood (Gabay and Kushner, 1999). Changes in nonprotein plasma constituents, such as hypoferremia, hypozincemia, hypercupremia, and increased plasma retinol concentrations, occur during the APR (Gabay and Kushner, 1999).

The systemic response to an acute inflammatory stimulus is the alteration of the synthesis of specific liver proteins such as APPs (Gabay and Kushner, 1999). APPs are defined as a set of plasma proteins whose concentrations increase or decrease by over 25 per cent during the APR (Kushner, 1982; Eckersall and Bell, 2010).

The immune system is closely integrated with the neuroendocrine system; therefore, non-infectious stress factors can also modulate the immune response (Yun *et al.*, 2014). Physical and physiological stress reactions can influence the APP levels in animals. Glucocorticoids can stimulate the expression of some APPs by enhancing transcriptional induction (conducted by pro-inflammatory cytokines) (Uhlar and Whitehead, 1999).

In addition to initiation and maintenance of the APR, downregulating mechanisms of this process are especially important for restoring homeostasis after the inflammation process. The pro-inflammatory cytokines IL-1, IL-6, and TNF-α also promote the resolution of the APR by initiating negative feedback for their synthesis (Baumann and

Gauldie, 1990; Baumann and Gauldie, 1994). The activation of the hypothalamic-pituitary-adrenal (HPA) axis is considered the main negative feedback loop of inflammation. Glucocorticoids exerts an anti-inflammatory effect by downregulating cytokine production in macrophages, monocytes, and stromal cells (Ray *et al.*, 1990; Gruys *et al.*, 2005; Borghetti *et al.*, 2009). This negative feedback response for damage control has been suggested to be one of the most important pathways in the homeostatic response (Baumann and Gauldie, 1994).

To prevent exaggerated APR reactions, anti-inflammatory cytokines, such as IL-10, IL-4, IL-13, and TGF-β, are released from T cell lymphocytes, macrophages, and other immune cells (Baumann and Gauldie, 1994; Burger and Dayer, 2002). Soluble cytokine receptors or receptor agonists remove the excess of pro-inflammatory cytokines (Koj, 1998). It has been found that insulin can act as an inhibitor of the activation of most APP genes as well (Campos *et al.*, 1994).

The APR is considered to be a short-period survival mechanism, as the process starts shortly after the injury or insult and ends within a few days. Nevertheless, the APR can extend and convert to a chronic phase of inflammation, which has negative consequences on the organism (Uhlar and Whitehead, 1999). Causes for a prolonged APR are the persistence of the stimulation factor or disruptions of normal APR control mechanisms (Baumann and Gauldie, 1994).

2.3. Acute-phase proteins

APPs are key components of the APR and have many different physiological roles in the organism during the APR (Cray, 2012). APPs can be grouped as negative (downregulated during the APR) or positive (upregulated during the APR) (Gruys *et al.*, 2005). Negative APPs include Alb, transferrin (TF), transthyretin (TTR), retinol-binding protein 4 (RBP4), and corticosteroid-binding globulin (CBG) (Murata *et al.*, 2004; Schrödl *et al.*, 2016). Positive APPs include Hp, C-reactive protein (CRP), SAA, ceruloplasmin (Cr), Fib, and alpha 1-acid glycoprotein (AGP) (Heinrich *et al.*, 1990; Murata *et al.*, 2004). At least 40 APPs have been identified (Schrödl *et al.*, 2016).

Another way to classify APPs is by the amplitude of concentration increase (APP response) during the APR: major, moderate, and minor.

APPs exist in all animal species, but the types of major APPs are species-specific (Cray, 2012). APPs of the major groups are characterised by a rapid serum concentration increase up to 1000-fold, peaking within 24–48 h and decreasing rapidly. The concentrations of APPs of the moderate group increase 5–10-fold, peaking after 2–3 days, and decreasing slower than the major APPs. Minor components, with slightly increase concentrations by 50% and 100% of their resting levels (Gabay and Kushner, 1999; Gruys *et al.*, 2005; Eckersall and Bell, 2010). The downregulation of negative APPs and increased synthesis of positive APPs alter plasma protein patterns during the APR (Uhlar and Whitehead, 1999). The concentrations of APPs in the affected animal depend on the severity of the disorder and tissue damage extend (Murata *et al.*, 2004).

The synthesis of APPs in the liver is induced by pro-inflammatory cytokines (TNF-α, IL-1, and IL-6) within a few hours after the initiating event, which activates hepatocyte receptors and induces the synthesis of APPs (Petersen *et al.*, 2004; Cerón *et al.*, 2005). This production is regulated by the activation of intracellular signalling pathways and transcription factors. The Kupffer cells of the liver play an intermediate role in the production of APPs, as they synthesise IL-6 after stimulation by pro-inflammatory cytokines, which are then presented to hepatocytes (Gruys *et al.*, 2005).

In addition to their synthesis in the liver, APPs are produced in peripheral tissues in response to focal injuries, to provide immediate protection from further damage and initiate the repair process (Schrödl *et al.*, 2016).

2.3.1. Acute-phase protein functions

The general roles of APPs involve binding foreign substances, opsonization and modulating phagocytic cell functions, activating the complement system, scavenging cellular remnants and free radicals, and neutralising proteolytic enzymes (Baumann and Gauldie, 1994; Gruys *et al.*, 2005). These proteins modulate the inflammatory process by interacting with defensive cells and pathogens (Ceciliani *et al.*, 2012).

SAA is a highly conserved protein which regulating genes have existed for at least half a billion years (Kisilevsky and Manley, 2012). SAA has

been identified in all vertebrates and even in protostomes (Uhlar and Whitehead, 1999; Rosani *et al.*, 2016). SAA is a 12–14 kDa protein and belongs to the family of homologous proteins (Frame and Gursky, 2017). The expression of SAA is induced by pro-inflammatory cytokines, mainly IL-1β, IL-6, and TNF-α, and occurs primarily in hepatocytes (Uhlar and Whitehead, 1999). However, SAA can also be synthesised extrahepatically in local tissues. Locally produced SAA isoforms interact with different cells through SAA receptors and play a physiological role at the site of expression (Urieli-Shoval *et al.*, 2000; Ye and Sun, 2015). There are numerous studies on the physiological functions of SAA; however, the details still need to be resolved (Sun and Ye, 2016). SAA is associated with high-density lipoproteins (Benditt and Eriksen, 1977) and binds to cholesterol via scavenger receptor and modulating the homeostasis (Liang and Sipe, 1995). SAA has immunomodulatory activities (Uhlar and Whitehead, 1999; Ceciliani *et al.*, 2012). It is an inhibitor of neutrophil respiratory burst (Linke et al., 1991) and is a chemoattractant for monocytes and polymorphonuclear leukocytes (Badolato *et al.*, 1994). During infections, retinol-binding and transport properties have been observed in SAA (Derebe *et al.*, 2014). One of the main properties is the opsonisation of gram-negative bacteria (Shah *et al.*, 2006). In the gastrointestinal tract, SAA upregulates MUC2 expression and mucin production (Larson *et al.*, 2003; Mack, 2003; Shigemura *et al.*, 2014), intestinal epithelial SAA has bactericidal properties (Eckhardt *et al.*, 2010), and is a predictive factor for intestinal epithelial wound recovery (Hinrichs *et al.*, 2018). The anti-inflammatory properties (downregulation of inflammation) of SAA are an important issue, as SAA concentrations in the serum during inflammation modulate neutrophil functions (Gatt *et al.*, 1998).

Hp is a glycoprotein with a molecular mass of 1,000–2,000 kDa (Morimatsu *et al.,* 1991; Ceciliani *et al.*, 2012) and belongs to a group of transport or metal-binding plasma proteins (Iliev and Georgieva, 2018). The main production sites of Hp are hepatocytes, and extrahepatic synthesis occurs in the lungs, skin, spleen, kidneys (D'Armiento *et al.*, 1997), and mammary glands (Thielen *et al.*, 2007). The main role of Hp is to form complexes with haemoglobin; it is, therefore, the principal scavenger of haemoglobin from the blood to prevent the loss of iron (Petersen *et al.*, 2004; Ceciliani *et al.,* 2012). Other functions of Hp include the regulating white blood cells during immune reactions, preventing cells from oxidative damage, exerting bacteriostatic effects

by restricting the available iron for bacterial growth, and enhancing angiogenesis and chaperone activity (Eaton *et al.*, 1982; Gabay and Kushner, 1999; Murata *et al.*, 2004; Petersen *et al.*, 2004; Ceciliani *et al.*, 2012). Bovine haptoglobin, at least in part, as an immunomodulator in serum suppression of lymphocyte proliferation (Murata and Miyamoto, 1993). Hp has been found to have an inhibitory effect on Th2 cytokine release but only weak inhibition of Th1 release, thereby modulating the Th1/Th2 balance (Arredouani *et al.*, 2003).

Alb, the main constitutive plasma protein, is considered to be a negative APP in all animal species (Murata *et al.*, 2004). Alb is synthesised in the liver, and its main functions are maintaining oncotic pressure and transportation of different substances such as amino acids. Fib is an acute-phase reactant and coagulation factor found in the serum of all vertebrates (Cray, 2012).

2.3.2. Acute-phase proteins as inflammatory markers

APPs are extensively used as non-specific quantitative biomarkers in medicine and veterinary medicine for diagnosis, prognosis, and monitoring of treatment during inflammation and infection (Alsemgeest *et al.*, 1994; Eckersall, 2000; Eckersall and Bell, 2010; Thomas *et al.,* 2016). In production animals, APPs can be used as markers of clinical or subclinical diseases and to monitor animal health and welfare (Skinner, 2001). It has been suggested that the general health status and management conditions in ruminant herds may be evaluated by determining APPs at the herd level (Gånheim *et al.*, 2007).

The production of pro-inflammatory cytokines and APPs varies under different inflammatory conditions (Gabay and Kushner, 1999). Owing to the expression of different cytokines, bacterial infections cause a more severe systemic APR than virus-induced infections because of the stronger reaction of the mononuclear-phagocytic system's cells (Gruys *et al.*, 2005). Bacterial and viral co-infections can result in prolonged APR (Gånheim *et al.*, 2003).

In particular, SAA is a sensitive biomarker during acute inflammation processes (Horadagoda *et al.*, 1999). SAA and Hp are useful markers for evaluating stress reactions in cattle (Lomborg *et al.*, 2008). The increased concentrations of SAA in physically stressed 4-month old calves

compared with those in a control group have been demonstrated (Alsemgeest *et al.*, 1995a). Likewise, APP (SAA, Hp) concentration changes have been shown to occur after transport stress in ewes (Piccione *et al.*, 2012).

Viruses, such as bovine respiratory syncytial virus (BRSV), can influence the increase in SAA and lipopolysaccharide-binding protein (LBP) concentrations, which has been demonstrated in calves (Orro *et al.*, 2011). Parasite infections can induce the production of APPs in ruminants as well, and an experimental study in lambs infected with *Cryptosporidium parvum* showed changes SAA and Hp concentrations (Dinler *et al.*, 2017). Another study did not find remarkable associations between APP concentrations and infections with *Cryptosporidium* spp. in dairy calves (Seppä-Lassila *et al.*, 2015). An observational field study in dairy calves indicated that *Cryptosporidium* shedding on the second week of life, with a higher number of oocysts in calves faecal samples, was associated with higher serum concentrations of SAA and Hp at that time of (Niine *et al.*, 2018).

2.4. Acute-phase proteins in ruminants

SAA and Hp are the major APPs in bovines (Conner *et al.,* 1988*;* Ceciliani *et al.,* 2012). The moderate bovine APPs are LBP, AGP, and Cr, and the minor ones are Fib and alpha 1-antiproteinase (α1-AP) (Ceciliani *et al.*, 2012). SAA is also expressed locally in certain bovine organs at moderate levels. These include the adipose tissue, colon, jejunum, skeletal muscles, synovial membranes, thymus, thyroid gland, uterus, and mammary gland (Berg *et al.*, 2011) and forestomach (Dilda *et al.,* 2012). SAA and Hp levels have been shown to increase during respiratory diseases in calves (Gånheim *et al.*, 2003; Nikunen *et al.*, 2007; Tóthová *et al.*, 2012). Hp and Fib levels can increase during respiratory tract infections and umbilical cord infections (Seppä-Lassila *et al.*, 2015). Similarly, SAA concentrations are higher during diarrhoea episodes in calves (Tóthová *et al.*, 2012). Hp has been demonstrated to have a seasonal effect, as Hp concentrations were higher in January than in July in a study using cows in Italy (Giannetto *et al.*, 2012). Respiratory diseases in calves have an influence on Fib concentrations, which are higher than those in healthy calves (Tóthová *et al.*, 2012). Fib concentrations have been shown to increase after turpentine injection (Conner *et al.,* 1988). In adult cows APPs are increased during the mastitis, endometritis,

respiratory disease, during the period of starvation and other inflammatory conditions (Eckersall *et al.*, 2001; Eckersall and Bell, 2010; Ceciliani *et al.*, 2012).

In small ruminants, particularly sheep, SAA and Hp are the major APPs; AGP is the moderate one, and Fib and Cr are the minor ones (Gomez-Laguna *et al.*, 2011; Iliev and Georgieva, 2018). In goats, SAA and Hp are also major APPs, while AGP and Fib are the moderate ones (Iliev and Georgieva, 2018). SAA and Hp concentrations increase during sheep scab disease (caused by *Psoroptes ovis*) (Wells *et al.*, 2013). During various bacterial and viral diseases, such as caseous lymphadenitis, endotoxemia, and scrapie, SAA and Hp concentrations increase as well (Eckersall *et al.*, 2007; Meling *et al.*, 2012; Chalmeh *et al.*, 2013).

There are few studies on APPs in reindeer (*Rangifer tarandus tarandus*). A challenge with *Escherichia coli* (*E. coli*) lipopolysaccharide (LPS) caused a remarkable increase in SAA concentration, compared with that in the control group, which peaked at 48 h. Hp concentration increased for the first 8 h and was elevated until 48 h, but there were no remarkable differences in Hp concentration in the *E. coli* endotoxin challenge. Therefore, SAA was a more sensitive indicator of bacterial infections in reindeer than Hp after the LPS challenge (Orro *et al.*, 2004). Fib concentration, increases in reindeer during inflammatory or infection (Catley *et al.,* 1990).

2.5. Changes in the concentrations of acute-phase proteins in neonatal ruminants

The neonatal period in ruminants is considered to be 1–28 days after birth while the haematological, biochemical, and metabolic parameters change (Knowles *et al.*, 2000; Piccione, 2011). Some of these parameters are characterised by higher postpartum values that decrease over time and others by lower values after birth that increase. Similar to other parameters, the levels of different APPs change during the first weeks of the life of a ruminant, which are termed age-related changes in APPs. In newborn calves, Alsemgeest *et al.* (1995b) demonstrated that SAA concentrations were lower immediately after birth than in adult cows. In a study of Finnish dairy calves, the mean serum concentration of SAA increased quickly after birth and reached the highest at 7 days of age, decreased after 10 days of age, and stabilization occurred after 3 weeks

of life (Orro *et al.*, 2008). The same tendency was shown in a study of seven clinically healthy calves, where the lowest mean value of SAA concentration was found at birth, and the SAA concentration increased from the first day of life, peaked on the seventh day, and decreased from the fourteenth day (Tóthová *et al.*, 2016). In beef calves, mean SAA concentrations have been shown to decline during the first month of life, and the concentrations were higher on day 3 (first sample) than on day 16 (Seppä-Lassila *et al.*, 2017).

The highest Hp mean concentrations were measured on day 10 and the lowest on day 21 in a study of dairy calves in Finland (Orro *et al.*, 2008). Tóthová *et al.* (2016) observed the lowest Hp concentrations in calves after birth, followed by a substantially gradual increase up to day 7 of life, and until the end of 30 days, the mean values were higher than those on the first day after birth. The highest Hp concentrations during the 68-day observation period were on days 10–13 in Holsten heifer calves (Ramos *et al.,* 2021). In beef calves, the concentrations of Hp showed no remarkable changes over the sampling period during the first 30 days, while approximately two-thirds of the samples had Hp concentrations below the detection limit (60 mg/l) (Seppä-Lassila *et al.*, 2017). In calves, Hp levels were low during the first 4 days after birth compared with those of adult cows (Alsemgeest *et al.*, 1995). Another study also found low Hp concentrations in the early stages of the lives of calves; only a few samples showed higher values than the reference range (determined as 0–400 mg/l) before 9 days of age and mean levels within the reference range after 9 days (Knowles *et al.*, 2000). However, the reference values for neonatal calves were established as <196 mg/l (Seppä-Lassila et al., 2013).

Alb, a negative APP, is the most prominent serum protein fraction at birth in calves (Tóthová *et al.*, 2016). Its relative concentrations decreased one day after birth with colostrum intake, but gradually increased from the second day during the first month (Tóthová *et al.*, 2016). Other studies have confirmed a progressive increase in Alb levels after birth in calves (Bertoni *et al.*, 2009; Piccione *et al.*, 2009). It has been previously shown that IL-6 inhibits the production of albumin (Tanaka et al., 2014). During the first week of life, increased expression of the Fib gamma-β chain was observed in the plasma (Herosimczyk *et al.*, 2013).

In lambs, SAA concentrations increased during the first week of life, started to decrease around the second week, and stabilised at the end of the third week of life (Dinler *et al.*, 2020). A study of goat kids showed that SAA concentration increased after birth, reaching the highest level at the end of the first week, after which it gradually decreased (Ulutas *et al.*, 2017). This pattern of change was similar to the changes in SAA concentration in neonatal lambs. In newborn lambs, serum Hp levels (baseline in lambs 0.9 ± 0.46 g/l) were constant during the first days of life (Samimi, 2019). In goat kids, no remarkable changes were found in serum Hp levels during the first 3 weeks of life (Ulutas *et al.*, 2017).

In neonatal reindeers, only one study, conducted in Finnish Lapland, described the APP concentration changes after birth, indicating that SAA concentrations were the lowest on the day of birth, peaked during the second week of life, and started to decrease during the third week of life (Orro *et al.*, 2006). SAA levels in neonatal fawns were considerably higher than those in adult white-tail deer (Cray *et al.*, 2019). This indicates that after the increased concentrations of SAA in the neonatal period, a considerable decrease was observed. In reindeer calves, Hp concentrations were the lowest at birth, increased during the second week of life, and decreased at the end of one month (Orro *et al.,* 2006).

2.6. Colostrum cytokines and acute-phase proteins

Bovine colostrum contains of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, and interferon gamma (IFN-γ), which synergistically mediate inflammation. Their concentrations are remarkably higher in colostrum than in mature milk (Goto *et al.,* 1997; Hagiwara *et al.*, 2000). To reduce the effects of pro-inflammatory cytokines, inhibitors, like the interleukin 1 receptor antagonist (IL-1ra), are present in colostrum (Hagiwara *et al.*, 2000). The association between colostrum cytokines and calf serum cytokines IL-1β, IL-6, TNF-α, IFN-γ, and IL-1ra has been demonstrated in a study where the highest concentration of pro-inflammatory cytokines was determined 24 h after birth after colostrum consumption in calf serum and become almost undetectable four weeks after the birth (Yamanaka *et al.*, 2003). The immunostimulatory effect of orally administrated IL-1β in newborn calves has been found to affect the proliferation of T cells and activation of neutrophils (Hagiwara *et al.*, 2001). The colostrum pro-inflammatory cytokines (IL-1β, TNF-α and

IFN-γ) improve the mitogenic response of peripheral blood mononuclear cells (Yamanaka *et al.,* 2003). Therefore, colostrum cytokines contribute to the maturation of neonatal immune functions (Yamanaka *et al.*, 2003).

In addition to the cytokines, cow colostrum contains several APPs, such as SAA, Hp, CRP, and AGP (McDonald *et al.*, 2001; Ceciliani *et al.*, 2005). The SAA isoform SAA3 is expressed in the mammary gland (synonym mammary-associated serum amyloid A 3 (M-SAA3)) (Mcdonald *et al.*, 2001; Larson *et al.*, 2005; Weber *et al.*, 2006; Molenaar *et al.*, 2009). This process is regulated by cytokines produced by cells in milk (Keller *et al.*, 1982), predominantly macrophages, which are part of the healthy lactating mammary gland. Furthermore, cytokines are released from neutrophils, which are predominant during infections (Wheeler *et al.*, 2007).

SAA3, from here forward named SAA, has been found in bovine, equine, and ovine colostrum (McDonald *et al.*, 2001), and its concentration is upregulated by prolactin during lactation (Larson *et al.*, 2005). SAA levels remain highly elevated in cow colostrum until 4 days postpartum, which is considered to be a non-pathological process in the udder at the beginning of lactation (McDonald *et al.*, 2001; Thomas *et al.*, 2016). Likewise, moderately elevated Hp concentrations are detectable in cow colostrum, and concentrations decrease around day 4 after calving (Hiss *et al.*, 2004; Thomas *et al.*, 2016). Similar to SAA, Hp is synthesized in the mammary gland (Hiss *et al.*, 2004; Thielen *et al.*, 2005). Bovine mammary gland produces also Alb (Shamay *et al.*, 2005).

The inflammation-regulatory functions of SAA are also true for locally expressed SAA, as it is detectable in the milk of cows and ewes with mastitis (Winter *et al.*, 2003; Jacobsen *et al.*, 2005). SAA has an important immunological role in the protection of the mammary gland against both gram-positive and gram-negative pathogens in cattle (Larson *et al.*, 2005; Molenaar *et al.*, 2009; Murata *et al.*, 2020). The expression of APPs at the beginning of lactation has a protective function for the udder.

The colostrum intake probably influences the concentrations of APPs in calf serum, as the SAA concentrations were low before colostrum consumption and increased during the first 24 h after birth. However,

no correlation was found between the elevated levels of SAA in the colostrum and SAA levels in the serum in calves (McDonald *et al.*, 2001).

No direct transfer of SAA isoforms from colostrum to calves was found in the study by Orro *et al.* (2008). Thus, it was assumed that SAA only has a local influence on the gastrointestinal tract in calves and/or that highly alkaline isoforms are removed from circulation (Orro *et al.*, 2008).

The influence of colostrum APPs on the concentration of APPs in offspring may vary depending on the APP and animal species. The possible influence of colostrum proteins on neonate plasma protein levels has been investigated in a study of sheep, which indicated that out of the 70 examined proteins, the levels of 8 increased in the blood plasma of lambs after colostrum consumption (Hernández-Castellano *et al.*, 2015). Another proteomics study in sheep demonstrated the effect of colostrum on SAA concentrations in the sera of lambs (Hernández-Castellano *et al.*, 2014).

Studies on the relationship between Hp concentrations in calves and colostrum consumption are inconclusive. Dairy calves that received milk-based formula had higher serum Hp concentrations than colostrum-fed calves, until 4 days of life respectively (Sadri *et al.*, 2020). In contrast, the trend for elevated plasma Hp concentrations were found in the group that received colostrum than in the group that received milk-based formula during the first 9 days of life in another study (Liermann *et al.*, 2020). In neonatal lambs, the colostrum deprived lambs had Hp concentrations detectable in plasma 14 hours after birth , compared with colostrum fed ones (Hernández-Castellano *et al.,* 2015). In neonatal pigs, it has been shown that colostrum Hp is transferred to piglets, and the endogenous Hp synthesis is also stimulated by colostrum (Hiss-Pesch *et al.*, 2011).

In conclusion, the effects of colostrum cytokines and APPs on offspring need further clarification.

2.7. Associations between acute-phase proteins and weight gain in young animals

The health and well-being of young animals are commonly measured by their ability to grow, gain weight, and develop according to species and

breed. Animal growth depends on many factors, such as nutrition, genetic background, disease and stress level (Seppä-Lassila *et al.*, 2018). During the neonatal period, the available resources for adequate development have a long-term effect on future performance and growth. The resources needed for growth and development are often depleted owing to infectious diseases and injuries.

Infections and accompanying inflammatory conditions elicit an APR and the production of APPs, protecting against invading pathogens and re-establishing homeostasis in the organism. However, if the inflammation period is prolonged (in cases of subclinical infections, chronic inflammation, and chronic stress) and homeostasis is not achieved soon after the disturbance, the chronic condition can cause loss of body weight or growth disturbances, and the well-being of young animals is impaired for a longer period (Gruys *et al.*, 2005). More specifically, if the APR continues, anorexia and cachexia negatively affect the nitrogen balance, and catabolism of muscle cells occurs (Gruys *et al.*, 2005).

Pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) that upregulate the inflammatory reaction contribute to the loss of body mass (Gabay and Kushner, 1999). It has been demonstrated that pro-inflammatory cytokines induce skeletal muscle protein breakdown (Goodman, 1991, 1994).

Postnatal growth is greatly controlled by GH and insulin-like growth factor 1 (IGF-1) (Strle *et al.*, 2004). Overexpression of IL-6 in transgenic mice led to lower plasma concentrations of IGF-1, and these animals were smaller than normal mice *(*De Benedetti *et al.,* 1997). In *in vitro* studies, GH-stimulated synthesis of IGF-I was inhibited by IL-1β and TNF-α (Wolf *et al.*, 1996), and pro-inflammatory cytokines induce resistance to the major growth factor, IGF-1 (O'Connor *et al.*, 2008). The increased production of the cytokines IL-1, IL-6, and TNF-α causes sickness behaviour and alters protein metabolism. During inflammation, nutrients are redirected to the liver for APP production. Specifically, during infection and inflammation, redirection of nutrients from protein production occurs towards liver anabolism of APP production (Colditz, 2002). Metabolic changes modify protein and amino acid requirements, and therefore, reduced food intake, decreased growth, and muscle wasting are frequently observed during inflammation (Le Floc'h *et al.,*

2004). Therefore, the influence of pro-inflammatory cytokines and APPs on long-term growth can be hypothesised.

The elevated levels of APPs (Hp) in neonate calves during the first week of life have been associated with higher morbidity and mortality rates until 4 months of age (Murray *et al.,* 2014). Studies in reindeer calves and beef calves, have demonstrated the association between elevated SAA concentrations in approximately the second week of life and poorer growth rate measured approximately at the age of 200 days of life in Finnish beef calves and at the age of 4 month in reindeer calves (Orro *et al.,* 2006; Seppä-Lassila *et al.,* 2017).

In a study of dairy calves reared for meat, the increased concentration of SAA at the day they arrived new farm (average age 24.1 days), had an association with a poorer growth rate at the age of approximately 73 and 224 days and the carcass weight at slaughter at 15-18 months of age (Seppä-Lassila *et al.,* 2018).

3. AIMS OF THE STUDY

The main aim of this study was to evaluate the changes in the concentrations of APPs after the birth of neonatal ruminants (reindeer calves, lambs, and dairy calves) and to determine the effect of colostrum intake on detected changes. Additionally, the growth rate of a young animals was assessed to evaluate the long-term associations with neonatal-period APP levels.

Specific aims of the study were as follows:

1. To describe systemic changes in the concentration of APPs (SAA, Hp, and Alb) in the blood serum of neonatal ruminants (reindeer (**I**), lambs (**II**–**III**), and calves (**IV**)) during the neonatal period of the first 3 weeks of life.

2. To study and describe the influence of ingested colostrum cytokines (IL-1β, IL-6, and TNF-α (**IV**)) and APPs (SAA (**II**–**IV**) and Hp (**IV**)) on the concentrations of APPs (**II**–**IV**) and cytokines (**IV**) in the blood serum of neonatal ruminants.

3. To study and describe the influence of colostrum intake and serum APP concentrations on the further performance of the animal via measured growth rates (**I**–**IV**).

4. MATERIALS AND METHODS

4.1. Study design and population

Reindeer (**I**) were kept in the Kaamanen experimental herd of the Reindeer Herders' Association (Rovaniemi, Finland) in Finnish Lapland. The total area of the herd is 48 km^2. The number of reindeer in the herd was 58 female reindeer. The study population comprised 54 semi-domesticated reindeer calves (28 males and 26 females) born between the 9th and 22nd of May, 2004. The hints in the herd had been given ivermectin as antiparasitic treatment in the previous autumn.

Lambs (**II**–**III**) were kept in an organic sheep farm located in Southern Estonia, specialised in meat production. The herd was composed of Estonian black-headed, Dorper, Gotland, Suffolk, Merino, and mixed-breed sheep. The main herd included approximately 250 ewes.

The lamb study (**II**) conducted in 2011 and 2012 included 322 lambs (163 females and 159 males) and 197 ewes. The lambs were born between April and May. The second lamb study (**III**), conducted in 2014, included 269 lambs (124 females and 145 males) and 187 ewes. The lambs were born between April and May.

In the first lamb study (**II**), the animals were housed in a barn with *ad libitum* access to water, silage, and hay and access to outdoor fenced grasslands. During the summer period, the lambs were kept outside the grassland near the farm. In the second lamb study (**III**), the animals were kept indoors in a single barn during the sampling period because of sub-freezing outdoor temperatures. They had *ad libitum* access to hay, silage, and fresh water. During the summer period, most sheep were taken to a small islet in the Baltic Sea near Western Estonia, where they stayed until weaning (27 July 2014) with minimal monitoring.

All lambs (**II**–**III**) received colostrum immediately after birth from their dams, and later the milk of the ewe was the main source of their nutrition. The herds were not vaccinated. Antiparasitic treatment, either albendazole or ivermectin, was performed in separate animal groups at risk, which was evaluated through faecal sample parasitology results. The lambs in the risk group were dewormed for the first time at weaning or soon after weaning.

The study of dairy calves (**IV**) was conducted on a large dairy farm in Central Estonia. During the study, there were approximately 1,800 dairy cows on the farm, whose average yearly milk production per cow was 10,000 kg. The herd was composed of Holstein cows. A total of 143 female calves born between 21 January and 16 March 2015 were included in this study. The calves were separated from their mothers immediately after birth and bottle-fed 3 litres of quality-controlled colostrum once within two hours after birth (median (±SD), 61 ± 30 min). The colostrum given to the calves was collected from the dam and the quality examined visually and with a colostrum densimeter (Jørgen Kruuse A/S, Langeskov, Denmark). In two cases colostrum quality was poor (specific gravity <1035, or total protein <50 g/l), so deep-frozen colostrum from another cow was used. Obstetric aid at birth were recorded as spontaneous delivery (n = 85), aid by one person (n = 46), and by two persons (n = 12). 76 mothers of the calves were primiparous (first time calving) and 67 multiparous (second time 28, third time 22, and 17 fourth time or more).

During the first 4 weeks, the calves were kept in individual pens. After that, they were moved to group pens (8–10 calves per pen). The calves were fed 2–3 kg warmed unpasteurised raw milk twice per day with free access to hay and starter feed (Prestarter; Agrovarustus OÜ, Tartu, Estonia) up to 15–17 days of age. Afterwards, a milk powder (Josera Golden Spezial; Josera GmbH & Co. KG, Kleinheubach, Germany) solution was fed while the calves had free access to starter feed and hay.

The calves were vaccinated against parainfluenza virus type 3 and BRSV (Rispoval®, Zoetis Belgium SA, Louvain-la-Neuve, Belgium) on the second day of life and against bovine herpesvirus-1 (Hiprabovis, Laboratorios HIPRA, S.A., Girona, Spain) at three months of age. Toltrazuril (Cevazuril, Ceva Santé Animale, Libourne, France) was used once at the age of 25-65 days as prophylactic treatment against *Eimeria* spp. infection. Owing to the outbreak of cryptosporidiosis, halofuginone lactate (HL; Halocur; Intervet International B.V., Boxmeer, the Netherlands) was used. For data analysis, the calves were retrospectively divided into three groups based on the HL treatment regimen: 1) no treatment, 2) incorrect treatment (treatment started >48 h after birth and lasted <7 days), and 3) correct treatment (according to the manufacturer's instructions; started <48 h after birth and lasted ≥7 days).

All the studies (**I**–**IV**) were observational field studies.

4.2. Sampling: blood samples, colostrum samples, and weight measurements

Reindeer calves (**I**) were sampled seven times, 3–5 days apart (days 0, 4–6, 8–10, 12–14, 16–17, and 20–22) from birth until the end of the study period. Blood was collected from 51 calves at 23–33 days of age.

In total, 370 serum samples, 210 ethylenediaminetetraacetic acid (EDTA) blood samples, and 366 faecal samples were collected from 54 reindeer calves. Blood samples were collected into 10 ml vacuum tubes (BD Vacutainer, New Jersey, USA), and EDTA blood samples were collected into 2 ml EDTA-coated tubes (BD Vacutainer). Serum was separated, divided into aliquots, and stored at -18 °C for further analysis. EDTA blood samples were analysed for Fib concentration on the day of collection.

Reindeer calves were weighed using a digital scale (Adam Equipment Co. Ltd., Milton Keynes, UK) at three time points: immediately after birth, at 20 or 21 days of age, and at 114–127 days of age.

The sample collection times for lambs were planned at the height of the lambing season (**II**–**III**). Blood samples from lambs in 2011 and 2012 (**II**) were collected once per week for 1–3 times during the lambing period. Blood samples from 176 lambs were sampled once, 90 lambs twice, and 56 lambs thrice, and the proportion of samples was almost equally divided between both years (248 samples in 2011 and 276 samples in 2012). In 2011, sampling was performed during the first 25 days of the lives of the lambs. In 2012, the lambs were sampled on days 0–13 (day 0 was counted as the day of birth).

Blood from the lambs was sampled by puncturing the jugular vein with a vacutainer using a 4 ml serum clot tube (Vacuette, Greiner Bio-One, The Netherlands). Serum was separated, frozen in portions and stored at -20 °C until further analysis.

In the second study in lambs (**III**), blood samples were taken at 7 days intervals four times (during 0–21 days of life), and 692 serum samples were collected. Serum samples were collected from the jugular vein into sterile evacuated 10 ml plastic tubes with an 18 G needle. Serum was

separated, and stored at -20 °C until further analysis. Additionally, 141 faecal samples were collected in Study **III**.

Individual colostrum samples from ewes (n = 197 (**II**) and n = 187 (**III**)) were collected 1–2 h after lambing by milking the ewes with hands into sterile 10 ml plastic tubes. The samples were immediately placed at -20 °C storage until further analysis.

In lamb study **II**, the body weight of the lambs was measured once at the age of 3–4 months (78–130 days of age) on the farm using a digital scale (precision of ± 50 g). To determine the daily weight gain (g/day) of the lambs, the estimated average weight of the lambs at birth (fixed at 4000 g) was subtracted from the weight measured at 3–4 months of age and divided by the age (days) of the lambs at weighing. The average birth weight of the lambs was estimated using a separate subset of lambs (n = 30) at the same farm in the birthing seasons from 2014 to 2015 when the lambs were weighed on the first day of their life. The ewe structure, feed, and management of the farm were similar to those of the study period from 2011–2012. Sixteen lambs were weighed in 2014 (**III**) (eight females and eight males; eight singles and eight twins), and 14 lambs were weighed in 2015 (nine females and five males; eight singles and six twins). The birth weight of the 30 lambs was 3998 ± 981 g (mean ±SD).

The weight of lambs in the second lamb study (**III**) was measured during the first week of life and at approximately 4–5 months of age (131 ± 10.2 days). The weight of the lambs was measured using a digital scale (precision of ± 50 g) on 28 April (n = 230) and 5 May (n = 159) 2014. To assess the average daily weight gain, additional measurements were performed using a digital scale (precision of ±100 g) on 18 August (n = 238). The decrease in the number of animals weighed at consecutive times was because some had already been sold for slaughter.

In the study of dairy calves (**IV**), serum and faecal samples collected from 143 female calves once a week during the first 3 weeks of life (1–3 samples per calf) were used in the present study. To avoid excessive stress on the calves, the sampling was sometimes skipped, based on the overall demeanour of the animal. Thus, not every calf included in the study had samples from all 3 weeks available. In total, 103 samples were available from the first week, 112 from the second, and 114 from the third, making for an overall sample size of 329. Mean sampling age

(±SD) was 4.3 (±2.0) days in the first week, 11.0 (±2.0) days in the second week, and 18.0 (±2.0) days in the third week.

Blood samples were collected from the jugular vein into sterile evacuated test tubes using an 18 G sterile needle. Samples were centrifuged (1,800 × *g*, 10 min), and the serum was separated and stored in aliquots at -20 °C until further analysis. A sample of colostrum was collected before it was provided to the newborn. These samples were collected into sterile 10 ml vials and stored in aliquots at -20 °C until further analysis. Before the laboratory analyses, the colostrum samples were skimmed via centrifugation (7,840 × *g*, 10 min), followed by removal of the fat layer.

The calves were weighed with a digital scale MS4 PW (Excell Precision Co., Ltd, Vilnius, Lithuania) immediately after birth, at approximately one month of age (29.6 ± 4.5 days (mean ± SD)) and at 9 months of age (264 ± 6.5 days). At one month of age, a digital scale (KERN EOS 150K100NXL, Kern & Sohn GmbH, Balingen, Germany) was used. At 3 months of age (101.4 ± 10.6 days), the weight of the calves was estimated using a measuring tape (ANImeter, Albert Kerbl GmbH, Buchbach, Germany) as the digital scale was not available. Average daily weight gain (ADWG, g/day) was calculated for the period from birth to one month of age (n = 122), from birth to 3 months of age (n = 120), and from birth to 9 months of age (n = 121).

The total number of the samples collected and analysed within the studies (**I–IV**) is presented in Table 1.

Table 1. Serum and colostrum samples collected and analysed during the studies

Animals	No. of animals	No. of serum samples	No. of colostrum samples	Evaluated parameters	Weight measurement
Reindeer calves (I)	54	370	-	SAA, Hp, Alb, γ-glob, and Fib	Birth weight Weight at 3–4 months of life
Lambs (II)	322	524	197	SAA, Hp, Alb, and Glob	Weight at 3–4 months of life
Lambs (III)	269	692	187	SAA, Hp, Alb, and Glob	Birth weight Weight at 4–5 months of life
Dairy calves (IV)	143	329	143	SAA, Hp, Alb, Glob, IL-6, TNF-α, and IL-1β	Birth weight Weight at 1, 3, and 9 months of life

4.3. Laboratory analyses

Descriptions of the analytical methods of other parameters and information about parasitic evaluation can be found in the corresponding sections of the articles (**I–IV**).

Serum (**I–IV**) and colostrum (**II–IV**) SAA concentrations were determined using a commercial kit for indirect enzyme-linked immunosorbent assay (ELISA) (Phase SAA Assay; Tridelta Development Ltd., Maynooth, Co. Kildare, Ireland), according to the manufacturer's instructions. When SAA concentrations exceeded the standard value, the serum samples were diluted with 0.9% NaCl solution and tested again. Detection limits for Hp and SAA were 0.06 g/l and 0.3 mg/l, respectively.

Serum Hp concentrations (**I–IV**) were determined using a haemoglobin binding assay method described by Makimura and Suzuki (1982), with modifications, using tetramethylbenzidine (0.06 mg/ml) as a substrate (Alsemgeest *et al.,* 1994). Pooled and lyophilised aliquots of bovine acute-phase serum were used to plot the standard curves. To calibrate the assay, a bovine plasma sample with a known Hp concentration provided by the European Commission Concerted Action Project (number QLK5-CT-1999-0153) was used (Skinner, 2001).

Cytokine concentrations in the colostrum and serum samples (**IV**) were determined using bovine IL-1β, IL-6, and TNF-α ELISA kits (Cusabio Biotech, Wuhan, Hubei, China) according to the manufacturer's instructions. The detection limits for IL-1β, IL-6, and TNF-α were 15.6 ng/l, 2.5 ng/l and 50.0 ng/l, respectively.

Fib concentration was measured using a heat precipitation method described in details in the respective publication. The Alb concentration in the serum was determined using a commercial photometric colorimetric method based on bromocresol green dye binding (ACCENT-200 Albumin II Gen, PZ Cormay S.A., Poland (**I**) and Photometric Colorimetric Test for Albumin Liquicolor, Human Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany (**II**-**IV**)).

4.4. Statistical analyses

Linear mixed regression models were used to investigate age-dependent changes in APP and serum immunoglobulin G (IgG) concentrations in reindeer calves and lambs (**I–III**). To compare the differences in concentrations of serum inflammatory markers (APPs and cytokines) and IgG in dairy calves (**IV**) by study week, linear mixed models (for SAA, Hp, Alb, IgG, IL-6, IL-1β, and TNF-α) and a mixed Tobit model (for IL-1β) were used.

Linear mixed regression models were used to investigate associations between serum protein concentrations and colostrum protein concentrations (APPs, cytokines, and IgG) in lambs and dairy calves (**II, III, and IV**). Pearson correlations were used to study the correlations between the measured variables in the colostrum and serum samples of dairy calves (**IV**).

Mixed linear regression models were used to study the associations between serum protein concentrations of different lambs (**II–III**) and colostrum SAA concentrations during the first 3 weeks of age with ADWG recorded approximately 3 months later. To investigate associations between the ADWG of the dairy calves (**IV**) and the concentrations of IgG, APPs, and cytokines in the colostrum and associations between ADWG and calf serum concentrations of proteins by study week, linear regression models were used. ADWG at 1, 3, and 9 months of age were used as response variables in each model. Linear regression models were also used to investigate the association between short-term (birth to 20–21 days) and long-term (birth to 114–128 days) ADWGs with area under the curves (AUCs) of serum concentrations of APPs and proteins (total protein and γ-globulins) during the neonatal period in reindeer calves (**I**).

In linear mixed models, ewes, lambs, reindeer calves, and dairy calves were included as random factors, and an isotropic spatial exponential covariance structure was used to model the correlation between repeated samples. All non-normally distributed response variables were logarithmically transformed (all APPs except Alb and cytokines). Stepwise backward elimination of the explanatory variables was used for the final models. Biologically meaningful interactions and linear relationships between the dependent and explanatory continuous

variables were tested. All model assumptions were verified using scatter plots and normality plots of the standardised residuals.

Stata/IC 13.1 (**I and II**) and Stata/IC 14.1 (**III and IV**) for Windows (StataCorp LP, Texas, USA) were used for all statistical analyses. The statistical significance level was set at $p \leq 0.05$.

5. RESULTS

5.1. Changes in acute-phase protein serum concentrations

In all the studies (**I–IV**), newborn serum SAA concentrations started to increase from a low base value on the day of birth. In lambs, SAA peaked during the first week of life, in reindeer and dairy calves during the second week of life. After that period, the concentration of SAA decreased in all the animal species. See the following subchapters on the exact values for newborns of different study species.

The serum Hp concentrations of newborns increased from a relatively low concentration in all species and peaked at 12–14 days in reindeer, at 4–7 days in lambs, and 7–11 days in dairy calves and decreased afterwards.

Serum Alb concentrations increased in all species during the study period, while serum globulin (Glob) concentrations decreased constantly during the first months of life.

5.1.1. Changes in acute-phase protein concentrations in reindeer (I)

In the study of reindeer, the mean SAA concentrations were lowest at the day of birth (3.1 ± 3.8 mg/l (mean ± SD) increased significantly up to 8–10 days (65.9 ± 41 mg/l) of age ($p < 0.01$), and peaked at 12–14 days (80.9 ± 49.9 mg/l). SAA concentrations decreased and stabilised at the end of the study period, at 33 days. Hp concentration was the lowest on the day of birth (0.412 ± 0.087 g/l), low on days 4–6 (0.433 ± 0.087 g/l), increased on days 8–10 (0.518 ± 0.056 g/l), peaked on days 12–14 (0.583 ± 0.057 g/l), and decreased at the end of the study period (22–23 days; 0.516 ± 0.098 g/l) (Figure 1).

Alb concentrations were lowest at birth (25.35 ± 2.86 g/l) and then increased and stabilised by the end of the third week of the life (22–23 days; 37.22 ± 2.56 g/l). Fib concentration was the lowest on the first day (2.44 ± 0.54 g/l) and increased and peaked between 4 and 14 (3.79 ± 0.84 and 3.75 ± 0.55 g/l, respectively) days before decreasing.

Mean γ-globulin concentrations were higher in the first 2 days (15.51 ± 4.76 g/l). Concentrations subsequently decreased, being the lowest at the age of 23–33 days (2.84 ± 0.56 g/l).

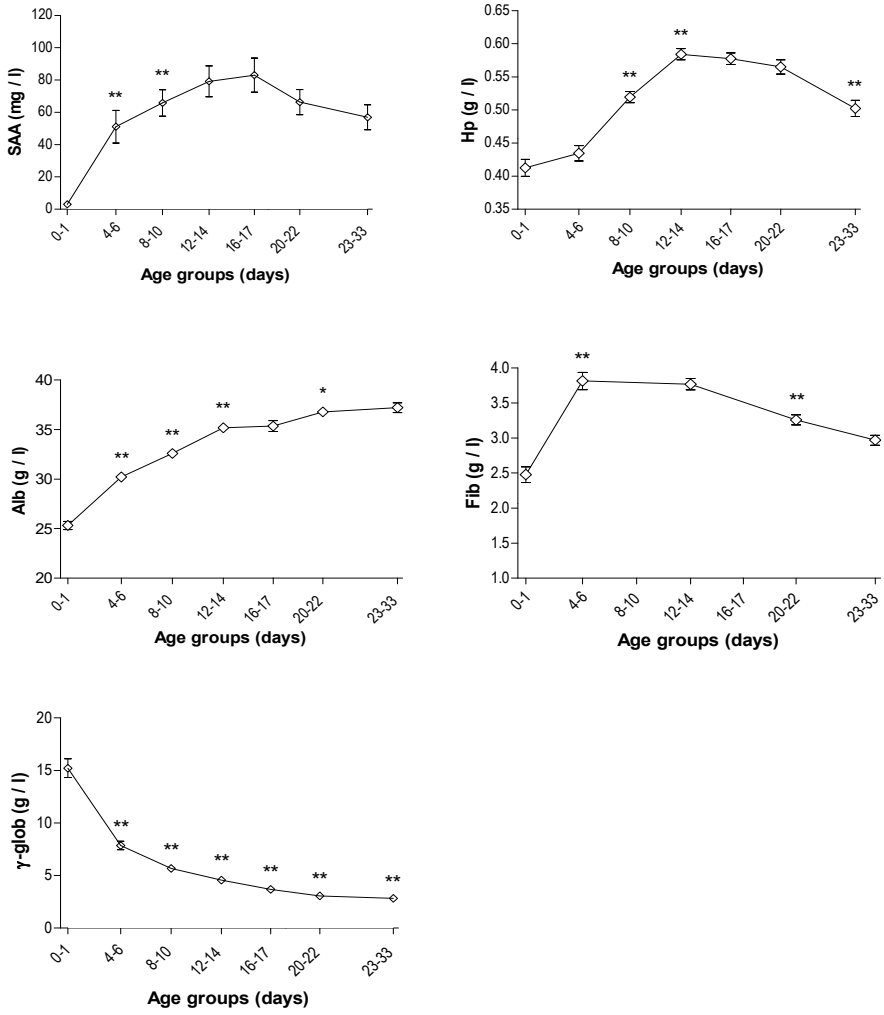

Figure 1. Mean (± SEM) serum amyloid A (SAA), haptoglobin (Hp), albumin (Alb), fibrinogen (Fib), and γ-globulin (γ-glob) concentrations in the sera of reindeer calves (n = 54) sampled during the study period (0–33 days of age). Exact sample size of serum samples for age groups 0–1 (n = 48, Fib n = 21), 4–6 (n = 53, Fib n = 46), 8–10 (n = 54), 12–14 (n = 54, Fib n = 48), 16–17 (n = 54), 20–22 (n = 54, Fib n = 51), and 23–33 (n = 51, γ-glob n = 48, Fib n = 23)

* Significantly different from the previous age group ($p < 0.05$)

** Significantly different from the previous age group ($p < 0.01$)

5.1.2. Changes in acute-phase protein concentrations in lambs (II–III)

The concentration changes of SAA in the blood serum of lambs were similar in both studies (**II**–**III**). More specifically: in the first lamb study (**II**), serum SAA concentrations were the lowest shortly after birth (0 day: median 2.2; min–max 1.3–14.3 mg/l; n = 5) but showed a significant increase on the first day of life ($p = 0.009$), when the values were also highest (median 20.9; min–max 1.5–200.5 mg/l; n = 39). Serum SAA concentrations remained elevated until 5 days of age (median of 2–5 days 18.7; min–max 0.3–216.4 mg/l; n = 160) and started to decrease from 6 days of age (median 11.9; min–max 0.3–164.4 mg/l; n = 46) ($p = 0.048$) and then remained stable until the end of the study period.

The concentrations of Hp were higher (linear trend $p = 0.012$) in the first week (median 151; min–max 60–3342 mg/l) and started to decrease during the second week of life (median 130; min–max 60–4678 mg/l; all samples after 7 days of age) (Figure 2). There was a difference between study years, as Hp concentrations were significantly higher in 2012 ($p < 0.001$) than in 2011.

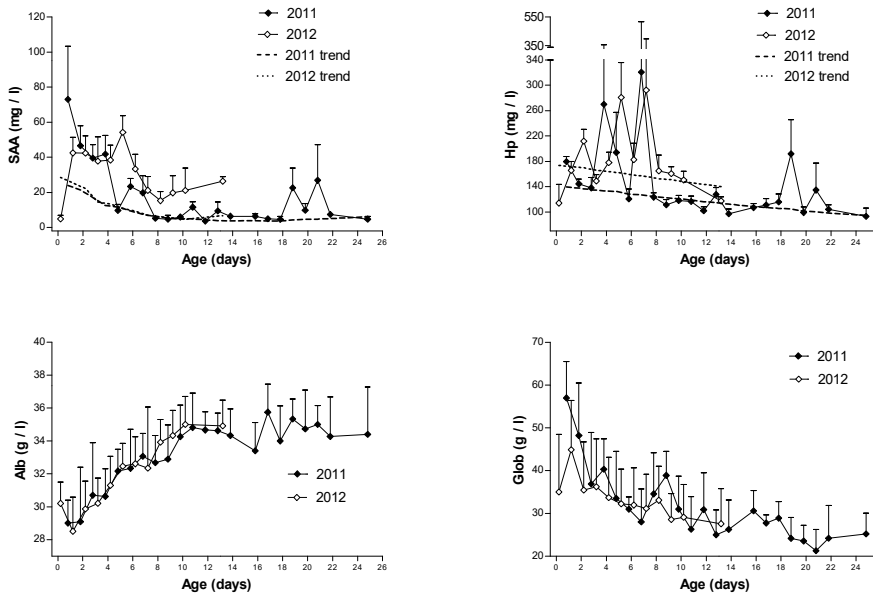

Figure 2. Mean (± SEM) serum concentrations of serum amyloid A (SAA), haptoglobin (Hp), albumin (Alb), and globulins (glob) in lambs during the first 25 days of life by study year. Dotted lines represent random regression models of time changing trend predictions by study years back-transformed from the logarithmic scale. Total number of samples n = 524; n = 248 in the year 2011 (111 lambs, 1–3 samples per lamb); n = 271 in the year 2012 (208 lambs, 1–2 samples per lamb)

In the second lamb study (III), SAA concentration was low on the day of birth (mean 21.1 mg/l, n = 27) and peaked at the age of 3-4 days (mean 118.7 mg/l, n = 66). Hp mean concentrations were 0.21 g/l on the day of birth (n = 27) and peaked at 4–8 days (mean 0.711 g/l, n = 161) (Figure 3).

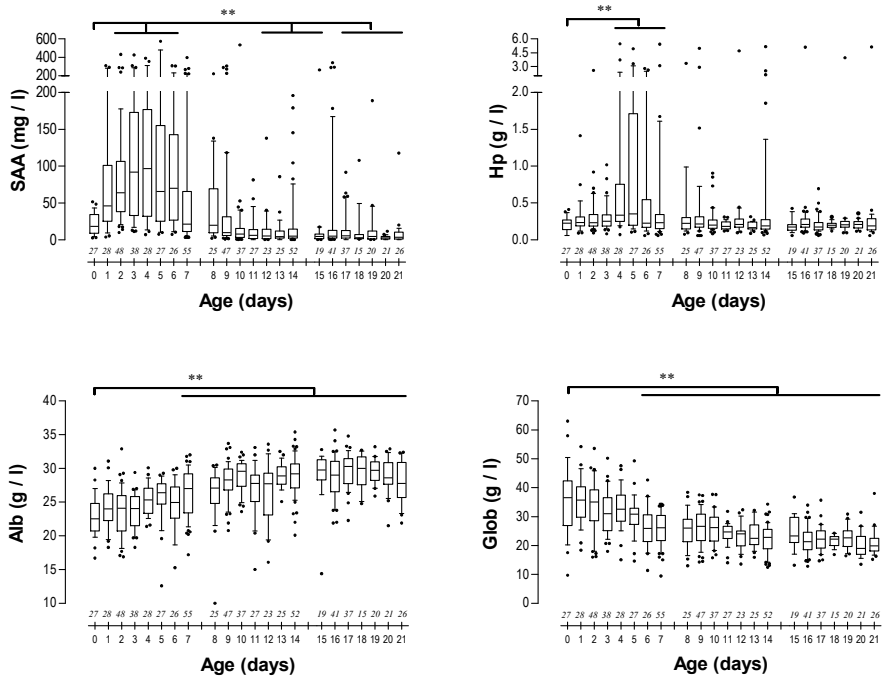

Figure 3. Concentrations of serum amyloid A (SAA), haptoglobin (Hp), albumin (Alb), and globulins (Glob) in lambs during the study period (0–21 days of age). The whiskers of the box plot represent 10–90% of the results. The numbers under the box plot bars represent the number of lambs at different sampling ages
** Significant differences (Bonferroni's corrected $p < 0.01$) between the first samples (age 0 days) and all other samples taken at different ages are indicated

5.1.3. Changes in acute-phase proteins and cytokines in dairy calves (IV)

Changes were observed in the serum APP concentrations of the calves during the first 3 weeks of life (Table 3 and Figure 4). SAA concentrations were elevated during the first 2 weeks and then declined in the third week. Hp concentrations were highest in the second week compared to the first and third weeks. Alb concentrations were lowest in the first week and then increased and stabilised during the second and third weeks. Serum IgG was highest in the first week and declined constantly over the three-week period investigated.

Age-dependent changes in the serum cytokine concentrations are presented in Table 3 and Figure 5. Serum concentrations of IL-6 in the

first week were higher than those in the second and third weeks. TNF-α concentrations were highest during the first week of life and then declined. IL-1β concentrations were lower during the second week than during the first and third weeks.

Serum concentrations of IL-6 and TNF-α in calves were positively correlated during all 3 weeks ($r = 0.79$, 0.92, and 0.64, respectively; all $p < 0.001$). Serum SAA and Hp concentrations were positively correlated during the second and third weeks of life ($r = 0.45$ and $r = 0.36$; both $p < 0.001$). All other pairwise correlations between serum protein concentrations during the first 3 weeks of age were not significant ($r < 0.3$).

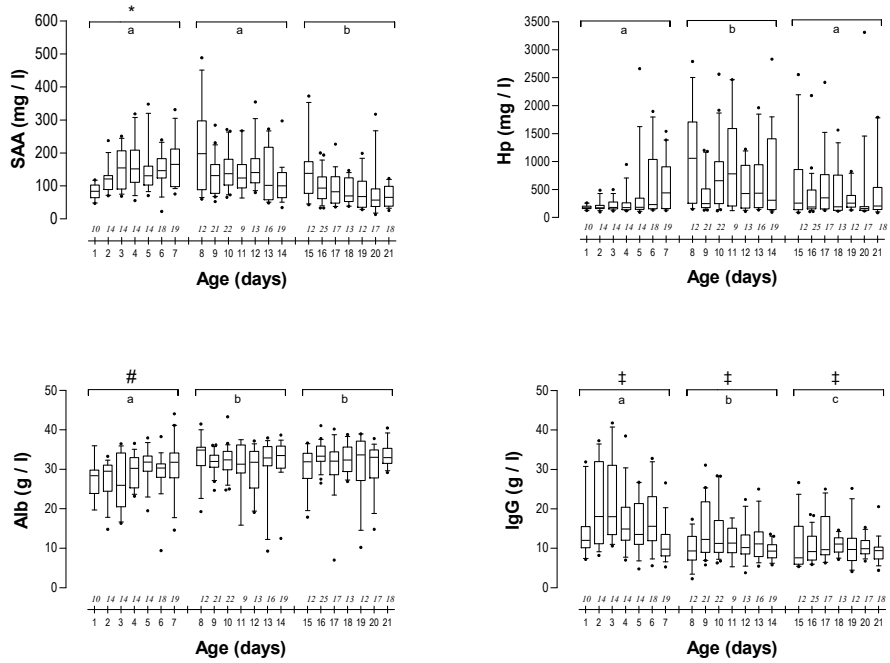

Figure 4. Concentrations of blood serum amyloid A (SAA), haptoglobin (Hp), albumin (Alb), and immunoglobulin G (IgG) of calves (n = 143; sampled 1–3 times during the first 3 weeks after birth). The whiskers of the box plot represent 10–90% of the results. Numbers under the columns represent the sample size for every day of age

* Negative association with colostrum SAA concentrations ($p = 0.038$) evaluated using linear regression models

Negative association with colostrum immunoglobulin (Ig) G concentrations ($p = 0.021$) evaluated by linear regression models

‡ Positive association with colostrum IgG concentrations ($p < 0.001$) evaluated using linear regression models

a, b, c Different letters indicate significantly ($p < 0.001$) different concentrations by week evaluated with mixed linear regression models

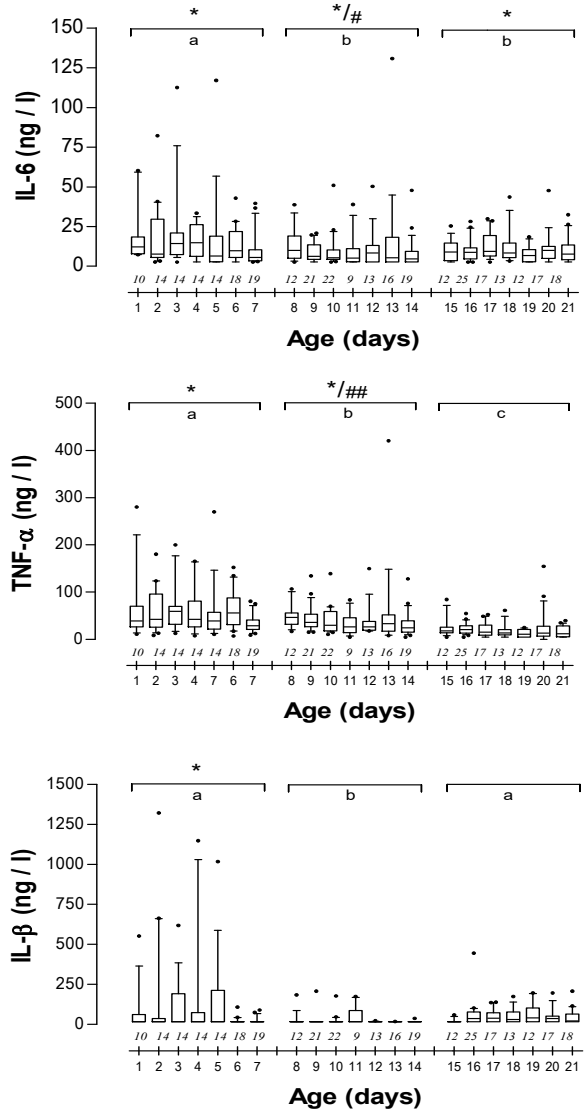

Figure 5. Concentrations of serum interleukin (IL)-6, tumour necrosis factor (TNF)-α, and IL-1β of calves (n = 143; sampled 1–3 times during the first 3 weeks after birth). The whiskers of the box plot represent 10–90% of the results. Numbers under the columns represent the sample size for every day of age

* Positive association with the same cytokine colostrum concentrations ($p < 0.001$) evaluated by regression (IL-6 and TNF-α) and Tobit regression (IL-1β) models

Positive association with colostrum immunoglobulin (Ig) G concentrations ($p = 0.031$) evaluated by regression model

Positive association with colostrum IgG concentrations ($p = 0.008$) evaluated by regression model

a, b, c Different letters indicate significantly ($p < 0.001$) different concentrations by week evaluated using mixed linear regression (IL-6 and TNF-α) and mixed Tobit regression (IL-1β) models

5.2. Colostrum and acute-phase proteins (II–IV)

In the first lamb study (**II**), a higher concentration of SAA in ewe colostrum was associated with a higher SAA concentration in lamb serum at 1–5 days of age (p = 0.009; Table 2).

Table 2. Association of colostrum serum amyloid A (SAA) concentrations and the number of siblings with serum SAA concentrations (logarithmically transformed) in 1–5-day old lambs (n = 169) in the model, where ewe (n = 120) was added as a random

Variable (n of samples)	Estimate	95% CI of estimate	p-value	Wald test p-value
Colostrum SAA (mg/l)	0.02	0.006; 0.039	0.009	
No. of siblings:				0.134
Single (n = 86)	0			
Twins (n = 82)	-0.44	-0.88; -0.01	0.045	
Triplets (n = 3)	-0.33	-3.15, 2.48	0.817	
Intercept	2.95	2.6; 3.29	<0.001	

CI – confidence interval

In the second study of lambs (**III**), higher colostrum SAA concentrations were also associated with higher SAA concentrations in the serum of their lambs at 2–4 days of life (n = 109; p = 0.042). Higher serum Hp (2–4 days) concentrations in lambs were positively associated with higher colostrum SAA concentrations in their ewes (n = 81; p = 0.013).

In the first lamb study (**II**), the mean (±SD) SAA concentration in ewe colostrum was 10.8 mg/l (±14.9), median 5.1 mg/l, and min–max 0.3–75 mg/l (number of ewes 197).

In the second lamb study (**III**), the mean (±SD) SAA concentration in ewe colostrum was 20.47 mg/l, median 5.43 mg/l, min–max 0.3–478.8 mg/l (number of ewes 187).

In the study of dairy calves (**IV**), the associations between cow colostrum pro-inflammatory cytokines and SAA and Hp concentrations and calf serum SAA, Hp, Alb, Glob, and cytokine IL-6, TNF-α, and IL-1β concentrations at different periods are presented in Figures 4 and 5. Colostrum concentrations of IL-6 and TNF-α were correlated with each other (r = 0.69, p < 0.001; n = 143).

The concentrations of other inflammatory markers and IgG in the colostrum did not correlate with each other ($r < 0.2$). Colostrum inflammatory marker concentrations had some associations with all measured protein concentrations in the calves' serum (Figures 4 and 5). Colostrum SAA had a negative association with calves SAA serum concentration in the first week of life ($p = 0.018$).

Colostrum IL-6 concentration was positively associated with serum IL-6 concentrations during all three weeks (all $p < 0.001$). Colostrum TNF-α concentration was positively associated with the same cytokine serum concentrations during the first two weeks ($p < 0.001$), and colostrum IL-1β concentration was associated with serum IL-1β during the first week ($p < 0.001$). Colostrum IgG concentration was positively associated with calves' IL-6 ($p = 0.031$) and TNF-α ($p = 0.008$) concentrations in the second week, and negatively with serum Alb concentration during the first week ($p = 0.021$). Colostrum IgG concentration was positively associated with serum IgG concentrations during all weeks ($p < 0.001$) (Figures 4 and 5). The concentrations of APPs (SAA, Hp, and Alb), cytokines (IL-6, TNF-α, and IL-1β), and IgG in the colostrum and serum (by weeks of age) are presented in Table 3.

Table 3. Colostrum and serum sample concentrations of pro-inflammatory cytokines, acute-phase protein (APP), and immunoglobulin G (IgG) in 143 dairy calves sampled during the first 3 weeks of life (1–3 samples per calf)

Variables	Statistics	Colostrum	Serum samples (days of age)		
			1-7	8-14	15-22
		(n = 143)	(n = 103)	(n = 112)	(n = 114)
SAA (mg/l)	mean (±SD)	65.7 (47.1)	146.6 (66.5)[a]	142.3 (78.8)[a]	93.0 (61.6)[b]
	median	52.1	128.9	125.3	79.0
	(min–max)	(7.5–277.0)	(22.4–347.7)	(34.0–487.9)	(13.2–371.9)
Hp (mg/l)	mean (±SD)	190 (62)	376 (430)[a]	701 (658)[b]	451 (553)[a]
	median	175	186	408	201
	(min–max)	(104–480)	(97–2,662)	(95–2,830)	(85–3,310)
Alb (g/l)	mean (±SD)	NA	29.1 (5.8)[a]	31.7 (5.4)[b]	32.1 (5.4)[b]
IgG (g/l)	mean (±SD)	55.1 (12.0)	16.8 (8.7)[a]	12.2 (5.7)[b]	10.7 (4.5)[c]
IL-6 (ng/l)	mean (±SD)	55.3 (53.5)	16.5 (19.5)[a]	10.5 (14.8)[b]	11.0 (8.4)[b]
	median	43.2	9.8	5.9	10.0
	(min-max)	(10.2–501.4)	(2.5–117.0)	(2.5–130.7)	(2.5–47.5)
TNF-α (ng/l)	mean (±SD)	5,126 (3,681)	578 (486)[a]	410 (445)[b]	215 (197)[c]
	median	4040	450	300	160
	(min-max)	(1,290–28,620)	(70–2,820)	(50–4,200)	(50–1,550)
IL-1β (ng/l)	mean (±SD)	551.0 (1,142.1)	102.8 (227.6)[a]	24.2 (33.3)[b]	49.8 (56.4)[a]
	median	115.9	15.6	15.6	34.3
	(min–max)	(15.6–5,445.1)	(15.6–1,321.5)	(15.6–207.4)	(15.6–444.8)

SAA, serum amyloid A; Hp, haptoglobin; Alb, albumin; IL-6, interleukin-6; TNF-α, tumour necrosis factor-α; IL-1β, interleukin-1β; NA, not analysed

[a, b, c] Different letters indicate significantly ($p < 0.001$) different concentrations by week evaluated using mixed linear regression and mixed Tobit (for IL-1β) models

5.3. Acute-phase proteins as predictors of ruminant growth (I–IV)

In the study of reindeer (**I**), male calves gained more weight (mean = 0.039 kg/day, 95% CI: 0.021–0.058; $p < 0.001$) in the long-term (from birth to 112 days) than female calves. No remarkable associations were found between APP concentrations and weight gain in reindeer calves.

In the first study of lambs (**II**), the mean (±SD) weight gain of the lambs at 3–4 months (78–130 days of age) was 25.34 (±6.16) kg. The daily weight gain of lambs until the age of 3–4 months in both years (2011 and 2012) was 200.8 g/day (±53.7; n = 259). Being a twin or a triplet was associated with a lower daily weight gain than being a single offspring. Older ewes had lambs with higher weight gain than primiparous ewes. On average, male lambs gained more weight daily than female lambs. There was no significant difference in weight gain of lambs between the study years. The lambs that were weighed later had a smaller ADWG (Table 4). There was a significant negative association between serum SAA concentrations at 6–12 days of age and average weight gain of the lambs at 3–4 months of age (n = 194; $p < 0.006$). Weight gain was not associated with serum Hp level at any of the weeks of age tested.

Table 4. Association of serum amyloid A (SAA) concentration, age of ewe, number of siblings, sampling year, and gender with weight gain (g/day) of 6–12-days old lambs (n = 194) in the random linear regression model, where ewe (n = 131) was added as a random factor

Variable (n of samples)	Estimate	95% CI of estimate	p-value	Wald test p-value
SAA (log mg/l)	-5.84	-10.04; -1.65	0.006	
Age at sampling (days)	-3.16	-6.44; 0.13	0.059	
Sample year:				
2011 (n = 108)	0			
2012 (n = 86)	-137.16	-406.68; 130.85	0.315	
Age at weighing 2011 (days)	-2.03	-3.64; -0.41	0.014	
Age at weighing 2012 (days)	1.55	-0.84; 3.94	0.204	
Age of ewe:				>0.001
1 year (n = 48)	0			
2 years (n = 61)	34.42	18.22; 50.62	>0.001	
3–4 years (n = 31)	65.54	42.69; 89.19	>0.001	
5–6 years (n = 32)	42.05	20.20; 63.89	>0.001	
7–8 years (n = 22)	40.05	13.04; 67.06	0.004	
No. of siblings:				>0.001
Single (n = 93)	0			
Twins (n = 95)	-52.29	-67.20; -37.38	>0.001	
Triplets (n = 6)	-103.8	-146.38; -61.22	>0.001	
Gender:				
Male (n = 107)	0			
Female (n = 87)	-23.22	-34.48; -11.96	>0.001	
Intercept	431.91	291.26; 572.26	>0.001	

CI – confidence interval

In the second study of lambs (**III**), ADWG (±SD) at 124 days of age (±6.9 days) was 208 ± 50 g/day (n = 239). A positive association was found between the ADWG of the lamb at 122 days of age and the serum concentrations of APPs (Hp and Alb) in the lambs during the second week of life and IgG concentrations on the first and second days of life. A tendency towards a significant association of ADWG with colostrum SAA concentration was found but not with serum SAA concentration (Table 5). No significant associations were found between serum APP concentrations of/during the first and third weeks of age and ADWG at 122 days.

Table 5. Results of mixed linear regression model (**III**) for average daily weight gain (ADWG) (kg/day) at 122 ± 5 days of age (n = 53) associated with concentrations of acute-phase proteins (APPs) serum amyloid A (SAA), haptoglobin (Hp), and albumin (Alb) during/measured in/of the second week of life. Immunoglobulin G (IgG) serum concentration of/measured on the first and second days of life and colostrum SAA concentration were added to the model to indirectly control the effect of colostrum

Variable (n of samples)	Estimate	95% CI of estimate	p-value	Wald test p-value
IgG (mg/dl)[a]	0.00002	0.000002; 0.00004	0.030	
SAA (log mg/l)[1, b]	-0.0075	-0.018; 0.003	0.163	
SAA colostrum (mg/l)	-0.0003	-0.001; 0.00001	0.068	
Hp (g/l)[b]	0.0112	0.002; 0.020	0.018	
Alb (g/l)[b]	0.0057	0.001: 0.011	0.025	
TP (g/l)[b]	-0.0025	-0.005; -0.0001	0.040	
No. of siblings:				
Singlet (n = 19)	0			
Twins (n = 34)	-0.0639	-0.090; -0.038	>0.001	
Ewe age:				0.256
1 year (n = 3)	0			
2 years (n = 10)	0.0410	-0.011; 0.093	0.124	
3–4 years (n = 15)	0.0209	-0.037; 0.079	0.479	
≥ 4 years (n = 25)	0.0622	0.010; 0.115	0.020	
Gender:				
Female (n = 21)	0			
Male (n = 32)	-0.0037	-0.026; 0.019	0.750	
Intercept	0.1693	0.075; 0.263	> 0.001	

CI – confidence interval, TP = total protein

[1] Log transformed in order to reduce skewness

[a] Calculated from gamma glutamyltransferase (GGT) activity at age 1 and 2 days using the formula: ln (IgG) (mg/dl) = 2.251 + 0.700 × ln [GGT] (IU/l) + 0.378 lamb age (days), where age 1 (day) = 0 and age 2 (days) = 1 (Britti *et al.*, 2005)

[b] Measured in the second week of life (age 8–14 days)

In the study on dairy calves (**IV**), the colostrum APP concentrations did not show considerable associations with ADWG at any age period, and the serum protein concentrations were not associated with the ADWG of the calves at one month of age.

The average birth weight of the calves was 41.2 ± 5.8 kg (ranging from 27 to 52 kg). At the age of one month, ADWG (g/day) was 419.5 ± 149.2 (n = 121), at the age of three months 783.8 ± 130.8 (n = 119) and at 9 months 688.8 ± 166.9 (n = 120).

Serum Hp concentration in the second week was borderline significantly negatively associated with three-month ADWG ($p = 0.067$; n = 98) and in the third week significantly negatively associated ($p = 0.025$; n = 104). Age at the blood sampling time, HL treatment group, *Cryptosporidium* oocyst level group and parity of the cow were included in these multiple linear regression models as possible confounders (Table 6).

Serum concentrations of SAA and IL-6 during the second week of life were negatively associated with ADWG at 9 months of age (Table 7). A significant correlation was found between serum SAA and Hp concentrations ($r = 0.36$) as well as between IL-6 and TNF-α concentrations ($r = 0.92$); thus, the associations between Hp and TNF-α concentrations and weight gain were evaluated separately from SAA and IL-6 concentrations in different models. In these separate models, significant negative associations were found between serum Hp concentration and 9-month ADWG ($p = 0.025$), between serum TNF-α concentration and 9-month ADWG ($p = 0.041$) in second week of life. In the third week of age negative association between SAA concentration and 9-month ADWG ($p = 0.010$; n = 105) was found. Age at the time of blood sampling, age at weighing, HL treatment group, and *Cryptosporidium* oocyst level group were included in these multiple linear regression models as confounders for 9-month ADWG (Table 7).

Table 6. Results of a linear regression model for detecting association of serum pro-inflammatory cytokine and acute-phase protein (APP) concentrations of calves (n = 104) at the third week of life (15–21 days of age) with average daily weight gain (ADWG; g/day), measured approximately at 3 months of age (mean (±SD), 101.4 ± 10.6 days)

Variable (n of samples)	Coef.	SEM	p-value	Wald test p-value
Age at sampling (days)	-0.39	5.77	0.946*	
Serum Hp conc. (mg/l)	-0.06	0.03	0.025	
HL treatment group:**				0.032
No treatment (n = 16)	0			
Incorrect treatment (n = 41)	-43.17	35.76	0.230	
Correct treatment (n = 47)	-95.16	37.03	0.012	
Cryptosporidium* group:**				0.583*
No oocysts found (n = 35)	0			
Low oocyst level (below the median) (n = 36)	-18.16	29.31	0.537	
High oocyst level (above the median) (n = 33)	-35.45	34.14	0.302	
Parity of the cow:				
Multiparous (n = 54)	0			
Primiparous (n = 50)	-44.47	23.59	0.062	
Intercept	914.06	111.81	<0.001	

SEM, standard error of the mean; Hp, haptoglobin; HL, halofuginone lactate

* Nonsignificant variable was retained in the model because of the confounder effect

** Incorrect treatment - treatment started >48 h after birth and lasted <7 days. Correct treatment - treatment started <48 h after birth and lasted ≥7 days

*** Positive samples were divided into low- and high-oocyst count categories using median values of the same week

Table 7. Results of multivariable linear regression model for detecting association of serum pro-inflammatory cytokine and acute-phase protein concentrations of calves (n = 100) at the second week of life (8–14 days of age) with average daily weight gain (ADWG; g/day) measured approximately at 9 months of age (mean (± SD), 264 ± 6.5 days)

Variable (n of samples)	Coef.	SEM	p-value	Wald test p-value
Birth weight (kg)	-5.06	2.02	0.014	
Age at sampling (days)	-9.19	4.84	0.061	
Age at weighing (days)	4.48	1.49	0.003	
SAA serum conc. (mg/l)	-0.36	0.12	0.004	
IL-6 serum conc. (ng/l)	-1.48	0.63	0.021	
HL treatment group*:				<0.001
No treatment (n = 16)	0			
Incorrect treatment (n = 37)	-96.61	27.47	0.001	
Correct treatment (n = 47)	-152.41	30.47	<0.001	
***Cryptosporidium* group**:**				0.158***
Negative (n = 50)	0			
Low oocyst count (n = 50)	-19.12	25.04	0.447	
High oocyst count (n = 29)	33.69	26.19	0.201	
Intercept	-9.57	387.22	0.980	

SEM, standard error of the mean; Hp, haptoglobin; SAA, serum amyloid A; IL-6, interleukin-6; HL, halofuginone lactate

* Incorrect treatment – treatment started >48 h after birth and lasted <7 days. Correct treatment – treatment started <48 h after birth and lasted ≥7 days

** Positive samples were divided into low- and high-oocyst count categories using median values of the same week

*** Nonsignificant variable was retained in the model because of the confounder effect

6. DISCUSSION

6.1. Changes in acute-phase protein concentrations in neonatal ruminants

After birth and during the neonatal period of reindeer calves, lambs and dairy calves changes in serum SAA, Hp, Alb (**I**–**IV**) and Fib (**I**) concentrations and cytokine (IL-1β, IL-6, and TNF-α) concentrations in dairy calves (**IV**) were notable for certain time periods. There were similarities in the changes in APP concentrations in the neonatal period between different animal species; however, to some extent there was species-specific variation.

The lowest SAA value was detected on the day of birth for all species. During the APR, the serum concentration of SAA increased markedly within 16–24 h (Cray, 2012; Cray *et al.,* 2019). Therefore, the low levels of SAA on day zero can be explained by the delay in APP synthesis in the liver (Cray *et al.*, 2019).

The SAA concentration changes in reindeer calves in this study (**I**) are comparable with the SAA concentration changes in the study by Orro *et al.* (2006), where the concentrations of SAA decreased from the third week of life. The serum concentration changes in SAA reported in this study in lambs (**II**–**III**) are comparable with those in other studies conducted in lambs (Dinler *et al.*, 2017; Samimi, 2019; Dinler *et al.,* 2020). In a study by Dinler *et al.* (2020), the reference intervals for neonatal lambs were established, and the concentration of SAA was given as the minimum-maximum because of the higher concentration during the first week of lamb life (reference interval of SAA 0.34–55.12 mg/l). This indicates the importance of higher SAA concentrations in the first week of life for lambs. In dairy calves, previous studies have demonstrated that the SAA concentration at less than 2 weeks of age was higher than that in calves over 14 days of age, which is comparable with the SAA concentration changes in study **IV** (Orro *et al.,* 2008; Seppä-Lassila *et al.,* 2013; Tóthová *et al.,* 2016).

The SAA concentrations increased in all studied species from the first day of life and decreased in the second or third weeks of life. While no association was found with the *Cryptosporidium* infection studied in the

first week of life, it probably indicates a physiological increase in SAA concentrations as part of the innate immune protection process.

In reindeer calves (**I**), Hp concentrations peaked at days 12–14, which is similar to the peaking time of SAA concentrations. In the study by Orro *et al.* (2006), the Hp concentrations in reindeer calves were the lowest at birth and thereafter increased constantly during the neonatal period.

The Hp concentrations were lower after birth in lambs (**II**, **III)**, peaked at the end of the first week of life, and stabilised to the basic level thereafter. Interestingly, in the first study of lambs (**II**), Hp concentrations differed between the two study years, indicating that Hp might react to some kind of overall e.g., environmental and/or management changes at the herd level. When the reference interval of Hp concentration to neonate lambs was established, not so apparent effect as that for SAA concentration was noted (Hp reference interval 0–0.4 g/l) (Dinler *et al.*, 2020). For comparison: the reference interval for healthy adult sheep was reported to be 0.05–0.18 g/l (Nowroozi-Asl *et al.*, 2008). In the study of dairy calves (**IV**), the Hp concentrations were low after birth, but compared with the concentrations at the first and third weeks of life, the highest concentrations were observed in the second week. In this study, *Cryptosporidium* oocysts were found in 85.3% (122/143) of the calves. However, the effect of *Cryptosporidium* was considered in the models, and thus should not be the main reason for the higher concentrations of Hp in the second week of life.

The levels of Alb concentrations were the lowest in the first week and increased and stabilised during the second and third weeks (**I**–**IV**), while the concentrations of globulins gradually decreased throughout the study in all study species (**I**–**IV**). Based on the studies (**I**–**IV)** it is to assume that Alb changes can be rather the result of the liver function development during the neonatal period and not influenced by the APR. Alb concentration fluctuations differ from changes in other APPs and are considered as part of the normal physiological processes after birth.

It is accepted in ruminant medicine that APPs are sensitive biomarkers of inflammatory and infectious processes (Eckersall and Bell, 2010; Ceciliani *et al.,* 2012). Nevertheless, the concentration changes in APPs in neonatal ruminants, as seen in the current studies with reindeer calves,

lambs, and dairy calves, raise the speculation that the APPs concentration changes have a protective role for newborns. It might be because the different functions of APPs can help the neonatal ruminant to have better protection against encountered pathogens and possibly play a role during the gut microbiota colonization.

All of the studies were observational field studies, and thus not controlled like experimental studies. It was not possible to control all of the potential influencing factors, which cover the specific management regime and stress responses to the specific disease status of the herd. It can be suggested that adaptation to the new environment after birth is a stressful situation for an organism. During that period an APR takes place. However, this is not necessarily an expression of infection, rather part of innate immune protection and, therefore, helps to achieve homeostasis in the newborn. In particular, the higher SAA concentrations in the first week of life have physiological and protective roles for the newborn and help it adapt to harmful environmental factors. A study by Jawor *et al.* (2018) also demonstrated that healthy dairy calves had higher levels of SAA than perinatal mortalities ones.

The factors that influence the APP concentration changes in newborn animals remain to be investigated. It has been shown that SAA concentrations are low before parturition in calf foetuses (Alsemgeest, 1993). In humans, it has been demonstrated that SAA, CRP, and IL-6 are not transferred to the foetus through cord blood, as their concentrations are lower than those in the maternal blood (Cicarelli *et al.,* 2005; Lannergård *et al.,* 2005).

Despite the absence of strong pro-inflammatory signals from cord blood, the low postnatal values of SAA and CRP concentrations in infants increase during the first days of life, when higher concentrations can be measured (Cicarelli *et al.,* 2005; Lannergård *et al.,* 2005; Chavez-Bueno *et al.,* 2011). In a study by Cicarelli *et al.* (2005), the increased CRP and SAA levels of newborn infants were speculated to be due to infection or trauma. The birth process itself, which is traumatic and causes tissue damage, could elicit the APR, which would explain the changes in the APPs concentrations (Marchini *et al.,* 2000; Lannergård *et al.,* 2005). Bracci *et al.* (2000) have concluded that slightly increased APPs in the absence of an active infection during the perinatal period might

be a part of the normal adaptation process related to the maturation of the immune system.

SAA's specific role is to provide protection against LPS-induced inflammation and tissue injury by binding directly to LPS to form a complex that promotes LPS uptake by macrophages and therefore reduces serum endotoxin levels (Cheng *et al.,* 2018). Likewise, SAA binds directly to the outer membrane protein A and acts as opsonin for Gram-negative bacteria (Shah *et al*., 2006). These beneficial effects of SAA can help the animal to survive also during the critical neonatal period. Supposedly, the increased concentration of SAA after birth can characterize as a physiological change.

Therefore, the immunomodulatory properties of SAA have a beneficial influence on neonatal animals regardless of pathogenic processes. The neonates need protection from harmful environmental factors, especially invading microbes, and the higher concentrations of SAA during the first week of life can give the animal an advantage in survival. Hp on the other hand might be more dependent on the inflammation process. It could be difficult to differentiate the physiological and pathological factors, which induce the Hp concentration changes in neonatal ruminants. In the study of dairy calves, it was found that *Cryptosporidium* spp. associated with Hp level in the first week of dairy calf's life, while the same association with SAA was evident during the second week of life (Niine *et al.,* 2018). In the first study of lambs, serum Hp concentration changes differed between the years, but this was not seen in SAA. It suggests that the activation of the inflammatory process has a greater influence on Hp in neonatal ruminants and changes in SAA concentrations are more related to physiological changes than Hp.

As pro-inflammatory cytokines induce APP synthesis, the postnatal changes in their concentrations must be described. The fluctuations in the concentrations of these cytokines were not similar to the changes in APP concentrations. Cytokines are often produced in a cascade where one cytokine stimulates the target cells to release additional cytokines (Zhang and An, 2007). IL-1β and TNF-α are stimulated on their own and can stimulate the synthesis of each other, and they both stimulate the synthesis of IL-6. IL-6 can induce its own production and can inhibit the synthesis of IL-1β and TNF-α (Ceciliani *et al.,* 2002). Constantly elevated cytokine concentrations in the study of calves (**IV**) indicate a

persistent stimulation, as cytokines only have a half-life of a few hours. In newborn infants, higher concentrations of IL-6 and TNF-α were demonstrated in blood serum during the first week of life compared to their cord blood (Angelone *et al.,* 2006). After an *in vitro* endotoxin challenge of monocytes, infants displayed a higher percentage of IL-6 and IL-8-positive cells (Schultz *et al.*, 2002), which indicates greater pro-inflammatory responses than that in adults. If the response to anti-inflammatory cytokines is weak, a more pronounced pro-inflammatory cytokine response occurs.

The changes in APP concentrations after birth that are unrelated to infection may be beneficial for neonates. APPs and cytokines fluctuate during the first week of life, unrelated to (clinical) inflammation, so their role in the development and maturation of the immune system seems to be substantial; and beneficial to the neonate. Not only is the innate immunity the main defence of neonates, those results furthermore indicate its relevance in important adaptation processes during the first 3 weeks of life. Nevertheless, these processes are useful only for a short and definite period of life, as prolonged inflammatory processes (such as subclinical or chronic diseases) could harm the organism in various ways. A balance between disease tolerance versus resistance is important in neonatal animal survival, and causative or influencing factors for neonatal ruminant APR are questions for future research.

6.2. Influence of colostrum on the systemic acute-phase proteins and cytokines of neonatal ruminants

The effect of colostrum on the APR of neonatal ruminants was studied in lambs and dairy calves (**II–IV**). Specifically, concentrations of the colostrum pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) in study **IV**; SAA in studies **II**, **III**, and **IV**; and Hp **IV** were evaluated.

The mean SAA concentrations in ewe colostrum in both lamb studies (**II–III**) were much lower than in a study by McDonald *et al.* (2001) where the SAA concentration in healthy ewe's (n = 6) colostrum was 62.8 mg/l. In addition to the number of studied animals, the management regime might also have an overall influence, as has been demonstrated in ewes whose malnutrition influenced the APR of their lambs (Eckersall *et al.,* 2008).

In the dairy calf study (**IV)**, the mean concentrations of SAA and Hp in cow colostrum samples were 65.7 mg/l and 190 mg/l, respectively. The levels of APPs in colostrum are comparable with the results during mastitis when the median SAA is 43.3 mg/l (Kalmus *et al.*, 2013). The median levels of Hp during the mastitis, in a study by Kalmus *et al.* (2013), was 214.1 mg/l.

The studies (**II**–**III**) demonstrated a positive association between SAA concentrations in the colostrum of ewes and the blood serum SAA concentrations of their lambs on days 1–5 (**II**) and 2–4 (**III**). It has also been shown that the concentrations of some low-abundance proteins (apolipoprotein A-IV, plasminogen, SAA, and Fib gamma chain) increased in lamb serum after colostrum intake (Hernández-Castellano *et al.*, 2014); hence, their direct absorption is possible after colostrum consumption in lambs. In dairy calves (**IV**), no positive association between colostrum SAA concentration and calf serum SAA concentration was detected. The same outcome was demonstrated in a study by McDonald *et al.* (2001) on calves (based on three samples), where no correlation was found between elevated levels of SAA in colostrum and SAA levels in calf serum. It has been shown that colostrum SAA isoforms do not directly cross the intestinal barrier of calves (Orro *et al.,* 2008). The different immunomodulatory effects of colostrum APPs on calves and lambs are probably different due to differences in their management conditions.

In the study of dairy calves (**IV**), the negative association between colostrum SAA concentration and calf blood SAA content during the first week of life was determined. This might be explained by the local role of SAA in the gastrointestinal tract. Non-immunoglobulin proteins in colostrum play an important role in the activation and attraction of immune cells in neonatal ruminants (Hernández-Castellano *et al.,* 2014). In newborn piglets, a higher SAA content in sow colostrum led to a fever occasions of piglet diarrhoea during the first days of life than a lower SAA content (Hasan *et al.*, 2019). It is assumed that SAA plays a protective role in tissues and organs that are exposed to the external environment like intestinal tract (Sun and Ye, 2016). One role of colostrum APPs (especially SAA) is the effect during and after microbiota colonisation. Following microbiota colonisation, SAA gene transcription is increased in the digestive tissues (Murdoch *et al.*, 2019).

In the calf study (**IV**), positive associations were found between the concentrations of colostrum cytokines IL-1β, TNF-α, and IL-6 and calf serum IL-1β (first week), TNF-α (first and second weeks), and IL-6 (during the entire 3 weeks). This is an important finding, which indicates that colostrum cytokine levels influence calf serum cytokine levels. The levels of IL-6 and TNF-α were also correlated in the colostrum, suggesting that the production of these cytokines is regulated by similar processes.

In the study (**IV**), the Alb concentration increased during the first three weeks of life, and a negative association between colostrum IgG and Alb during the first week of life was evident. This emphasizes the inhibiting effect of colostrum globulins on neonatal albumin production.

Colostrum pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) have a direct influence on the immune system of calves during the first week of life. Thus, it should be further emphasised that colostrum pro-inflammatory cytokines play a role in activating the innate immune response in newborns. As colostrum cytokine concentrations were positively associated with calf serum cytokine concentrations in study **IV**, the influence of colostrum on the innate immunity of the offspring could be determined by the concentrations of these cytokines.

The question remains whether lambs benefit from the effect of higher colostrum SAA concentrations and thus the higher SAA concentration in their serum during the first week of life. On the other hand, negative association between colostrum SAA content and blood SAA concentration during the first week of life was observed in calves. When comparing the feeding protocols, lambs and reindeer calves had an opportunity to consume colostrum and milk *ad libitum*, whereas dairy calves had only one feeding event of the colostrum of their dams from a bottle, and the calves were separated from their mothers immediately after birth. One possible explanation is that when the newborn receives less SAA through colostrum, the organism is pressured to produce it more by itself. The differences in colostrum management regimes have been demonstrated to affect early immune development in calves, as dairy calves had a peak of pro-inflammatory cytokine expression (immune transcription analysis) compared with that of beef calves who had *ad libitum* access to colostrum and milk (Vlasova and Saif, 2021). Hence, it would be better for the development of the immune system of

calves and their future well-being if the newborns had *ad libitum* access to colostrum. Previous studies have shown that suckling calves in cow-calf-operations show considerable metabolic and endocrine differences compared to calves fed restrictively (Egli and Blum, 1998).

In summary, colostrum has a direct influence on the newborn innate immune system and immunomodulatory effects through pro-inflammatory cytokines and APPs in the first weeks of ruminant life and possibly longer, as in the case of colostrum IL-6.

6.3. The influence of acute-phase proteins during the neonatal period on weight-gain

To evaluate the general health status and the influence of the neonatal period on the growth of animals, connections between neonatal-period APPs (evaluated by weeks) (**I–IV**) and pro-inflammatory cytokines (**IV**) and ADWG were studied.

An increased SAA concentration of 10 mg/l in the second week of life in lambs (**II**) was associated with a 2.9 g/day decrease in ADWG, and on average, the weight of this lamb at 200 days was 0.6 kg lower. As 10 mg/l is an interquartile range in the SAA concentrations of 2-weeks old lambs, the weight loss can be attributed to at least 25% of the lambs or more. The same tendency was seen in dairy calves (**IV**), as the 80 mg/l (interquartile range in 2-weeks old calves) higher SAA concentration in the second week of life meant 29 g/day less weight gain and an average 10.4 kg lower weight at the age of one year.

No significant associations were observed between weight gain and APP concentrations at different ages/time points within the first 0–22 days of life in reindeer (**I**). This differs from the results of a previous study of reindeer calves by Orro *et al.* (2006), where higher SAA concentrations at the age of 8–14 days were associated with smaller daily weight gain at 111–125 days of age. In the first study of lambs (**II**), a significant negative association was found between serum SAA concentrations at second week of life and ADWG of lambs evaluated at approximately 3–4 months of age. In contrast, no association was found between lamb serum SAA concentrations and weight gain at the age of approximately 4 months in the second lamb study (**III**).

The studies on reindeer calves (**I**) and those by Orro *et al.* (2006) were conducted in the same herds, but in different years. The reindeer management conditions were similar in study **I** and the study by Orro *et al.* (2006). The studies in lambs (**I–II**) were also conducted in one herd but in different years.

Possibly, a varying general herd health and management status between these years led to the different results in the studies on lambs (**II–III**). Nevertheless, the period between studies **II** and **III** was only 2 years, and feeding and disease prophylaxis on the farm were similar. The only variation in management was in study **III** when the lambs were kept inside the barn during the lambing season owing to the weather conditions, whereas in study **II**, they were allowed to enter the pasture earlier. In study **III**, the effects of the protozoa were evaluated for SAA and Hp concentrations. However, there was not a remarkable association between SAA, Hp, and Alb concentrations and protozoan infections in lambs.

When comparing the management regimes of different ruminants in these studies, management conditions of reindeer calves and lambs were more similar than those in dairy calves. The reindeer calves and lambs were kept in a herd, while dairy calves were separated from the cows and kept in individual pens. In addition, dairy calves received colostrum only once, whereas reindeer calves and lambs could consume colostrum and milk from their dams *ad libitum*. The second important difference is that dairy calves were kept inside a barn under similar conditions, but reindeer calves were kept outside, and lambs were free to go outside. Therefore, the effects environmental conditions (depending on the year) of reindeer calves (**I**) were different from those in a previous study by Orro *et al.* (2006), and the same were observed in the lamb studies (**II–III**); where the environmental differences between the years might have had an influence. Neonatal dairy calves are affected more on the presence of infectious and parasitic diseases and the overall hygiene and management of a farm. Avoiding the factors that are causing increased inflammation possibly decreases the risk of poor growth in dairy calves (Graham *et al.*, 2010).

In dairy calves (**IV**) the second and third week of life had long-lasting effects on animal growth. The SAA concentration during those weeks and IL-6 during the second week were negatively associated with

ADWG at 9 months of age. The negative association between Hp concentrations during the third week of life were associated with a poorer growth rate at three months of age and Hp levels at the second week of life was associated with poorer growth at nine months of age. The TNF-α level at the two weeks of life had the same effect on weight gain at 9 months of age. This supports the importance of the second and third weeks for animal further performance. The difference between **IV** study in dairy calves and the studies by Seppä-Lassila *et al.* (2017; 2018) was that the studied dairy calves in Finland were born in multiple different farms before being brought together at the rearing unit and beef calves were from six different herds. This further indicates, that the association between APPs and poorer growth rate is not the effect of one specific farm.

While no colostrum pro-inflammatory cytokines or APPs influence on weight gain was detected, during the second and third week of life some infections causing inflammatory response may occur and the elevated levels of SAA and Hp associations with long-term performance may be associated with this activation of APR. Our research group has previously demonstrated that infection with *Cryptosporidium* has a positive association with SAA and Hp concentrations during the second week of life in calves and with the Hp during the first week of life (Niine *et al.*, 2018). In the current study (**IV**), the *Cryptosporidium* effect was taken into account and found that additional factors, besides the protozoa, must have influenced the APR and weight gain of calves. Therefore, other factors like microbiota colonization, management conditions, feeding and different infectious diseases should be evaluated.

There was a positive association between the lambs' ADWG measured at 4 months of age and their Hp and Alb serum concentrations at the second week of life (**III**). A positive association between Alb and growth rate is difficult to explain. A positive association of albumin and globulin with early growth has been found in beef calves (Seppä-Lassila *et al.*, 2017). In contrast, in the study in dairy calves, albumin had no associations with ADWG (**IV**). Similar to lamb study (**III**), a positive association between Hp serum concentration at 2-3 weeks of age and carcass weight at slaughter at 16 months of age of calves was found (Seppä-Lassila *et al.*, 2018). Arthington *et al.* (2003) reported lower Hp concentrations in transported calves compared to non-transported calves which indicates the acute stress reaction has influence to Hp

response negatively. Comparable associations were reported in the study by Seppä-Lassila *et al.* (2015) where weak signs of eimeriosis had decreased Hp and increased Fib concentrations compared to *Eimeria* free calves. The positive association of Fib in this study supports the hypothesis that subclinical, mild or early-stage inflammatory response can reflect initially in lower circulating Hp concentrations. Hp binds to free haemoglobin and thus acts as a haemoglobin scavenger reducing the availability of iron to microorganisms and explaining its important role (Eaton *et al.,* 1982). Both mild eimeriosis and transportation (stress) will probably result in haemorrhaging tissue damage, increased binding of haemoglobin by Hp and lower circulating Hp. When the inflammatory response escalates further, the pro-inflammatory cytokine cascade will initiate liver production of APPs, including Hp; and Hp concentrations will increase. This indicates that the underlying cause for this positive association with weight gain in the lamb study (**III**) is probably also related to the activation of the innate immune response like the negative association of SAA concentrations during the second week of age and weight gain in lambs and dairy calves (**II, IV**).

There are differences between individual animals in their immuno-competency. It is important to establish the health status of the whole herd as well as introduce prophylactic treatments. More extensive or time-dependent changes in APP concentrations seem to have a negative effect on the animals' future weight-gain. If reference intervals for younger animals are established or have already been determined, the exceptional values should be taken into account when interpreting these results.

Nevertheless, the processes which are influencing neonate ruminants during the second and third week of life have a longer lasting effects on animal further performance. Therefore, the evaluation of the relationship between APPs and animal growth can have benefits in farm level management improvement. The environmental factors can cause long-lasting immunomodulatory effects; that impact the negative weight gain associations more strongly. This is especially likely during the second (and third) week of life, which is considered to be an important adaptation period during which the colostrum effect declines and responses to pathogens happen during that time.

The studies included in this thesis were all observational field studies describing the real-life situations of farms and different management conditions. However, such kinds of studies can rise more questions. These studies are influenced by different factors which are not controllable in all. These studies can help to provide some more study hypothesis and even if those results are not directly transferable to farm management practice those can have value in the academic world and for further pathophysiology studies. However, these studies can eventually lead to results that can be directly transferable to farm management practices.

The role of pro-inflammatory cytokines and APPs (SAA, Hp) in the colostrum for offspring APR needs further clarification. There is a need to evaluate the possible association between ewe's colostrum cytokines and lamb serum cytokines and APPs. In future studies, SAA isoforms from sheep colostrum should be researched, as their functions may be different than in calves. Future studies should examine environmental factors (e.g., pathogen exposure) and microbial colonization during the neonatal period more closely, since they may have a long-term impact on animal health and production.

7. CONCLUSIONS

APPs have age-dependent concentration changes in reindeer calves, lambs and dairy calves during the neonatal period defined as the first three weeks of an animal's life. The most prominent changes are the elevated concentrations of evaluated positive APPs at the first two weeks of life in all investigated ruminant species.

Especially, fluctuations in SAA concentrations during the first weeks of life might reflect physiological changes in the organism and the importance of the adaptation processes during the neonatal period to cope with challenges arising from the external environment (**I–IV**). Increased knowledge about physiological changes after birth allows for better evaluation of the adaptation and further development of neonate ruminants. Reliable markers of the physiological process are important tools for increasing this knowledge.

Colostrum APPs and pro-inflammatory cytokines have a potentially beneficial influence on the systemic APR in neonatal animals. This is especially clear during the first week of life, which may indicate mainly local protective effects of these immunomodulating ingredients of colostrum on the neonate ruminants. However, there are differences between lambs and dairy calves in the effect of colostral SAA on the newborns. Colostrum SAA concentration has a direct positive association with lamb serum SAA concentrations during the first days of life, which is not the case in calves. This suggests different roles and action mechanisms of colostrum SAA in these ruminant species (**II–IV**).

In contrary to the other colostral pro-inflammatory cytokines, IL-6 seems to have a more pronounced and longer lasting effect to the dairy calves APR, suggesting an important role of IL-6 in the adaptation period of neonates (**IV**).

The activation of the APR in response to any environmental stressor in neonate ruminants has a long-lasting effect on their growth (**II–IV**). Production animal species with outdoor access had variations in the fluctuations of APP concentrations between different study years and thus, it can be speculated that environmental factors have a significant effect on the APR.

Increased knowledge of early developmental processes of immune function allows providing a better growth environment for the neonatal ruminants in order to promote their welfare and limit the spread of pathogens.

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SUMMARY IN ESTONIAN

Ägeda faasi valkude kontsentratsiooni muutused, nende seosed ternespiima ja massi-iibega vastsündinud mäletsejalistel

Sissejuhatus ja teema ülevaade

Noorloomade kasvuperiood on eriti tähtis arenguetapp, sest see ajajärk määrab terve täiskasvanud looma jõudluse (Aghakeshmiri jt, 2017). Patogeenid ning stress avaldavad kohanemisperioodi jooksul looma arengule ja kasvule negatiivset mõju. On leitud, et täiskasvanud loomadega võrreldes esineb neonataalperioodil (esimesel kolmel elunädalal), suurem haigestumus- ja suremusrisk (Svensson jt, 2006; Raboisson jt, 2013). Tänapäevane intensiivne loomapidamisviis tähendab tihti seda, et loomi peetakse karjas tihedalt koos, mis võimaldab haigustekitajate hõlpsamat levikut ja kehva hügieeni lisandudes mõjutab see negatiivselt looma arengut.

Sünni järel on mäletsejalised väga vastuvõtlikud haigustekitajatele, sest sünepiteliokoriaalset tüüpi platsenta ei võimalda antikehade ülekannet emaloomalt lootele (Wooding, 1992; Barrington ja Parish, 2001).Vastsündinud mäletsejaliste jaoks on elumuse suhtes väga tähtis ternespiimaga antikehade saamine (Chase jt, 2008). Viimase kümnendi jooksul on hakatud rohkem uurima ternespiimas sisalduvate spetsiifiliste valkude osa vastsündinu immuunsuse kujunemisel, ent nende täpsem bioaktiivne toime vajab veel väljaselgitamist (Hernández-Castellano jt, 2015). Neonataalperioodil on passiivse immuunsuse kõrval tähtis ka kaasasündinud ehk loomulik immuunsus. Seega on oluline mõista paremini mäletsejaliste immuunsüsteemi arengut vastsündinuperioodil. Selle töö fookuses on vastsündinud mäletsejaliste areng, mis on seotud ägeda faasi vastuse (*acute-phase response,* APR) ja ägeda faasi valkudega (*acute-phase proteins*, APP).

Ägeda faasi vastusena mõistetakse organismi süsteemset reaktsiooni koekahjustusele, mida kutsuvad esile nakkuslikud tegurid, vigastused, stressireaktsioonid ja kasvajalised protsessid (Baumann ja Gauldie 1994; Gruys jt, 2005). Selle protsessi täpsem eesmärk on patogeenide isoleerimine ning hävitamine, koekahjustuse süvenemise pidurdamine ja tervenemise soodustamine, et taastada organismi tasakaal (Baumann ja Gauldie, 1994; Uhlar ja Whitehead, 1999). Ägeda faasi valkude

sünteesimine maksas toimub mõne tunni jooksul pärast ägeda faasi vastuse põhjustanud teguri toimet. Maksa retseptoritele toimivad proinflammatoorsed tsütokiinid, peamiselt TNF-α, IL-1, IL-6 (Petersen jt, 2004; Cerón jt, 2005). Ägeda faasi valke sünteesitakse mõningal määral ka paiksetes kudedes, et võimaldada viivitamatut kaitset edasise kahjustuse eest ja alustada paranemisprotsessi (Berg jt, 2012).

Ägeda faasi vastuse jooksul on APP-del organismis palju erinevaid füsioloogilisi ülesandeid, sh seondumine võõrkehadega, opsoniseerimine, fagotsütaarsete rakkude funktsioonide moduleerimine, komplemendi aktiveerimine, rakujäänuste eemaldamine ning vabade radikaalide ja proteolüütiliste ensüümide neutraliseerimine (Baumann ja Gauldie, 1994; Gruys jt, 2005; Cray 2012).

Mäletsejaliste tähtsamad APP-d on seerumi amüloid A (*serum amyloid A*, SAA) ja haptoglobiin (*haptoglobin*, Hp). SAA ülesanne on kolesterooli sidumine (Liang ja Sipe, 1995). Samuti on sellele valgule omane immunomoduleeriv toime (Uhlar ja Whitehead, 1999; Ceciliani jt, 2012). Hp on glükovalk, mis seondub hemoglobiiniga, vältides nii oksüdeerivaid kahjustusi kui ka organismi rauakadu. Samuti on sellel valgul immunomoduleeriv toime (Murata jt, 2004; Petersen jt, 2004; Ceciliani jt, 2012).

Ägeda faasi valke kasutatakse meditsiinis kvantitatiivsete biomarkeritena haiguse diagnoosimisel, haiguskulu prognoosimisel ja ravivastuse jälgimisel põletike ja nakkushaiguste korral (Alsemgeest jt, 1994; Eckersall 2000; Eckersall ja Bell 2010; Thomas jt, 2015).

Mäletsejalistel on APP-sid uuritud erinevate haigusseisundite ja stressireaktsiooni korral (Ceciliani jt, 2012). Varasemalt on teada, et ägeda faasi valkude kontsentratsioon muutub mäletsejaliste vereseerumis esimestel elunädalatel (Orro jt, 2006; Orro jt, 2008; Tóthová jt, 2015).

Vastsündinud mäletsejaliste APRi puudutavaid uuringuid on vähe ning seetõttu ei ole lõpuni teada, mis on sellel perioodil APPde kontsentratsioonides toimuvate muutuste põhjuseks. Käesolev doktoritöö selgitas APPde kontsentratsioonide muutuseid kolme liiki mäletsejaliste vastsündinute vereseerumis. Selgitamist vajas ka muutuste võimalik

pikaajaline mõju loomade edasisele kasvule, mida hinnati massi-iibe kaudu.

On teada, et lehmade ternespiim sisaldab tavalise piimaga võrreldes suuremas kontsentratsioonis proinflammatoorseid tsütokiine IL-1β, IL-6, TNF-α ja IFN-γ (Hagiwara jt, 2000). Lehma ternespiim sisaldab peale tsütokiinide ka ägeda faasi valke SAA, Hp, C-reaktiivne valk ja alfa-1 glükoproteiin (Mc Donald jt, 2001; Ceciliani jt, 2005). Suurenenud kontsentratsiooniga SAA-d on ternespiimas leitud kuni neljanda poegimisjärgse päevani ja laktatsiooni alguses ei loeta seda udara patoloogiliseks protsessiks (McDonald jt, 2001; Thomas jt, 2016). Hp mõõdukas suurenemine on samuti tuvastatav kuni neljanda poegimisjärgse päevani (Hiss jt, 2004; Thomas jt, 2016). Nii SAA kui ka Hp sünteesitakse udarakoes (Hiss jt, 2004).

Uuringus, milles käsitleti ternespiimast pärit SAA mõju vasika ägeda faasi vastusele, leiti, et ternespiima SAA isovormid ei kandu otse üle vasika vereringesse (Orro jt, 2008). Sellest järeldati, et SAA-l on vasika mao-soolekulglas ainult paikne toime või eemaldatakse väga aluselised isovormid kiiresti ringlusest (Orro jt, 2008). Samas võib ternespiima APP-de toime järglastele loomaliigiti erineda. Ternespiima valkude võimalikku mõju vastsündinu valkude profiilile on uuritud lambatalledel ja on leitud, et pärast ternespiima joomist oli tallede vereplasmas suurenenud apolipoproteiin A-IV, plasminogeeni, seerum amüloid A ja fibrinogeeni kontsentratsioon (Hernández-Castellano jt, 2014).
Uuringud põhjapõdravasikate, lihaveisevasikate ja piimalehmavasikatega on näidanud seost teise elunädala vereseerumi SAA suurenenud kontsentratsiooni ja vähenenud massi-iibe vahel mõõdetuna kolmandal-neljandal elukuul (Orro jt, 2006; Seppä-Lassila jt, 2017, 2018).

Uurimistöö eesmärgid

Väitekirja eesmärk oli selgitada ägeda faasi valkude kontsentratsioonide sünnijärgseid muutuseid põhjapõdravasikatel, lambatalledel ja piimalehmavasikatel kolmel esimesel elunädalal ehk neonataalperioodil ning hinnata ternespiima mõju vastsündinu ägeda faasi vastusele. Neonataalperioodi ägeda faasi vastuse pikaajalise mõju selgitamiseks uuriti selle seoseid loomade massi-iibega.

Uurimistöös püstitati järgmised ülesanded:

- hinnata ägeda faasi valkude seerumi amüloid A (SAA) (**I**-**IV**), haptoglobiini (Hp (**I**-**IV**)), albumiini (Alb) (**I**-**IV**) ja fibrinogeeni (**I**) kontsentratsioonide muutusi põhjapõdravasikate (**I**), lambatallede (**II**–**III**) ja piimalehmavasikate (**IV**) vereseerumis esimese kolme elunädala jooksul;

- uurida tarbitud ternespiima, tsütokiinide (**IV**) ja ägeda faasi valkude SAA (**II**–**IV**) ja Hp (**IV**) mõju vastsündinud mäletsejaliste ägeda faasi vastusele hinnatuna APPde sisalduse kaudu vastsündinu vereseerumis;

- hinnata tarbitud ternespiima ja neonataalperioodi ägeda faasi valkude kontsentratsioonide ning hilisema massi-iibe vahelisi võimalikke seoseid (**I**-**IV**).

Uurimistöö materjal, metoodika ja tulemused

Selles töös olid uuritavad mäletsejad põhjapõdravasikad, lambatalled ja piimalehmavasikad. Poolkodustatud põhjapõdrad elasid suurel territooriumil (48 km^2) Soomes Lapimaal, kuuludes eksperimentaalkarja. Põhikarja kuulus 58 emaslooma ja poegimisperiood oli kevadel. Pärast sündimist jäid vasikad emasloomade juurde kuni võõrutamiseni. Lammaste uuringud (**II**, **III**) toimusid Lõuna-Eesti ühes lambafarmis, mis on spetsialiseerunud liha tootmisele. Põhikarja suurus oli ligikaudu 250 utte. Kuni võõrutamiseni jäid lambatalled uttede juurde. Lambad elasid laudas, kuid neil oli väljapääs õue ja hiljem viibisid nad rohumaal. Piimalehmavasikate uuring (**IV**) toimus Kesk-Eesti suures farmis; uurimisperioodil oli karjas ligikaudu 1800 lehma. Vasikad eraldati kohe pärast sündi emast ja neile anti emalooma ternespiima umbes kahe tunni jooksul pärast sündimist. Vasikaid peeti laudas.

APPde ja seerumi immunoglobuliinide ning vanusega seotud muutuste uurimiseks kasutati lineaarseid regressiooni segamudeleid (**I–III**). Selleks, et võrrelda seerumi põletikumarkerite kontsentratsioonide erinevusi nädalate kaupa, kasutati samu mudeleid (SAA, Hp, Alb, IgG, IL-6, IL-1β ja TNF-α) ning lisaks Tobit regressioonianalüüsi IL-1β hindamiseks (**IV**). Vastsündinud looma seerumi valkude ja ternespiima valkude kontsentratsioonide vaheliste seoste uurimise jaoks kasutati

lineaarseid regressiooni segamudeleid (**II**–**IV**). Samuti kasutati lineaarseid regressiooni segamudeleid uurimaks seoseid seerumi valkude ja massi-iibe vahel kolmandal elukuul (**II**, **III**) ja lineaarseid regressiooni mudeleid piimalehmavasikatel ühe, kolme ja üheksa elukuu vanuses (**IV**). Põhjapõdravasikate puhul kasutati APPde kontsentratsioonide sünni järgsete muutuste kõvera alust pindala seerumi proteiinide ja massi-iibe vahelise seose uurimiseks lineaarseid regressioonimudeleid.

Kõikidel uuritud liikide vastsündinutel (**I**–**IV**) oli SAA sisaldus vereseerumis kõige madalam sündimise päeval, seejärel hakkas tase suurenema. Vereseerumi SAA kõrgeim tase mõõdeti lambatalledel esimesel elunädalal, piimalehmavasikatel ja põhjapõdravasikatel teisel elunädalal. Pärast seda vähenes SAA kontsentratsioon kõikidel loomaliikidel. Vereseerumi Hp kontsentratsioon oli samuti kõige väiksem sündimise päeval, mille järel see suurenes. Põhjapõdravasikatel oli kõige kõrgem kontsentratsioon 12.–14. elupäeval, lambatalledel 4.–7. elupäeval, vasikatel 7.–11. elupäeval, seejärel vähenes Hp sisaldus kõikidel loomaliikidel. Kogu uurimisperioodi jooksul (kolm esimest elunädalat) suurenes ühtlaselt Alb ning vähenes globuliinide (Glob) kontsentratsioon vereseerumis.

Esimene uuring (**I**) oli põhjapõdravasikatega (n = 54), kellelt koguti ja analüüsiti seerumiproove (n = 370) ning EDTA vereproove (n = 210). Uuriti APP-de seerumi kontsentratsiooni muutusi vasika sünnist kuni 33. elupäevani ning võimalikke seoseid nädalate kaupa APP-de ja hilisema (114–127 elupäeva) massi-iibe vahel. Kõige madalam SAA vereseerumi tase oli põhjapõdravasikatel sünni päeval (0-päev, keskmine 3,1 ±3,8 mg/l), seejärel suurenes see kuni 8.–10. päevani (65,9 ±41 mg/l); ($p < 0,01$) ning kõige kõrgem SAA sisaldus mõõdeti 12.–14. päeval (80,9±49,9 mg/l). SAA stabiliseerus 33. päevaks. Hp väikseim kontsentratsioon oli sünni päeval (0,412 ±0,087 g/l), madal 4.–6. päeval (0,433 ±0,087 g/l) ning suurenes 8.–10. päeval (0,518 ±0,056 g/l), haripunkt saavutati 12.–14. päeval (0,583 ±0,057 g/l) ja stabiliseerumine toimus 22.–23. päeval (0,516 ±0,098 g/l). Albumiini kontsentratsioon oli väikseim sünni päeval (25,35 ±2,86 g/l), seejärel suurenes ning stabiliseerus kolmanda elunädala lõpuks (22.–23. päeval; 37,22 ±2,56 g/l). Fibrinogeeni kontsentratsioon oli väikseim esimesel päeval (2,44 ±0,54 g/l), seejärel suurenes ja haripunkt tekkis 4.–14. päeval (3,79 ±0,84 ja 3,75 ±0,55 g/l), millele järgnes vähenemine. Selles uuringus ei leitud statistiliselt olulisi seoseid APP-de kontsentratsiooni ja

põhjapõdravasika hilisema juurdekasvu vahel. Isased vasikad võtsid pikemal ajaperioodil (sünnist kuni 112. päevani) kaalus rohkem juurde (0,039 kg päevas, 95% CI: 0,021–0,058; $p < 0,001$) kui emased. Seega ei kinnitanud tulemus seost teisel elunädalal suurenenud SAA kontsentratsiooni ja hilisema väiksema juurdekasvu vahel, mida on täheldatud varasemas põhjapõtrade uuringus (Orro jt, 2006).

Lambatallede uuringutes (**II**, **III**) olid SAA kontsentratsiooni muutused mõlemas sarnased. Üldistavalt: SAA tase oli kõige madalam sünni päeval, suurenes esimesel elupäeval ning seejärel esimese elunädala jooksul; vähenemine algas umbkaudu teisel elunädalal. Esimene lambatallede uuring (**II**) hõlmas kaheaastase uurimisperioodi jooksul 322 talle, kellelt koguti 524 seerumiproovi ning lisaks koguti 197 utelt ternespiima-proovid. Proovid koguti 2011. ja 2012. aastal. SAA kõige väiksem tase ilmnes pärast sündi (0-päev: mediaantulemus 2,2 mg/l; vahemik 1,3–14,3 mg/l; $n = 5$), esimesel päeval suurenes see märgatavalt ($p = 0,009$) ning siis mõõdeti ka suurimad väärtused (keskmine 20,9 mg/l; vahemik 1,5–200,5 mg/l; $n = 39$). Seerumi SAA kontsentratsioon oli suur kuni viienda elupäevani (keskmine 2.–5. päeval 18,7 mg/l; vahemik 0,3–216,4 mg/l; $n = 160$), vähenemine algas kuuendast elupäevast (keskmine 11,9mg/l; vahemik 0,3–164,4 mg/l; $n = 46$; $p = 0,048$) ja seejärel jäid väärtused uuringuperioodi lõpuni stabiilseks. Hp kontsentratsioon oli suurem (lineaarne trendijoon $p = 0,012$) esimesel elunädalal (mediaan 151 mg/l; min-max 60–3342 mg/l) ja vähenemine toimus teisel elu-nädalal (mediaan 130 mg/l; min-max 60–4678 mg/l; kõik proovid pärast 7. elupäeva). Uurimisaastate vahel täheldati Hp kontsentratsioonides erinevust: Hp oli märgatavalt kõrgem 2012. aastal ($p < 0,001$) võrreldes 2011. aastaga. Leiti positiivne seos ute ternespiima SAA sisalduse ja lambatalle seerumi SAA sisalduse vahel 1.–5. elupäeval ($n = 120$; $p = 0,009$). Uttede ternespiima ($n = 197$) SAA kontsentratsiooni keskmine (±SD) oli 10,8 mg/l (±14,9), mediaan 5,1 mg/l ja vahemik 0,3–75 mg/l. Lambatallede kaal 3.–4. elukuul (78–130 päeva vanuses) oli keskmiselt 25,34 (±6,16) kg. Päevane massi-iive oli talledel kuni 3.–4. elukuuni mõlemal aastal (2011, 2012) 200,8 g päevas (±53,7; $n = 259$). Üksikute talledega võrreldes oli kaksikute ja kolmikute juurdekasv väiksem. Vanemate uttede tallede juurdekasv oli suurem kui esmaspoegijate talledel. Märgatavaid erinevusi 2011. ja 2021. aasta vahel ei esinenud. Leidus oluline negatiivne seos seerumi SAA kontsentrat-sioonide vahel 6.–12. elupäeval ja tallede 3.–4. elukuu juurdekasvus ($n = 194$; $p < 0,006$).

Teine uuring lammastel (**III**) viidi läbi samas farmis 2014 aastal. Seerumiproovid koguti 269 lambatallelt, kokku oli proove n = 692 kolme esimese elunädala jooksul, 187 utelt koguti ternespiimaproovid. Seerumi SAA kontsentratsioon oli kõige madalam sündimise päeval (keskmine 21,1 mg/l, n = 27) ning kõige kõrgem 3.–4. elupäeval (keskmine 118,7 mg/l, n = 66). Hp kontsentratsioon oli sündimise päeval 0,21 g/l (n = 27) ja kõrgeim 4.–8. elupäeval (keskmine 0,711 g/l, n = 161). Ka siin tuvastati positiivne seos uttede ternespiima SAA ja tallede SAA vereseerumi kontsentratsioonide vahel 2.–4. elupäeval (n = 109; p = 0,042). Uttede (n = 187) seerumi SAA kontsentratsioon oli keskmiselt (±SD) 20,47 mg/l, mediaan 5,43 mg/l, min-max 0,3–478,8 mg/l. Lambatallede seerumi kõrgem Hp kontsentratsioon oli positiivses seoses uttede ternespiima SAA kontsentratsiooniga 2.–4. elupäeval (n = 81; p = 0,013). Lambatallede massi-iive mõõdetuna 124 (±6.9) päeva vanustel talledel oli 208 ±50 g päevas (n = 239). Teise elunädala Hp ja Alb olid positiivselt seotud lammaste massi-iibega 122 päeva vanuselt. Lisaks oli immuunglobuliinide (IgG) kontsentratsioon esimesel ja teisel elupäeval positiivses seoses massi-iibega 122 päeva vanustel talledel. Küll aga ei leitud selles uuringus seost tallede esimeste elunädalate vereseerumi SAA ja hilisema negatiivse massi-iibe vahel. See oli erinevus võrreldes tallede (**II**) ning samuti varem avaldatud põhjapõdra- ja piimalehmavasikate uuringutega (Orro jt, 2006; Seppa-Lassila jt 2018).

Neljas uuring (**IV**) hõlmas piimalehmavasikaid (n = 143), kellelt koguti esimese kolme elunädala jooksul ühenädalase vahega seerumiproove (n = 329). Lisaks koguti lehmadelt ternespiimaproove (n = 143). SAA kontsentratsioon oli suurem kahel esimesel elunädalal ja hakkas vähenema kolmandal nädalal. Vereseerumis oli Hp kõige kõrgem teisel elunädalal. Alb vereseerumi kontsentratsioon suurenes kolme elunädala jooksul. Neljandas uuringus hinnati kolmel esimesel elunädalal ka seerumi proinflammatoorsete tsütokiinide kontsentratsiooni. Tulemused olid järgmised: IL-6 oli kõrgeim esimesel elunädalal, TNF-α oli kõige kõrgem esimesel elunädalal ning edasi järgnes ühtlane vähenemine, IL-1β kontsentratsioon oli väiksem teisel elunädalal. Vasikate seerumi IL-6 ja TNF-α kontsentratsioonide vahel oli positiivne korrelatsioon esimesel kolmel nädalal (r = 0,79, r = 0,92 ja r = 0,64, kõik p < 0,001). Seerumi SAA ja Hp kontsentratsioon olid positiivses korrelatsioonis teisel ja kolmandal elunädalal (r = 0,45 ja r = 0,36, mõlemad p < 0,001). Kõik teised paarikaupa hinnatud korrelatsioonid seerumivalkude sisalduse

vahel esimesel kolmel elunädalal ei olnud märkimisväärsed ($r < 0,3$). Omavahel korreleerusid ternespiima IL-6 ja TNF-α ($r = 0,69$, $p < 0,001$); teised põletikumarkerid ja IgG ei korreleerunud omavahel ($r < 0,2$).

Ternespiima IL-6 ja järglase seerumi IL-6 kontsentratsiooni vahel esines kolme elunädala jooksul positiivne seos ($p < 0,001$). Ternespiima TNF-α oli positiivselt seotud sama tsütokiiniga seerumis esimese kahe elunädala jooksul (mõlema $p < 0,001$) ja ternespiima IL-1β oli seotud seerumiga IL-1β esimesel elunädalal ($p < 0,001$). Ternespiima IgG oli positiivselt seotud vasikate IL-6 kontsentratsiooniga ($p = 0,031$) ja TNF-α kontsentratsiooniga ($p = 0,008$) teisel elunädalal ning seerumi IgG kontsentratsiooniga kõigil kolmel nädalal ($p < 0,001$). Ternespiima IgG oli negatiivselt seotud vasikate seerumi Alb kontsentratsiooniga esimesel elunädalal ($p = 0,0021$). Ternespiimas sisalduva SAA ja vasika esimese elunädala seerumi SAA kontsentratioonide vahel esines negatiivne seos ($p = 0.018$). Ternespiima APP-de kontsentratsiooni ja vasikate massi-iibe vahel ei leitud märkimisväärset seost. Samuti ei esinenud seost esimese elukuu massi-iibe ja seerumi valkude kontsentratsiooni vahel. Seerumi Hp-l teisel elunädalal oli negatiivne tendents (koefitsient mg/l (±SEM): –0,03 ±0,02; $p = 0,067$; $n = 98$) ja kolmandal (koefitsient mg/l (±SEM) –0,06 ±0,03 $p = 0,025$; $n = 104$) elunädalal oli väljendunud oluline negatiivne seos kolmanda elukuu massi-iibega. SAA ja IL-6 seerumi kontsentratsioon teisel elunädalal oli negatiivses seoses massi-iibega üheksandal elukuul. Kuna nii SAA ja Hp vahel ($r = 0,36$) kui ka IL-6 ja TNF-α vahel esines tugev korrelatsioon ($r = 0,92$), siis hinnati seoseid Hp ja TNF-α ning massi-iibe vahel SAA-st ja IL-6-st eraldi erinevates mudelites. Nende mudelite põhjal leiti negatiivne seos seerumi Hp kontsentratsiooni ja üheksanda elukuuni massi-iibe vahel (koefitsient mg/l (±SEM): –0,035 ±0,015; $p = 0,025$) ning seerumi TNF-α ja üheksanda elukuu massi-iibe vahel (koefitsient ng/l (±SEM): –0,05 ±0,02; $p = 0,014$), samuti kolmanda elunädala SAA kontsentratsiooni ja üheksanda elukuuni massi-iibe vahel (koefitsient mg/l (±SEM): –0,45 ±0,17; $p = 0,010$; $n = 105$).

Järeldused

Käesoleva doktoritöö raames läbiviidud uuringud näitasid, et neonataalperioodil muutub põhjapõdravasikatel, lambatalledel ja piimalehmavasikatel vere SAA, Hp, Fib ning Alb kontsentratsioon. Uuritud

positiivsete APPde kontsentratsioon suureneb kahel esimesel elunädalal. Esimesel elunädalal tekkinud muutused kirjeldavad pigem füsioloogilisi protsesse (eriti SAA puhul) viidates vastsündinu kohanemisele väliskeskkonnaga. Seetõttu on APP-de osa neonataalperioodil looma immuunsüsteemi arengus ja küpsemises väga tähtis. Füsioloogilise protsessi kirjeldused biomarkerite kaudu võimaldavad paremini aru saada mäletsejaliste normaalsest arengust vastsündinuperioodil.

Mäletsejaliste ternespiima APPde sisaldus mõjutab järglase ägeda faasi vastust, eriti esimese elunädala jooksul. Ternespiim võib mõjutada vastavate ägeda faasi valkude jõudmist vereringesse otseselt või proinflammatoorsete tsütokiinide toimel kaudselt. Ternespiim mõjutab seega otseselt vastsündinu immuunsuse regulatsiooni, sest sellel on mäletsejaliste esimestel elunädalatel APR-i kaudu immunomoduleeriv toime. Lambatallede ja piimalehmavasikate vereseerumi SAA ja ternespiima SAA kontsentratsioonide vahel tuvastati esimestel elupäevadel vastupidine seos, talledel positiivne vasikatel negatiivne (**II–IV**)**.**

Ternespiima pro-inflammatoorsetest tsütokiinidest on IL-6 mõju piimalehmavasikate APRle suurim, mõjutades seega vasikate adaptsiooniperioodi kõige rohkem.

Teisel ja kolmandal elunädalal toimuv ägeda faasi vastus (vastusena mingile välisele tegurile) võib pikaajaliselt mõjutada looma arengut avaldudes hilisemas väiksemas massi-iibes (**II–IV**).Vastsündinud talledel varieerus aastate lõikes APR, mille põhjuseks võib olla keskkonnategurite mõju.

Põhjalikumad teadmised varajaste immunoloogiliste protsesside kujunemisest võimaldavad tootmisloomadele tagada parema arengukeskkonna, suurendades loomade heaolu ning piirates haigustekitajate levikut.

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I

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Full length article

Giardia and *Cryptosporidium* infections in neonatal reindeer calves: Relation to the acute phase response

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ABSTRACT

This longitudinal observational study was conducted to investigate the spontaneous effect of *Giardia* and *Cryptosporidium* infections on acute phase response (APR) in reindeer calves (*Rangifer tarandus tarandus*) in Finnish Lapland.

Serum (n = 609) and faecal samples (n = 366) were collected from 54 reindeer calves aged zero to 33 days. The samples were analysed for *Giardia*, *Cryptosporidium*, acute phase proteins (APP) and γ-globulins.

Linear regression models were used to investigate associations of early *Giardia* infection (before 12 days of life) with the response of APPs and acquiring of passive immunity.

Giardia was detected in 100% and *Cryptosporidium* in 23% of calves. There was a negative association between early *Giardia* infection and γ-globulin concentrations (p = 0.032) and a positive association with serum amyloid A (SAA) concentrations (p = 0.042). The results suggest a protective effect of colostrum against *Giardia* infection and that early infection may induce activation of APR.

1. Introduction

Reindeers (*Rangifer tarandus tarandus*) are semi-domesticated ruminants that live in the harsh Arctic environment and calve seasonally. Ensuring survival and good health of a calf is crucial to successful reindeer husbandry. A very important element of a calf's survival is the transfer of maternal immunity from the hind. The neonate gets almost all of its first immunoglobulins (Ig) from colostrum. As observed in other domesticated ruminants, female reindeers (hind) have syndesmochorial placentation, which prevents the transfer of Ig from hind to calf through the placenta. As a result, ingestion of Ig after birth (in colostrum) is important for the calf survival. The lowest level of Ig serum concentration occurs when the calf is 20 days old, which makes calves more susceptible to infections during this period [1]. Pathogenic infections during the neonatal period can have a negative impact on growth and development [2].

The acute phase reaction (APR) is an immunological reaction triggered by inflammatory processes following tissue damage. The specific proteins that increase in concentration during an APR are termed positive acute phase proteins (APP) [3]. Serum amyloid A (SAA) and haptoglobin (HP) act as positive APR markers in reindeer exposed to *Escherichia coli* lipopolysaccharide, and SAA seems to be the more sensitive APR marker of the two [4]. In reindeer, SAA concentrations peak around the second week of life while HP continues to rise until 3–4 weeks of life [5]. Higher SAA concentrations at the second week of life were negatively associated with daily weight gain at 4 months of age, suggesting that activation of APR early in life may influence negatively immunological development of new-born reindeer [5]. Concentrations of another APP, fibrinogen (FIB) increase in clinically affected reindeer [6] and red deer (*Cervus elaphus*) [7]. Albumin (ALB) is considered to be an important APP in ruminants, the concentration of which decreases during APR [3].

In dairy calves, *Giardia* stimulate the production of IgG2 and IgA antibodies [8]. These antibodies do not bind to *Giardia* very strongly in calves and simultaneously inflammation-related genes in the jejunum are down-regulated [9]. This may partly explain why there are no clinical signs of *Giardia* infection and why the infection is chronic in nature [10]. Dairy calves on average start to shed *Giardia* cysts at 31 days of age, which suggests colostrum is of passive protective value against the parasite infection [11]. Similar interactions relevant for the early life of reindeer calves may occur for pathogens including *Giardia* and *Cryptosporidium* and for innate and passive immunity.

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The aim of this study was to determine if spontaneously occurring *Giardia* and *Cryptosporidium* infections in neonatal reindeer have significant impact on the innate immune response.

2. Materials and methods

2.1. Animals

The study population comprised 54 semi-domesticated reindeer calves (28 male and 28 female) from initially born 56 calves between 9th and 22nd May 2004 in the Kaamanen experimental herd of the Reindeer Herders' Association (total land area 48 km^2), in Finnish Lapland. Reindeer hinds were treated with ivermectin in the previous autumn. Weighing was performed using a digital scale (Adam Equipment Co Ltd, Milton Keynes, UK) immediately after birth, at 20 or 21 days of age, and 114–127 days of age.

2.2. Sample collection

In total, 609 serum samples, 210 EDTA samples and 366 faecal samples were collected from 54 calves. Blood samples were collected into 10 ml vacuum tubes (BD Vacutainer, New Jersey, USA) at 7 time points from the time of birth: day 0, 4–6, 8–10, 12–14, 16–17, and 20–22 from all calves. Sampling was planned so that all the calves would be sampled within 3–5 days after the previous sampling during first weeks of life. In addition, blood was collected from a subgroup (n = 51) at 23–33 days. EDTA blood samples were collected into 2 ml EDTA-coated tubes (BD Vacutainer, New Jersey, USA) when calves were 0–1, 4–6, 12–14, 20–22 and 23–33 days of age.

Samples were stored at 6 °C for 30 min and at 21 °C for 15 min before serum separation. Serum was separated by centrifuging and as divided into aliquots and stored at −18 °C for further analysis. EDTA samples were analysed for FIB on the day of collection. Because of technical difficulties, approximately half of the EDTA samples from age groups 0–1 and 23–33 were analysed for FIB.

Faecal samples were collected simultaneously with blood samples directly from the rectum into disposable latex gloves and stored at 6 °C and then at −18 °C until further analysis.

2.3. Sample analysis

Sample total protein concentration was determined using a modified spectrophotometry method [12] in a clinical chemistry analyser (KONE Pro, Konelab, Thermo Clinical Labsystems Oy, Vantaa, Finland). ALB was measured using the bromocresol green method in a clinical chemistry analyser (Accent-200 Albumin II Gen, PZ Cormay S.A., Poland). γ-globulins were measured by serum protein electrophoresis of agarose gel using a Paragonw electrophoresis system (Beckman Coulter, Inc., Fullerton, CA, USA). γ-globulin fraction relative size (%) to the all proteins in the agarose gel was used to calculate γ-globulin serum concentrations (g/l) when serum total protein concentrations in the sample were 100%.

The concentration of SAA was measured using an indirect ELISA test (Phase BE kit, Tridelta Ltd., Ireland) according to the manufacturer's instructions for cattle.

HP was measured using a modified method based on the ability of HP to bind to haemoglobin [13] with modifications to the original protocol using tetramethylbenzidine (0.06 mg/ml) as the substrate and microtitration plates [14]. Lyophilized aliquots of acute phase bovine serum were used as standards. Standards were calibrated using samples provided by the European Union concerted action on standardization of animal APPs for cattle (number QLK5-1999-0153).

FIB concentration was measured using a heat precipitation method [15]. EDTA blood samples were centrifuged for 5 min in a microhaematocrit centrifuge 15000 times/min. From each sample 2 capillaries were prepared. Capillaries were placed in a water bath (56 °C) for 3 min to precipitate the FIB in the plasma. After 3 min of centrifugation, the heights of the FIB and serum column were measured (mm) and transformed into concentrations (g/l) by dividing the height of the FIB column by the height of the serum column and multiplying the result by 100. The final figure was the average of the results from two capillaries prepared from a single sample.

Faecal samples were analysed for *Giardia* cysts and oocysts of *Cryptosporidium* using an immunofluorescent staining method (Crypto/Giardia Cel, Cellabs Pty Ltd., Sydney, Australia) according to manufacturer's instructions. The numbers of cysts and oocysts in the samples per visual field at 200× magnification were ranked as: none (no cysts/oocysts found), low (1–5 cystskk/oocysts), medium (6–30 cysts/oocysts) and high (> 31 cysts/oocysts).

2.4. Statistical analyses

Previous studies demonstrated that dairy and beef calves that were naturally infected with *Giardia* started shedding cysts during the second week of life [11,16]. To investigate the association between serum proteins and APP concentration and early *Giardia* infection a new variable was constructed – "early *Giardia* infection". Calves were considered to be of the early infection group if they had a faecal sample positive for *Giardia* at ≤12 days of age (n = 21).

Logistic regression analysis was used to determine if γ-globulin and APP (SAA, HP, FIB or ALB) concentrations during first (age 0–1) and second samples (age 4–6) had an effect on the onset of early *Giardia* infection. The outcome variable was "early *Giardia* infection" and explanatory variables were γ-globulins and total protein concentrations from the first or second sample. Birth period was added as a three level categorical variable ("early birth period" 9th–14th May, n = 16; "middle birth period" 15th–17th May n = 17; "late birth period" 18th–22nd May, n = 23) to control for a possible confounding effect of birth period.

A linear mixed model was constructed to establish if APP (SAA, HP, FIB and ALB) or γ-globulin concentrations changed over the study period (0–33 days). Protein concentrations were used as response variables and age groups as a 7-level categorical variable (age groups: 0–1, 4–6, 8–10, 12–14, 16–17, 20–22 and 23–33 days of age), regarded as a fixed explanatory variable. Calf was included as a random factor and isotropic spatial exponential covariance structure was used to model correlation between repeated samples within reindeer calves. Statistical difference was evaluated between every consecutive age group and Bonferroni corrections were used for controlling multiple comparison bias. Logarithmical transformations of γ-globulin, SAA and HP data were used.

Linear regression models were used to determine if "early infection" was associated with protein or APP concentration levels through the study period (0–22 days of age). For every protein, area under the curve (AUC) was calculated for the period using the trapezoidal rule:

$$AUC = \Sigma\,[(t_i - t_{i-1})f_{i-1}] + [0.5(t_i - t_{i-1})(f_i - f_{i-1})],$$

Where t_i = time of observation, t_{i-1} = previous time of observation, f_i = APP concentration at the time, and f_{i-1} = APP concentration at previous time. AUC was used to summarize changes in serum proteins and APP concentrations over the study period. Because the sampling periods were not equal for all calves (difference of up to 2 days), AUC values were divided by period days (day AUC) in order to allow comparison of AUCs between calves with different sample periods.

Average protein AUCs were used as outcome variables in regression models. Predictor variables were "early *Giardia* infection" (2-level categorical variable), *Cryptosporidium* infection (2-level categorical variable), and other protein (γ-globulins, SAA, ALB, HP and FIB) day AUC values. A birth period categorical variable with three levels was included in all models and a manual step-wise backward elimination procedure was used. The variables used in the multiple regression

models were checked for collinearity using a threshold of 10 for the variance inflation factor (VIF), which none of the components exceeded [17]. FIB average AUC was initially added to γ-globulin and SAA models, but it was not statistically significant and was consequently removed from all models. HP day AUC was non-significant in the SAA model and was also excluded.

Linear regression models were used to investigate the association between daily weight gain (DWG) in the short-term (birth to 20–21 days) and long-term (birth to 114–128 days). Predicting variables were serum proteins (total protein, γ-globulins) and APPs (SAA, HP, FIB) day AUCs, sex and birth period and early *Giardia* infection and *Cryptosporidium* infection. A manual step-wise backward elimination procedure was used.

Normality scatter plots of model residuals were used for evaluating the linear regression model assumptions.

Basic data management was done using Excel 2010 (Microsoft, Redmond, USA). Data was analysed using Stata/IC 13.1 for Windows (StataCorp LP, Texas, USA). Statistical significance level was set as $p \leq 0.05$. Coefficient plot figures were made using Stata software package coefplot [18].

Results from calves with complete data (n = 48) were used in statistical analysis (48 calves from 54 initially included in the study).

3. Results

3.1. Clinical signs

During the calving season, 9 calves in the study were diagnosed with diarrhoea at the time of sample collection. Calves were diagnosed with diarrhoea if their faeces were thin and watery. Six calves had diarrhoea at the age of 9–16 days and one calf had diarrhoea at the age of 31 days. Two calves experienced diarrhoea for two consecutive sampling times (at the age of 9 and 13 in one calf and 13 and 17 days in the second) and both had *Cryptosporidium* in faecal samples at the later sampling times. All calves except one (diarrhoea once at the age of 10 days) belonged to the late *Giardia* infection group.

3.2. Weight gain

The median (± SD) weight of calves at birth (0), 3 weeks of age, and 21 weeks of age was 6.4 kg (± 0.65; range 4.5–7.6 kg), 15.9 kg (± 2.07; range 12–21 kg), and 49.5 kg (± 5.24; range 39–61 kg) respectively. The daily weight gain from birth to 3–4 weeks of age was 0.382 kg/d (± 0.047; range 0.290–0.500 kg/d) and from birth to approximately 4 months of age was 0.364 kg/d (± 0.037; range 0.281–0.435 kg/d). No significant associations were established between weight gain in early and late-term (respectively up to 33 and 112 days of age) with early *Giardia* infection, *Cryptosporidium* infection, protein concentrations at different age groups or average protein AUCs during 0–22 days. Male calves gained more weight (0.039 kg/d, 95% CI: 0.021–0.058; $p < 0.001$) in the long term (from birth to 112 days) than females.

3.3. Giardia and Cryptosporidium infections

All the calves in the study from which a faecal sample was collected were *Giardia* positive. The faecal sample of one calf was positive on day 0. At 2 weeks of age, more than 60% of calves in the study were infected with *Giardia*. The infection rate sharply increased after 2 weeks of age (Fig. 1). During the first 10 days 38.9% calves had a low infection level. During the entire study 67% of calves had at least one sample for which the cyst count was high. By the age of 16 days 83% (45/54) calves had already at least one positive sample. 12 calves (22%) had at least one positive *Cryptosporidium* faecal sample during the study, but the overall prevalence remained relatively low (Fig. 2). Only one calf had 2 positive *Cryptosporidium* samples (at day 13 and 17). That calf also had

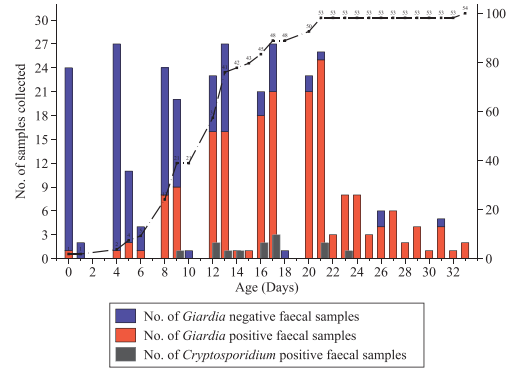

Fig. 1. Giardia positive faecal samples. The age of calf for first positive faecal sample and number samples collected in a day. Left hand y-axis shows no. of faecal samples collected on given day. Right hand y-axis shows% of calves that had at least one positive Giardia sample collected by that age. All the animals that had faecal sample collected tested positive (n = 54): in total 312 samples were collected.

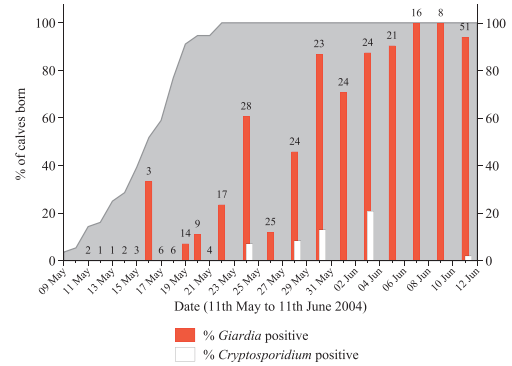

Fig. 2. *Giardia* and *Cryptosporidium* positive faecal samples (%) at given date. Left hand y-axis shows how many of the calves were born by a given date. All the calves were born between 9th May and 22nd May 2004. Right hand y-axis shows the% of how many of the collected faecal samples tested positive for *Giardia* and *Cryptosporidium*. Number on top of bar is the total no. of faecal samples collected on a specific date. The percent of calves born (54 = 100%) by given date presented in grey background.

diarrhoea at the later sample time. Six of the *Cryptosporidium*-positive calves were from the early *Giardia* infection group and 6 from the late *Giardia* infection group, 32% and 21% respectively.

3.4. γ-globulin and APP concentrations changes over time

Average γ-globulin concentrations were higher in the first two days (15.51 ± 4.76 g/l; n = 48). Concentrations subsequently decreased, being lowest at the age 23–33 days (2.84 ± 0.56 g/l; n = 45) (Fig. 3). There was significant decrease in average concentrations between every consecutive sample ($p < 0.01$). Four calves had γ-globulin concentrations below 10 g/l during the 24 h period after birth (all females). Three of them belonged to the early *Giardia* infection group and one to the late *Giardia* infection group (one with the lowest value, 3.57 g/l). None of those calves had diarrhoea episodes during the remainder of the study period.

Calf serum SAA levels started at a very low level after birth (0.31 ± 0.38 mg/dl; n = 48) and increased up to 8–10 days (6.59 ± 4.10 mg/dl) of age (change from first to second sample time and from second to third sample time $p < 0.01$). They peaked at

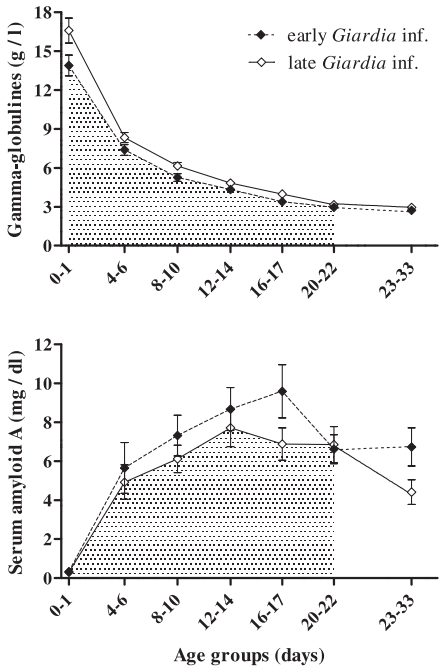

Fig. 3. Mean (± SEM) γ-globulin and serum amyloid-A (SAA) concentrations in serum of reindeer calves in early and late Giardia infection groups (from 0 to 22 days of age n = 19 and n = 29 respectively, at 23–33 age of age n = 19 and n = 26 respectively) during study period (0–33 days of age). Filled area represents time period where protein AUCs were calculated and used for studying differences in overall protein responses between early and late Giardia infection groups. Significant changes in protein concentrations after birth are presented in main text.

12–14 days of age (8.09 ± 4.99 mg/dl) and then began to decrease until the end of study period, but without statistically significant change (Fig. 3).

Median HP serum concentration levels were lowest at birth and at 4–6 days and then increased at 8–10 days and peaked at 12–14 days of age. Concentrations decreased again at 23–33 days (Fig. 4). ALB concentrations were lowest at birth, then increased and stabilised by the end of the third week of a calf's life (Fig. 4). FIB concentration was lowest on the first day, briefly increased and peaked between 4 and 6 and 12–14 days before decreasing (Fig. 4).

3.5. Associations between overall passive immunity and APP response with early Giardia infection

The logistic regression models for the onset of early *Giardia* infection did not indicate significant associations with γ-globulin and APP (SAA, HP, FIB or ALB) concentrations at the first (age 0–1 days) and second sampling times (age 4–6 days).

The multiple regression model was used to determine whether the early *Giardia* infection was associated with overall γ-globulin concentrations during the study period (Fig. 5). Early *Giardia* infection ($p = 0.032$) and ALB average AUC ($p < 0.001$) were negatively associated, whereas SAA average AUC ($p = 0.002$) and HP average AUC ($p = 0.017$) were positively associated with γ-globulin overall concentration.

Similar models were used to evaluate factors associated with overall SAA response during 0–22 days of age (Fig. 6). Early *Giardia* infection ($p = 0.042$), average γ-globulin AUC ($p = 0.001$) and ALB ($p = 0.015$) were positively associated with SAA overall response.

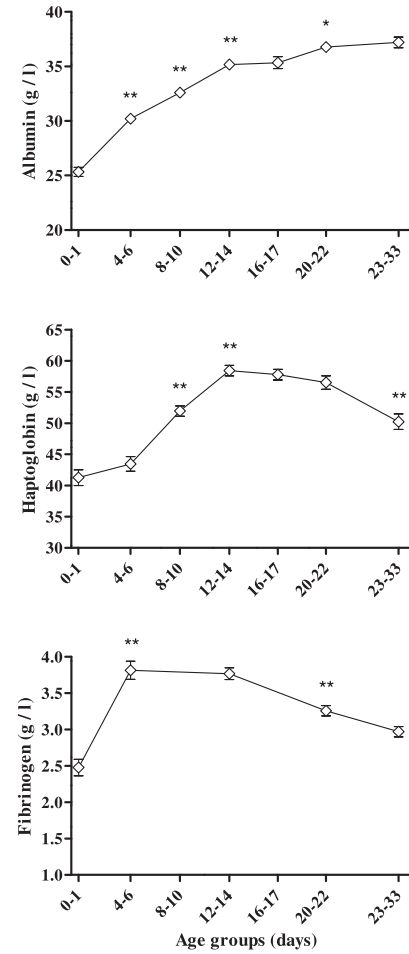

Fig. 4. Mean (± SEM) albumin (ALB), haptoglobin (HP) and fibrinogen (FIB) concentrations in serum of reindeer calves during study period (0–33 days of age). Sample size for ALB and HP at 0–22 days of age and at 23–33 days of age n = 48 and n = 45 respectively. For FIB at 0–1 days, 4–22 days and 23–33 days of age n = 21, n = 48 and n = 23 respectively.
*Significant difference from previous age group ($p < 0.05$)
**Significant difference from previous age group ($p < 0.01$)

Identical multiple regression models as described were constructed for HP, FIB, and ALB overall response, but there were no significant associations with parasite infections or average protein AUCs.

4. Discussion

This study describes *Giardia* and *Cryptosporidium* infection in semi-domesticated reindeer calves. *Giardia* and *Cryptosporidium* were found from wild reindeer faecal samples in Norway [19] and an epidemiologic study on reindeer in northern Finland and Norway was unsuccessful in detecting *Cryptosporidium* infection [20]. The role of *Giardia* as a pathogen in ruminants is still uncertain, although its importance as a potential zoonotic organism should not be underestimated [21].

In the harsh Arctic climate it is unlikely that *Giardia* or

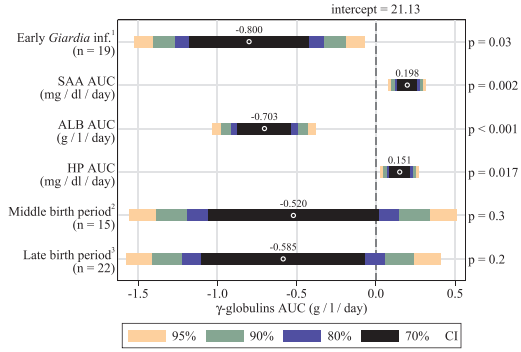

Fig. 5. γ-globulin AUC regression model coefficient plot (age 0–22 days). Model confidence intervals (CI) are presented as horizontal bars. Point estimates for variables are shown on top of the bars. AUC was calculated for each animal (n = 48) using a trapezoidal method for 6 time points and averaging for number of days (20–22 days of age).
[1] Compared to late *Giardia* infection group (n = 29)
[2] Middle birth period (15–17 May) compared to early birth period (9–14 May; n = 11)
[3] Late birth period (18–22 May) compared to early birth period (9–14 May; n = 11)

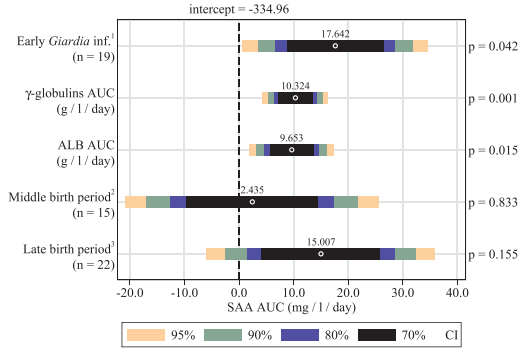

Fig. 6. Serum amyloid A (SAA) AUC regression model coefficient plot (age 0–22 days). Model confidence intervals (CI) are presented as horizontal bars. Point estimates for variables are shown on top of the bars. AUC was calculated for each animal (n = 48) using a trapezoidal method for 6 time points and averaging for number of days (20–22 days of age).
[1] Compared to late *Giardia* infection group (n = 29)
[2] Middle birth period (15–17 May) compared to early birth period (9–14 May; n = 11)
[3] Late birth period (18–22 May) compared to early birth period (9–14 May; n = 11)

Cryptosporidium can survive in soil over winter [22] because of physical damage from freeze-thaw cycles. They could survive in open water and in animals however or be transmitted by humans. In sheep it was established that *Giardia* shed from ewes reach peak levels at around parturition [23]. The same phenomenon could apply also to reindeer. It was demonstrated in cattle that *Giardia* infection can become chronic and persist for long time (over 7 months) [11,24]. It is unknown how long infection persisted in this study because sampling ceased when the animals were 33 days old. The greatest sources of *Giardia* infection of calves were probably the hinds and subsequently other calves.

A direct fluorescence antibody test for detecting *Giardia/Cryptosporidium* antigens from faeces is both sensitive and specific (over 90%), but is also sensitive to the concentration of oocysts before detection [25–27]. Some of the faecal samples could have been false negatives for *Giardia* and *Cryptosporidium* due to freezing of samples, which can damage the parasites and mask detection of very low numbers.

In this study the majority of *Cryptosporidium* infections were detected after 2 weeks of age. It is possible that colostrum provided sufficient protection to prolong the initiation of shedding, as was demonstrated in dairy calves [28].

Both *Giardia* and *Cryptosporidium* establish infection from very low levels (< 10 oocysts/cysts) [29], which could mean that once an animal becomes infected the entire herd is quickly infected, being exacerbated by the decrease in γ-globulin levels.

In this study, all the calves from which faecal samples were collected became infected with *Giardia*. Although dairy calves are kept under very different conditions, reindeer calves did appear to shed *Giardia* from an earlier age [9]. *Giardia* cysts were detected in faeces of neonatal dairy calves in the third week of life [30]. The concentrations of γ-globulins during the first 3 weeks of life were not negatively associated with the early infection. γ-globulin concentrations decreased as progressively more calves became infected, but the time of birth did not seem to contribute significantly to shedding of *Giardia* cysts during the first 12 days of the calf's life. This finding suggests that maternal antibodies provided some protection against *Giardia* infection. However, high γ-globulin concentrations at the first week of age were not associated with early *Giardia* infection, suggesting that late *Giardia* shedding calves may have developed an early humoral immune response, resulting in inhibition of *Giardia* shedding and higher overall γ-globulin response recorded in this group. The antibody interaction with a parasite's life cycle was demonstrated in murine *Giardia* infection models, which may indicate reduction of cyst shedding in infected animals [31].

At the same time, the innate immune system appeared to have responded to *Giardia* infections because there was positive association between SAA overall response and an early infection. The differences between *Giardia* infection groups were more evident at the end of second and at the beginning of the third week of age (Fig. 3). This supports the theory that early *Giardia* infection calves were not able to mount an immune response early and more severe infection pressure at the time when passive protective immunity declined quickly (as seen in Fig. 3) resulted in more pronounced activation of APR.

SAA levels in this study were comparable with those of a previous study on reindeer [5]. In both studies SAA concentrations peaked at around 2 weeks of age and were comparable with concentrations in dairy calves after birth [32]. In our previous study, reindeer calves with higher SAA at the second week of life had lower weight gain at 4 months of age [5]. Our research group has recently established the same phenomenon in lambs [33] and beef calves [34]. Those findings support the hypothesis that the second week of life in neonatal ruminants is important for immunological development and adaptation to the environment. Similarly, in the present study it could be speculated that calves infected sooner had weaker immune responses and were more susceptible to negative environmental factors, resulting in a lower growth rate. However, no evidence for this was forthcoming. Either the infection pressure from *Giardia* was insufficiently strong or it affected all the calves similarly. Overall, our results indicate that early *Giardia* infection cannot be related to the impaired adaptation or immunological development of reindeer calves. Higher *Cryptosporidium* infection rates at the time could potentially stimulate a more severe immunomodulatory effect, but there were only mild and rare infections established in this study.

HP and FIB were without significant positive associations with early *Giardia* infection, underlining the weak inflammatory stimulus of *Giardia* infection. A positive association with γ-globulin serum levels in early life and SAA concentrations supports the hypothesis that proteins from colostrum are transferred to the calf, as was demonstrated for lambs with SAA and FIB [35].

5. Conclusions

This study describes *Giardia* and *Cryptosporidium* infections in the neonatal period of reindeer calves. The early *Giardia* infection (before

12 days if age) was positively associated with lower overall γ-globulin intake/response and with higher overall SAA response, indicating interaction between host humoral and innate immune systems and *Giardia* infection.

Conflict of interest statement

None of the authors has any financial or personal relationship with organisations or people that could influence the content or conclusions reached in this the study.

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Short communication

Serum amyloid A and haptoglobin concentrations in relation to growth and colostrum intake in neonatal lambs

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ABSTRACT

Acute-phase proteins (APPs) can be used as sensitive quantitative biomarkers to study the inflammatory conditions and disease status of domestic animals. The variation in serum concentrations of two major APPs, namely serum amyloid A (SAA) and haptoglobin (Hp), and their association with weight gain were investigated in 322 newborn mixed-breed lambs at the age of 0–25 days. Serum SAA concentrations in lambs were the lowest on the day of birth, and it increased until day 5 after which the concentrations started to decrease. Hp concentrations were highest during the first week after birth and started to decrease in the second week. The lamb serum SAA concentrations were compared to the colostrum SAA concentrations of their mothers. Higher colostrum SAA concentrations were associated with higher serum SAA concentrations of lambs at 1–5 days of age. Lambs serum SAA concentrations at second week of age (6–12 days) were negatively associated the average weight gain from birth to the 3–4 months of age. There were no associations of lambs serum Hp concentrations with colostrum SAA concentrations and with lambs weight gain.

Our study suggests that the composition of ingested colostrum, immune challenges during early life may contribute into the individual variation in SAA and Hp levels. However, more studies are needed to determine the exact mechanisms of the fluctuation of serum APP levels in newborn lambs.

1. Introduction

Immediately after birth and during the first weeks of their lives, newborn lambs need to physiologically adapt to extra-uterine environment. Acute-phase reaction is one of the first non-specific and innate protection mechanisms of the host's immune system that is activated in response to infection, inflammation or trauma (Baumann and Gauldie, 1994). Acute-phase proteins (APPs) can be used as sensitive quantitative biomarkers that are regulated by pro-inflammatory cytokines produced mostly in the liver and in local tissues (Baumann and Gauldie, 1994; Eckersall and Bell, 2010). Haptoglobin (Hp) and serum amyloid A (SAA) are the major APPs in ruminants, and their concentrations are shown to be significantly increased during different inflammatory processes (Ceciliania et al., 2012). Several studies have suggested that APPs play physiologically important role in the foetal and neonatal period, but the mechanisms remain unknown (Murata et al., 2004; Orro et al., 2006, 2008). Major APPs in sheep are not studied as profoundly as in cattle, but similar responses of Hp and SAA in immune challenges of domestic ovines have been shown (Eckersall and Bell, 2010).

High concentrations of SAA have been reported in the colostrum of healthy females, and it is suggested that it has a beneficial role for the newborn's health and well-being (McDonald et al., 2001). Hence, characterizing the changes in serum APPs during the first weeks of a lamb's life would help to clarify the role of inflammatory responses in the postnatal development of lambs. Studies describing the patterns of APPs and specifying their role in the development of newborn ruminants are scarce, which is why we have chosen the lamb model to describe the fluctuations of SAA and Hp early in life.

We also measured the colostrum SAA concentrations of the ewes for testing if it is (1) related to the serum SAA concentrations of lambs and (2) enhances growth of the lambs. We proposed that adaptation after birth might initiate inflammatory processes that in turn could affect the weight gain of lambs later in their developmental period.

2. Materials and methods

The study was conducted during 2011 and 2012 in April–May at organic sheep farm in Southern Estonia. The study herd of approximately 250 ewes included Estonian blackheaded and Dorper's mix sheep. All animals were housed in a barn with ad libitum access to water, silage and hay and access to outdoor fenced grassland.

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Immediately after birth, all the lambs received colostrum from their dams, and later they were fed with mother's milk. No vaccination was used and anti-parasitic treatment with abendazole or ivermectin was performed in bases of faecal investigation results separately in animals groups at risk. If needed, lambs were first time dewormed at the time of weaning or soon after that.

Blood from the lambs was sampled by puncturing the jugular vein with a Vacutainer using a 4-mL serum clot tube (Vacuette, Greiner Bio-One, the Netherlands) once per week for 1–3 times during the lambing period. In total, 322 lambs and 197 ewes were included in the 2-year study. The blood of 176 lambs was sampled once, 90 lambs twice and 56 lambs three times, and the proportion of samples were almost equally divided between both years (248 samples in 2011 and 276 samples in 2012). In 2011, sampling was performed during days 1–25 from the lambs' birth. In 2012, the lambs were sampled on days 0–13 (day 0 was counted as the birth day). The blood samples were centrifuged (at 4000 rpm for 5 min) for serum separation, which was then stored at −20 °C until further analyses.

Individual colostrum samples from ewes ($n = 197$) were collected shortly after parturition into sterile 10 mL plastic tubes and were stored at −20 °C prior to analyses.

The ewes' body condition was measured shortly after parturition, using a body condition scoring system from 1 to 5 according to Thomson and Meyer (1994).

The body weight of the lambs was measured once at the age of 3–4 months (78–130 days of age) on the farm using a digital scale (precision of ± 50 g). To determine the lamb's daily weight gain (g/day), estimated average weight of the lamb at birth (fixed at 4000 g) was subtracted from the weight measured at 3–4 months of age and divided by the age (days) of the lamb at weighing. The average birth weight of the lambs was estimated using separate subset of lambs ($n = 30$) at the same farm in the birthing seasons in 2014–2015. The lambs were weighed on the first day of their life. Farms' ewe structure, feed and management was similar to study period of 2011–2012. Sixteen lambs were weighed in 2014 (8 females and 8 males; 8 singles and 8 twins) and 14 lambs were weighed in 2015 (9 females and 5 males; 8 singles and 6 twins). The average birth weight of these 30 lambs was 3998 g (± SD 981).

The concentration of SAA in the lambs' serum and ewes' colostrum was determined using a commercial ELISA kit (Phase MAA Assay Kit; Tridelta Development Ltd., Maynooth, Co. Kildare, Ireland, the batch number: 21KT070) according to the manufacturer's instructions for cattle.

Serum concentrations of Hp were determined using a method based on the ability of Hp to bind to haemoglobin (Alsemgeest et al., 1994). Pooled and lyophilized aliquots of bovine acute-phase serum were used to plot standard curves. To calibrate the assay, a bovine plasma sample with a known Hp concentration provided by the European Commission Concerted Action Project (number QLK5-CT-1999-0153) was used (Skinner, 2001).

The intra- and inter-assay coefficients of variations for SAA were <12% and <14%, respectively, and for Hp, <15% and <9%, respectively.

2.1. Statistical analysis

Linear mixed regression models were used to investigate age-dependent changes in APPs serum concentrations as well as associations between APPs serum concentrations, colostrum SAA concentrations and average weight gain of lambs. Day 0-aged lambs were excluded from all statistical analysis (only five samples from 2011). Logarithmically transformed serum SAA and Hp concentrations were used in all models as outcome variables.

In first model APPs concentrations were outcome variables and age as categorical grouping explanatory variable (1 day of age as comparison category). In second model polynomials of age in increasing order were used to test differences between trends by study years (Figs. 1 and 2.). The ewe and the lamb within the ewe were included as random factors. Isotropic spatial exponential covariance structure was used to model the correlation between repeated samples within the lambs.

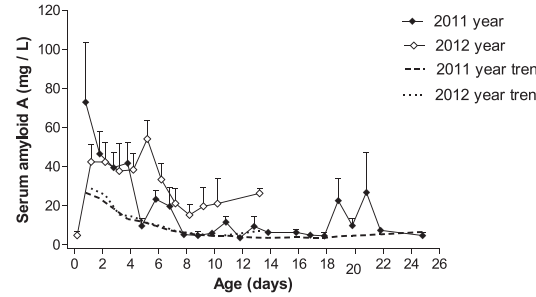

Fig. 1. Mean (± SEM) serum concentrations of serum amyloid A (SAA) in lamb blood during the first 25 days of life by study year. Dotted lines represents random regression models of time changing trend predictions by study years back-transformed from the logaritmic scale. Total number of samples $n = 524$: $n = 248$ in 2011 (111 lambs, 1–3 samples per lamb) and $n = 271$ in 2012 (208 lambs, 1–2 samples per lamb).

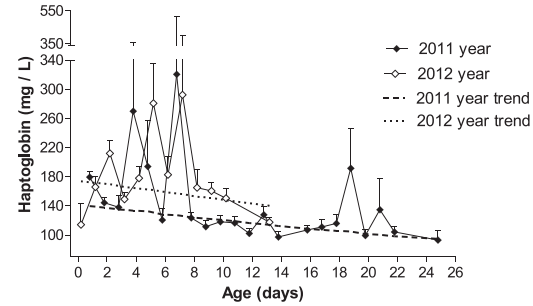

Fig. 2. Mean (± SEM) serum concentrations of haptoglobin of lambs during the first 25 days of life by study year. Dotted lines represent random regression models of time changing trend predictions by study years back-transformed from the logaritmic scale. Total number of samples $n = 524$: $n = 248$ in 2011 (111 lambs, 1–3 samples per lamb) and $n = 271$ in 2012 (208 lambs, 1–2 samples per lamb).

For investigating APPs associations with colostrum SAA and average weight gain analysis were performed separately by lamb's age groups (1–5, 6–12 and 13–19 days of age). Firstly linear mixed regression models where APPs concentrations were as outcome variables and colostrum SAA concentration and the age of the lambs at sampling were included as continuous independent variables were built. The number of siblings as three-level (single, twin, triple), the age of ewes as five-level (1 year old, 2 years old, 3–4 years old, 5–6 years old and 7–8 years old), the ewes' body condition score as three-level (scores 1.5–3, 3.5 and over 3.5), and gender and study year were included as categorical independent variables. Secondly similar models where average weight gain was outcome variable and the same explanatory variables plus APPs serum concentrations were conducted. Colostrum SAA concentration and age of the lambs at sampling were included as continuous independent variables and number of siblings, age-group of ewes, ewes' body condition score group, and gender and study year were included as categorical independent variables. Ewe was included as a random factor in all models.

Step-wise backward elimination of explanatory variables was used for the final models. Biologically meaningful interactions and linear relationships between dependent variables and explanatory continuous

variables were tested. As the serum SAA concentration association with weight gain at the second week of age was not linear, logarithmically transformed SAA concentrations were used as best fit. All model assumptions were verified by scatter plots and normality plots of standardized residuals.

The statistical analyses were performed using Stata/IC 13.0 statistical software (StataCorp LP, College Station, TX, USA). $P \leq 0.05$ was considered statistically significant.

3. Results

All the animals were clinically healthy at the time of sampling. The mean (± SD) age of ewes at giving birth was 3.2 (± 2.1) years. The mean (± SD) weight of lambs at 3–4 months was 25.34 (± 6.16) kg. The mean (± SD) daily weight gain of lambs until the age of 3–4 months in both years was 200.8 g/day (± 53.7; $n = 259$).

Serum SAA concentrations in lambs were the lowest shortly after birth (0 day: median 2.2 mg/L; range 1.3–14.3 mg/L; $n = 5$), but showed significant increase on the first day of life compared to day 0 ($P = 0.009$), when the measures were also the highest (median 20.9; range 1.5–200.5 mg/L; $n = 39$). Concentrations of serum SAA remained elevated until 5 days of age (median of 2–5 days 18.7; range 0.3–216.4 mg/L; $n = 160$), and started to decrease at day 6 of age (median 11.9; range 0.3–164.4 mg/L; $n = 46$) compared to day 1 ($P = 0.048$), and then remained stable (Fig. 1).

Hp concentrations were the highest during the first week after birth and started to decrease in the second week. The overall Hp concentrations were significantly higher in 2012 ($P < 0.001$) (Fig. 2).

The mean (± SD) colostrum SAA concentration was 10.8 mg/L (± 14.9) (median 5.1, range 0.3–75.0 mg/L; $n = 207$). Higher colostrum SAA concentrations of ewes were associated with higher serum SAA concentrations of their lambs at 1–5 days old ($n = 120$; $P = 0.009$).

There was a significant negative association between serum SAA concentrations and average weight gain of lambs during the 3–4 months of age in the model with lambs at 6–12 days of age ($n = 194$; $P < 0.008$; Fig. 1.). The number of siblings influenced the average daily weight gain as being a twin or a triplet was associated with lower daily weight gain compared to singly born lambs (Table 1.). The age of the ewe was an important factor as older ewes had lambs with higher weight gain compared to the 1 year old ewes. There was no significant difference in weight gain between the years, but older age of lambs at the time of weighing had a negative effect on the weight gain. Male lambs gained more weight daily on average than female lambs (Table 1). Weight gain was not related to the serum Hp level in any of the weeks of age tested.

4. Discussion

Our study shows that the higher colostrum SAA concentrations of ewes are associated with higher SAA concentrations of their lambs shortly after birth. McDonald et al. (2001) have shown in bovines that extra-hepatically, a mammary gland produces local mammary-associated SAA3 (M-SAA3). SAA has been demonstrated not to correlate in the serum and milk of ewes, which indicates that SAA is synthesized in the ewe's mammary tissue (Ceciliani et al., 2012). McDonald et al. (2001) demonstrated that the M-SAA3 concentrations were high after parturition in bovine, equine and ovine colostrum, and then started to decrease and reached the lowest level in milk on day 4. It is assumed that M-SAA3 might have an important and beneficial effect on the well-being of the new-born and/or the maintenance of the mammary gland (McDonald et al., 2001). Colostrum contains high quantities of free pro-inflammatory cytokines as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 (Sordillo et al., 1991; Hagiwara et al., 2000), which are regulating the synthesis of APPs in the hepatic tissue (Baumann and Gauldie, 1994). Yamanaka et al. (2003) have demonstrated that cytokines in new-born calves reached the highest levels by 24 h and then started to decrease. They assumed that those cytokines probably originated from colostrum.

Our results indicate that there might be a direct transfusion of SAA from colostrum to lambs. However, in dairy calves' serum samples after birth, no M-SAA3 isoforms were included, but SAA isoforms were produced by the liver (Orro et al., 2008). The other possibility is that this process is regulated by cytokines in ewe colostrum, which causes SAA production in ewe mammary glands and lamb livers. We assume that lambs will benefit from higher colostrum SAA content, but the exact mechanisms still remain unclear.

Our results revealed different patterns of variation in SAA and Hp concentrations during the first 3 weeks of life. There are very few other studies of APPs in neonatal lambs available with which to compare. Eckersall et al. (2008) have shown that lambs' SAA and Hp were moderately elevated on the day of birth compared to days 8–14.

Table 1
Association of serum amyloid A (SAA) concentration, age of ewes, multiple births, sampling year and gender with weight gain (g/day) of 6–12 days old lambs ($n = 194$) in the model, where ewes ($n = 131$) were added as a random factor.

Variable	No. of samples	Estimate	95% CI of estimate	*P*-value	Wald test *P*-value
SAA (log mg/L)		−5.71	−9.90; −1.51	0.008	
Age at the sample (days)		−2.99	−6.26; 0.28	0.073*	
Sample year:					
2011	108	0			
2012	86	32.40	−22.43; 87.24	0.247*	
Age at weighting (days)		−1.33	−2.54; −0.12	0.031	
Age of ewe:					<0.001
1 year	48	0			
2 years	61	34.16	17.91; 50.40	<0.001	
3–4 years	31	64.58	41.21; 87.95	<0.001	
5–6 years	32	43.42	21.46; 65.39	<0.001	
7–8 years	22	38.94	11.68; 66.21	0.005	
No. of siblings:					<0.001
Single	93	0			
Twins	95	−53.33	−68.29; −38.38	<0.001	
Triplets	6	−105.50	−148.43; −62.56	<0.001	
Gender:					
Male	107	0			
Female	87	−23.44	−34.69; −12.20	<0.001	
Intercept		371.63	265.56; 477.71	<0.001	

* Variables were retained in model as coufounders.

Considering other ruminant species, similar age-related changes in SAA and Hp concentrations have been found in reindeer (Orro et al., 2006) and dairy calves (Orro et al., 2008). Different changes of SAA and Hp after birth could be explained by experiments in cattle that have demonstrated SAA to be more sensitive to inflammatory response than Hp (Horadagoda et al., 1999; Heegaard et al., 2000). Orro et al. (2006) suggested that temporal patterns of both proteins indicate that they contribute to the development of defence and adaptation mechanisms of new-born calves, or reflect the role of the inflammatory process during the adaptation period of the immune system.

Interestingly, Hp values were significantly higher in 2012. These differences cannot be explained by herd management, which was identical between the years studied. Therefore, we could assume that Hp is more dependent on environmental conditions and is therefore a more inconsistent physiological marker than SAA. APPs response in ruminants can be influenced by a stress reaction. It has been shown that increased SAA and Hp responses after exposure to complex stress can be used as indicators of the presence of stress in adult cattle (Lomborg et al., 2008). As stress by several different means has an impact on the calf immune system, the same relations can be assumed in young animals (Cortese, 2009).

Parturition is assumed to be particularly stressful event for both mothers and neonates, with the accompanying release of corticosteroids and physiological acute phase response, as has been described by Humblet et al. (2006). Studies on humans have shown that normally delivered infants had twofold higher levels of SAA at birth compared to infants delivered by caesarean section, which suggests that trauma or physical stress during parturition may increase the levels of APPs (Buonocore et al., 1995; Marchini et al., 2000).

The characterization of changes in APP concentrations after birth could elucidate the role of inflammatory response in new-borns' adaptation to extra-uterine life (Orro et al., 2008). We could assume that changes in SAA concentrations after birth can influence the resistance to diseases until the new-born organism has adapted to novel environmental conditions.

We also showed that SAA concentrations at second week of life are negatively associated with weight gain of the lambs. It has also been found by Orro et al. (2006) that SAA concentrations in the second week of life had a negative association with weight gain in reindeer calves during the study period of 111–125 days. They suggested that there might be an exposure to infections at the second week of life which influences reindeer calves development throughout the first months of life. Recently, a similar negative association between the second week of life serum concentrations of SAA and weight gain throughout an animals life in beef calves was reported (Seppä-Lassila et al., 2017). The negative association between high serum SAA values during the second week of life and overall body weight gain may indicate that systemic inflammatory response was activated, but this resulted in an inadequate or inappropriate cytokine cascade for proper immunological development and maturation (Orro et al., 2006). However, there was no associations between lambs serum Hp concentrations and average weight gain and this associations were not found also in previous studies where serum SAA and weight gain were negatively associated in reindeer and beef calves (Orro et al., 2006; Seppä-Lassila et al., 2017). This can be explained by different sensitivity to stimulus of these APPs seen in bovines (Alsemgeest et al., 1996; Horadagoda et al., 1999; Heegaard et al., 2000).

In conclusion, our results show that there is a link between ovine colostrum SAA and lambs first week serum SAA levels and possibly the intake of colostrum through the presence of APP mediators may contribute to the individual variation in SAA and Hp levels in early life. We suggest that APPs, and SAA particularly, might have a role in the defence and environmental adaptation mechanisms of lambs and stimulation of SAA in second week of life may reflect negative adaptation to the extra uterine life. However, the exact mechanisms of the fluctuation of SAA and Hp levels at first weeks of life of lambs are still unclear and need to be studied in more depth.

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Acute phase response in organic lambs associated with colostrum serum amyloid A, weight gain, and *Cryptosporidium* and *Giardia* infections

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ABSTRACT

In neonatal period, lamb's immune system goes through rapid adaptation to the extra-uterine environment. Success of this process can influence the animal's future performance and, thus, the quantitative assessment of it would greatly benefit sheep producers. The current study was conducted to investigate the acute phase response (APR) (measured through serum amyloid A (SAA), haptoglobin (Hp) and albumin (ALB)) in relation to later life growth (measured at 122 days of age), and naturally occurring *Cryptosporidium* and *Giardia* infections in neonatal lambs grown in organic farm. Serum (n = 692) and faecal (n = 141) samples were collected from 269 lambs in their first 3 weeks of life. The ewes' colostrum (n = 181) SAA concentrations were positively associated with the lambs' serum SAA and Hp concentrations at 2 to 4 days of age. Hp and ALB concentrations at the second week of age were positively associated with the growth rate at 122 days of age. Lamb serum globulin (GLOB) concentrations and *Cryptosporidium*-positive faecal samples were negatively associated at the second and third weeks of life. These findings suggest the importance of interactions between the immune system and environmental factors at the second week of the lambs' lives and its association with future performance.

1. Introduction

Acute phase response (APR) refers to the activation of a series of systemic innate immune system defences in a situation where the animal is affected by physical trauma, infection, stress, or inflammation (Ceciliani et al., 2012). In sheep proteins, haptoglobin (Hp), and serum amyloid A (SAA) which concentrations increase significantly during APR (at least 10-fold) in serum are considered to be major positive acute phase proteins (APPs), while albumin (ALB) is considered to be a negative APP as its concentration significantly decreases in serum (Ceciliani et al., 2012).

In healthy lambs at the day of parturition, mean serum SAA has been found to be around 2.6 mg/l, and Hp concentration around 0.2 g/l (Dinler et al., 2017). In ovine colostrum SAA has been found as an acute phase and in a mammary gland-specific form, M-SAA3 (also known as milk amyloid A, MAA) (McDonald et al., 2001). The main immunological function of colostrum is to passively provide protection for lambs; including immune function-related low-abundance proteins to cross over to the serum during the consumption of colostrum (Hernández-Castellano et al., 2015). It has been suggested that some APPs could transfer directly to lambs via the colostrum and increase low-abundance protein concentrations (for example, SAA and fibrinogen [FIB]), thus modulating the immune response (Peetsalu et al., 2013; Hernández-Castellano et al., 2014).The exact mechanisms and effects of colostrum APPs on offspring's immune system require further studies.

Gamma-glutamyltransferase (GGT) activity in lamb serum during the first days after birth could theoretically be used to control the effects of colostrum on offspring APPs as it has been found to strongly correlate with the transfer of immunoglobulin G (IgG) from the colostrum to the lamb serum (also known as passive immune transfer) (Maden et al., 2003; Britti et al., 2005). While it has been demonstrated that ewe's parity and body condition score do not seem to significantly affect colostrum's IgG and lambs' IgG, total protein (TP) or ALB serum concentrations (Alves et al., 2015), the malnutrition of ewes nevertheless, in late pregnancy has been associated with lower SAA and Hp concentrations in neonatal lambs (Eckersall et al., 2008). Later on, lambs with higher SAA concentrations in their second week of life tend to have lower growth rates during the first months of their lives (Peetsalu et al., 2013). This suggests that while ewe's own body condition or parity does not seem to strongly affect the colostrum composition, it still could influence lamb through SAA or Hp during the first weeks of age.

Cryptosporidium and *Giardia* are gastrointestinal protozoan parasites that have worldwide distribution (O'Handley and Olson, 2006). Lambs infected with *Cryptosporidium* spp. start to shed oocysts 4 to 5 days post-

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infection and excretion continues on average for 9 to 11 days (Bukhari and Smith, 1997). *Cryptosporidium* infection can induce APR response, profuse diarrhoea and reduction in milk consumption, which starts 1–2 days after the initiation of shedding and ends around 15–20 days of age (Quílez et al., 2002; Dinler et al., 2017). In long-term perspective (7–8 months of age), *Cryptosporidium* infection has been shown to reduce lambs' hot carcass weight (6.6–9.7%) (Sweeny et al., 2011). Lambs infected with *Giardia* start to shed cysts 10–21 days after infection and often become chronically infected (Taminelli et al., 1989; O'Handley and Olson, 2006). *Giardia* infection in lambs has been associated with malodorous and poorly formed faeces, diarrhoea, decreased weight gain, impairment in feed efficiency, and decreased carcass weight at slaughter (Olson et al., 1995; Sweeny et al., 2011). *Cryptosporidium* itself and in coinfection with *Giardia* in lambs younger than 2 months old can cause soft-pelleted faeces, diarrhoea, and slower growth rates (Xiao et al., 1993; Muñoz et al., 1996; O'Handley and Olson, 2006).

The aim of this study was to investigate lambs' APPs concentrations in first weeks of life in relation to later life performance. The outcome variable was set average daily weight gain (ADWG) as it would one of the most important factors in measuring success of sheepherders focused on meat production. Additionally the APPs response was controlled for possible interaction with colostrum SAA, serum IgG and naturally occurring *Cryptosporidium* and *Giardia* infections.

2. Materials and methods

2.1. Animals

This study was conducted at a Southern Estonian organic sheep farm specialising in meat production. The herd was composed of Estonian black-headed, Dorper, Gotland, Suffolk, Merino, and mixed-breed sheep. A total of 193 ewes were lambing during the sample collection period in the spring of 2014. All the lambs (n = 269, 124 females and 145 males) in the study were born from April 4 to May 4 (Table 1). Animals were kept indoors in a single barn during the sampling period due to unfavourable weather conditions (sub-freezing temperatures and occasional blizzards) with ad libitum access to silage and fresh water. During the summer, most of the animals except for few ewes and all the rams were taken to small islets in the Baltic Sea near the coast of western Estonia where they remained until weaning (July 27, 2014) with minimal supervision. The lambs' weights were measured using a digital scale (precision of ± 50 g) on April 28 (n = 230) and May 5 (n = 159). For assessing the average daily weight gain, additional measurements were done using a digital scale (precision of ± 100 g) on August 18 (n = 238). A decrease in the numbers of animals weighed at consecutive time was because some were sold for slaughter.

2.2. Serum and colostrum sample collection

Sample collection times were planned at the height of lambing season. The farm was visited 4 times for sample collection with 7-day intervals (Table 1). Serum samples (n = 692) were collected from the jugular vein into sterile evacuated 10 ml plastic tubes with an 18 G needle. The samples were stored at room temperature for 2 h until transportation to a laboratory. The blood samples were centrifuged at 234 relative centrifugal force (RCF) for 10 min for serum separation and stored at −20 °C until further analysis. Colostrum samples (n = 187) were collected 1–2 h after lambing by milking the ewe by hand into sterile 10 ml plastic tubes. The samples were immediately placed into −20 °C storage until further analysis.

Table 1
Lambs' ages at sample collections in days.

Collection no.	Average age (± SD)	Range	No. of lambs sampled
1	3.8 ± 2.5	0–10	113
2	7.5 ± 4.6	0–17	190
3	11.8 ± 5.6	1–21	230
4	14.3 ± 5.2	1–21	159

2.3. Serum and colostrum sample analysis

Hp concentrations were measured by its ability to bind haemoglobin (Makimura and Suzuki, 1982). Tetramethylbenzidine (0.06 mg/ml) was used as a substrate for Hp and adapted for a microtitration plate (Alsemgeest et al., 1994). The calibration of the test was based on the European Union's animal acute phase proteins project (QLK5-1999-0153) (Skinner, 2001). SAA concentrations in the lambs' serum and the ewe's colostrum were tested using a commercial indirect ELISA test (Phase SAA Assay, Tridelta Development Ltd., Kildare, Ireland). When SAA concentrations reached over the standard value, the serum samples were diluted and tested again. Detection limits for Hp and SAA were 0.06 g/l and 0.3 mg/l, respectively, with intra- and inter-assay coefficients of variation lower than 15%.

GGT activity was determined by the kinetic method with L-γ-glutamyl-3-carboxy-4-nitroanilide using a commercial kit (Accent-200 GGT, PZ Cormay S.A., Łomianki, Poland). ALB concentrations in serum were measured via bromocresol green method using a commercial kit (Photometric Colorimetric Test for Albumin Liquicolor, Human Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany). TP was measured using a commercial kit (Photometric Colorimetric Test for Total Proteins Liquicolor, Human Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany). GGT, ALB, and TP levels were measured using an automatic liquid chemistry analyser (Mindray BS-200, Mindray Medical International Limited, Shenzhen, China). GLOB concentrations were calculated by subtracting ALB concentrations from TP concentrations.

The concentration of IgG was calculated using GGT activity, as it has been demonstrated to have very strong association, by formula: ln(IgG) (mg/dl) = 2.251 + 0.700 × ln[GGT] (IU/l) + 0.378 lamb age (days), where age 1 (day) = 0 and age 2 (days) = 1 (Britti et al., 2005). One and two days old lambs' IgG serum concentrations were considered as a strong proxy for controlling the effectiveness of adequate passive immune transfer (Alves et al., 2015).

2.4. Faecal samples collection and analysis

Faecal samples (n = 141) were collected directly from the rectums of 110 lambs into a clean plastic cup using a disposable latex glove. Each time the farm was visited faecal samples were retrieved, in total, a single sample from 80 lambs, two samples from 29 lambs, and three samples from 1 lamb. Faecal sample collection was attempted on each lamb, but if the rectum was found to be empty and rectal palpation and defecation stimulation using a finger was unsuccessful, then the lamb was freed to avoid causing it excessive discomfort. *Cryptosporidium* oocysts and *Giardia* cysts were detected in faecal samples using a commercial kit (immunofluorescence staining) according to the manufacturer's instructions (Crypto/Giardia Cel, Cellabs Pty Ltd., Sydney, Australia). All faecal samples went through only a single freeze-thawing cycle in order to avoid reduction of recognizable *Cryptosporidium* oocysts and *Giardia* cysts (Robertson and Gjerde, 2004). In order to calculate the approximate number of oocysts or cysts in a gram (OPG) of faeces, 0.1 g of deep frozen lamb faeces was mixed with 1 ml of tap water. The subsample was thoroughly stirred using a vortex and 20 µl of the subsample was fixed on glass slides (14 mm diameter latex well). *Cryptosporidium* oocysts and *Giardia* cysts were counted visually on the slides using an epifluorescence microscope Nikon Eclipse 80i at 200–400 × magnification. The sample was considered positive if at least 1 oocyst or cyst was found. The oocysts and cysts counted were used to calculate the approximate number of OPGs, which were corrected for the dilution of the subsample and the total area of the well

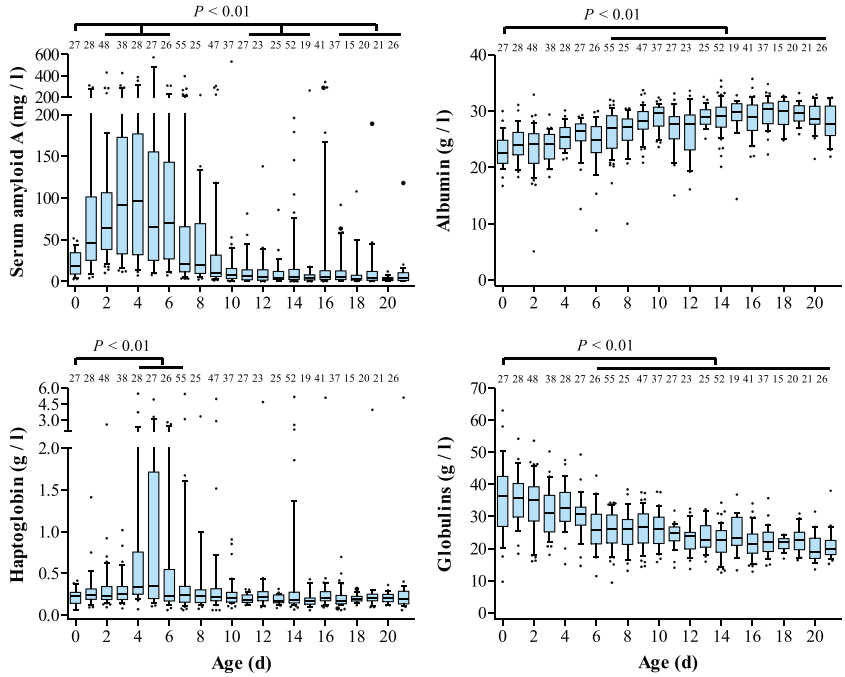

Fig. 1. Concentrations of serum amyloid A (SAA), haptoglobin (Hp), and serum proteins (albumin, ALB, and globulins, GLOB) in lambs during the first 3 weeks of age. The whiskers of the box plot represent 10–90% of results. The numbers on top of the box plot bars represent the number of lambs at different sampling ages. Significant differences (Bonferroni's corrected *P* value) between the first samples (age 0 d) and all other samples taken in different ages are indicated.

(De Waele et al., 2010). If a single well had too many oocysts or cysts to count, then 3 visual fields at random were counted (Lassen and Lepik, 2014). The 3 results were averaged and multiplied by the visual field area to calculate the approximate number of oocysts and/or cysts in a well.

2.5. Statistical analysis

Partial correlations were used to study the correlations between the lambs' serum protein concentrations and GGT activity. An analysis was performed separately on the study week and all 2 by 2 correlations were estimated taking into account all other variables (protein concentrations, age, and GGT activity). To test the effects of colostral SAA concentrations on the lambs' serum protein concentrations and GGT activity, a mixed linear regression model with protein concentrations and GGT activity during the first week in the lambs' serum were used as response variables and ewes were included as random factors. In current model and in following ones logarithmical transformation of SAA and Hp was applied if necessary in order to reduce skewness and to transform nonlinear relationship into linear. Colostrum SAA concentration, the lambs' ages (days), number of siblings (single or twins), and ages of the ewes (4 level category variables < 2, 2, 3–4, and > 4 years) were included in all models as explanatory variables.

Mixed linear regression models were used to analyse time-dependent changes in the lambs' serum protein concentrations. Logarithmically transformed SAA and Hp concentrations and ALB or GLOB concentrations were used as response variables. The ewes and lambs were included as random factors in all models. For modelling effect of repeated sampling in the lambs' isotropic spatial exponential covariance structure was used. The ages in days were included as categorical variables and all age groups were compared to the first age group (age 0 days).

Mixed linear models were used for testing the associations of parasite infections, protein concentrations, and GGT activity during the first 3 weeks of the lambs' lives. Protein concentrations (logarithmically transformed SAA and Hp) and GGT activity at the first week were used as response variables and *Cryptosporidium* or *Giardia* positivity (yes/no) as explanatory variables. An analysis was performed separately by study weeks for all response variables. The lambs' ages were included in all models and the ewes were used as random factors.

Mixed linear regression models were used to study the associations between different lambs' serum protein concentrations (IgG, SAA, Hp, ALB, TP) and colostrum SAA during the first 3 weeks of age with average daily weight gain (ADWG) recorded around 3 months later. For this model, a subsample of lambs was selected retrospectively and following inclusion criteria were applied: serum sample collected at first or second day of life, colostrum SAA measured and weighing done around 124 days of age. In total 53 lambs matched those criteria. Calculation of ADWG (kg/day) was done by subtracting earliest measured weight (kg) (average age: 12 ± 6 days) from measurement day weight (kg) and dividing it by age (days). ADWGs recorded on August 18, 2014 were used as response variables and ewes as random factors. Response variables were proteins concentrations by study week, IgG concentration at the first or second day of age, gender, number of lamb siblings (singleton, twin) and the ages of the ewes (< 2, 2, 3–4, and > 4 years).

The backward stepwise elimination procedure to fit best model was used in the mixed regression models. Assumptions of the linear association between continuous explanatory and response variables were checked and the logarithmic transformation of the SAA concentrations

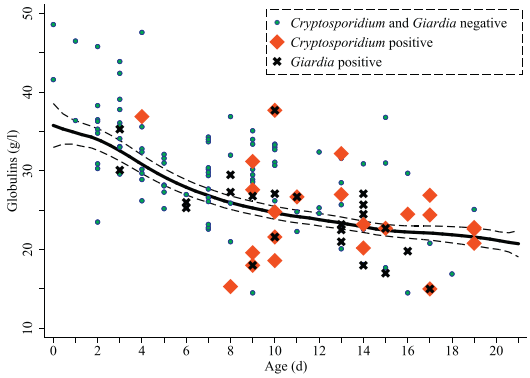

Fig. 2. Scatterplot of globulin (GLOB) serum concentrations in lambs with *Cryptosporidium* or *Giardia* positive (both $n = 23$) faecal samples ($n = 141$). The thick black line represents the relation/function of GLOB on age by local polynomial smooth. The degree of the polynomial used in the smoothing was set to 3. The dashed lines represent the 95% confidence interval. Lower GLOB serum concentrations were associated with *Cryptosporidium* positive faecal samples at the second and third weeks of age, $P = .033$ and $P < .001$, respectively.

in the ADWG model was used as explanatory variables. All model assumptions were verified by scatter and normal plots of standardised residuals. The Bonferroni correction was used in all mixed models and in partial correlation analysis to avoid repeated measuring bias. The significance level was set at $P \leq .05$ with tendency to the significance at level from 0.051 to 0.1. Statistical analysis was performed using Stata 14.1 (StataCorp LP, College Station, TX, USA).

3. Results

3.1. Acute phase proteins, globulins, and gamma-glutamyltransferase

SAA serum concentrations were lower at birth (average: 21.1 mg/l; $n = 27$) and increased during the first week of life, peaking around 3 to 4 days (average: 118.7 mg/l; $n = 66$). Hp concentrations were also lower at day 0 (average: 0.21 g/l; n = 27) and peaked at 4–8 days of age (average: 0.711 g/l; $n = 161$) (Fig. 1). GLOB concentrations were higher at day 0 (average: 35.5 g/l; n = 27) and then gradually decreased throughout the study period, with the lowest at 21 days of age (average: 21.0 g/l; $n = 26$) (Figs. 1 and 2). ALB concentrations were lower at day 0 (average: 22.9 g/l; n = 27) and then gradually increased during the first 2 weeks of life, peaking around 14–16 days of age (average: 28.8 g/l; $n = 112$) (Fig. 1). Serum average GGT activity in the lambs at age 0 to 13 days of age was 658 IU/l (range 50–2179; median = 422; $n = 269$) and GGT activity was negatively associated with age ($P < .001$). Mean GGT level in lamb serum was at day 0 1228 IU/l (range 50–2104; median 1284; n = 27) and at day 13 it was 368 IU/l (range 70–1996; median 226; $n = 25$).

At the first week (1–7 days of age; $n = 250$), serum SAA and GGT were significantly negatively correlated, while SAA and Hp and GGT and GLOB were positively correlated (Fig. 3). At the second week (8–14 days of age; $n = 236$) SAA and Hp, ALB and GLOB, and Hp and GLOB were positively correlated (Fig. 3). At the third week (ages 15 21 d; $n = 179$), GLOB and Hp, ALB and GLOB, and SAA and Hp were positively correlated (Fig. 3).

3.2. Colostrum and lamb serum acute phase proteins

Average (min-max) colostrum SAA concentration was 20.47 mg/l (0.3–478.8; $n = 181$, median = 5.43 mg/l). The lambs' ($n = 109$) serum SAA and Hp concentrations at days 2–4 of age ($P = .042$ and $P = .013$, respectively) were positively associated with their ewes' colostrum ($n = 81$) SAA concentrations. There were no significant associations found between the ewes' colostrum SAA concentrations and their lambs' serum GGT activity and ALB or GLOB concentrations during the first week of the lambs' lives.

3.3. Average daily weight gain and acute phase proteins

ADWG (± SD) at 124 days of age (± 6.9 days) was 0.208 ± 0.05 kg/day ($n = 239$). There was significant association between the lambs' ADWG at 122 days of age and lambs' APPs (Hp, ALB) serum concentrations at the second week of life and IgG concentration at first and second day of life (Table 2). A tendency of significant association was found with SAA colostrum, but not with serum SAA concentration. No significant associations were found with APPs serum concentrations at first and third week of age with ADWG at 122 days (data not shown).

3.4. Cryptosporidium and Giardia infection

From all the faecal samples ($n = 141$), 23 tested positive for both *Cryptosporidium* and *Giardia* during the first 3 weeks of life (Fig. 2) (Table 3). The median OPG in a positive *Cryptosporidium* and *Giardia* faecal sample was 97,500 and 162,000, with min-max of 500–23,021,500 and 500–9,914,500, respectively. GLOB concentrations and diagnosing a *Cryptosporidium*-positive faecal sample were negatively associated at the second ($P = .033$) and third weeks ($P < .001$). At the second and third weeks of life, *Cryptosporidium* was detected in 20.0% (13/65) and 42.1% (8/19) of faecal samples, respectively (Fig. 2). Higher serum GGT activity at the first week of age was positively associated with *Cryptosporidium* infection at the third week of age (estimate = 419 IU/l, intercept = 3298 IU/l, $n = 19$, $P < .001$). *Giardia* infection had no significant associations with GLOB concentrations and GGT activity (data not shown). No significant associations between SAA, HP and ALB concentrations and parasites infections were found (data not shown).

4. Discussion

4.1. Colostrum intake, acute phase proteins, and growth rates

GGT activity in lamb serum has been demonstrated to be a strong indicator for assessing the successful transfer of passive immunity (Maden et al., 2003; Britti et al., 2005). We saw significant positive correlation between GGT and GLOB in the first week of life but not in the second or third week (Fig. 3), suggesting the lambs' own antibody production picks-up as passive immunity gradually declines. This was further supported by a significant positive correlation between GLOB and Hp concentrations at the second ($r = 0.20$) and third ($r = 0.32$) weeks indicating the triggering of APR with concurrent immunoglobulin production increase. The latter was likely a response to environmental factors.

Mean GGT activity (1120 IU/l; $n = 28$) on the first day of life in our study was considerably lower than reported by other authors (mean values: 3605 to 4077 IU/l) (Maden et al., 2003; Britti et al., 2005). On the other hand, it was comparable to the study by Kerslake et al. (2009) where lambs whose ewes did not receive concentrated feed had significantly lower mean GGT activity (1451 IU/l). In addition, ewes during the current study did not have access to fresh pastures as the vegetative period had not yet started. Another contributing factor to lower GGT activity might have been the management system, as it was a certified organic farm where the ewes did not have access to concentrated feed (except grain) due to stricter regulations.

Using GGT activity as a proxy for assessing the transfer of APPs with

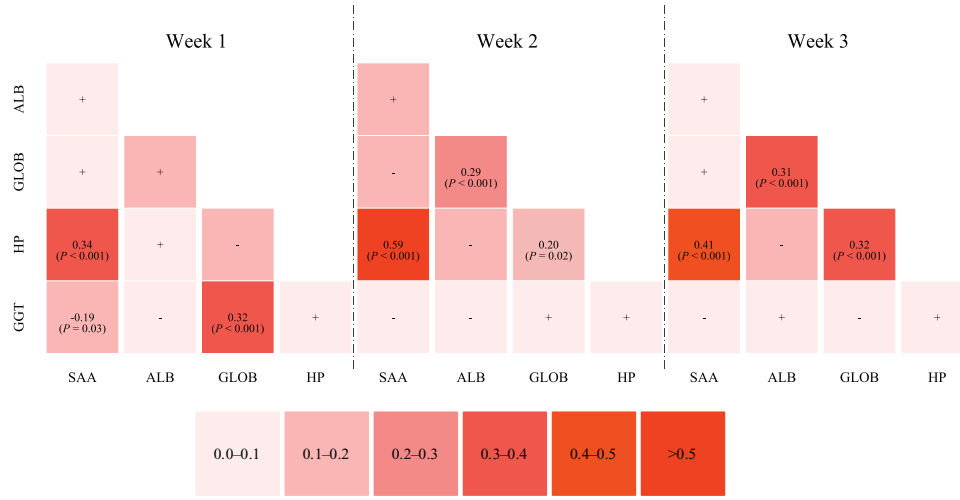

Fig. 3. Heat map matrix of partial correlations between serum amyloid A (SAA), haptoglobin (Hp), globulin (GLOB), and γ-glutamyltransferase (GGT) by week of age. The strength of the correlation is indicated by the colour tone. Only significant correlation coefficients are presented on corresponding squares with the *P* value (Bonferroni corrected). The age of lamb was added to the calculations as a possible confounder, but was not presented in the figure. The direction of correlation is indicated in the figure by + (positive) or - (negative).

Table 2
Results of the mixed linear regression model for average daily weight gain (ADWG) (kg/day) at 122 ± 5 days of age (n = 53) associated with acute phase proteins (APPs) – Serum amyloid A (SAA), haptoglobin (Hp) and albumin (ALB) at second week of life. Immunoglobulin G (IgG) serum concentration at first and second day of life and SAA colostrum concentration were added to the model for indirectly controlling effect of colostrum.

Variable (n = no. of observations)	Estimate	Confidence interval 95%	*P*-value
IgG (mg/dl)[a]	0.00002	0.000002; 0.00004	0.030
SAA log (mg/l)[1,b]	−0.0075	−0.018; 0.003	0.163
SAA colostrum (mg/l)	−0.0003	−0.001; 0.00001	0.068
Hp (g/l)[b]	0.0112	0.002; 0.020	0.018
ALB (g/l) [b]	0.0057	0.001: 0.011	0.025
TP (g/l) [b]	−0.0025	−0.005; −0.0001	0.040
Singletons (n = 19)	ref.	–	–
Twins (n = 34)	−0.0639	−0.090; −0.038	> 0.001
Ewe age 1 y (n = 3)	ref.	–	–
Ewe age 2 y (n = 10)	0.0410	−0.011; 0.093	0.124
Ewe age 3–4 y (n = 15)	0.0209	−0.037; 0.079	0.479
Ewe age ≥ 4 y (n = 25)	0.0622	0.010; 0.115	0.020
Female (n = 21)	ref.	–	–
Male (n = 32)	−0.0037	−0.026; 0.019	0.750
Intercept	0.1693	0.075; 0.263	> 0.001

TP = total protein, GGT = gamma-glutamyltransferase.
[1] Log transformed in order to reduce skewness.
[a] Calculated from GGT activity at age 1 and 2 days by formula: ln(IgG) (mg/dl) = 2.251 + 0.700 × ln[GGT] (IU/l) + 0.378 lamb age (days), where age 1 (day) = 0 and age 2 (days) = 1 (Britti et al., 2005).
[b] Measured at second week of life (age 8–14 d).

Table 3
Faecal samples collected.

Week	*Cryptosporidium* positive faecal samples (n)	*Giardia* positive faecal samples (n)	Total no. of faecal samples (n)
1	1	4	57
2	14	15	65
3	8	4	19
Total	23	23	141

colostrum did not provide significant results. This might be because the colostrum intake quantity and quality was not directly related to the lambs' serum APPs increase that was observed during the first 2 weeks of life (Fig. 1). Nevertheless, we found significant negative correlation (r = −0.19) between SAA and GGT in the first week (Fig. 3), but this correlation could be explained by opposite direction time-dependent changes of concentration and activity (Britti et al., 2005; Dinler et al., 2017). In addition, concentrations of APPs in the colostrum were likely strongly associated with the health status of the ewe rather than the colostrum's IgG concentration (Eckersall et al., 2008; Miglio et al., 2013).

Although we found a significant association between concentrations of SAA in the colostrum and the lamb serum, which has been shown previously (Peetsalu et al., 2013), there was a lack of information about whether it transfers to lamb serum and if and how it impacts the offspring's immune system. The source of SAA in the colostrum could be the udder itself whether it is healthy (Scumaci et al., 2015) or has a degraded health status (for example, subclinical mastitis) (Miglio et al., 2013). The mean concentration ± SD of SAA in the colostrum in the current study was 21 ± 53 mg/l, lower than reported by McDonald et al. (2001), 62 mg/l with SEM ± 64 mg/l (n = 6). We speculate that the considerable difference between these 2 studies could be attributed to differences in the study group sizes, breeds, or feeding. There was likely no direct transfer of SAA from the colostrum in large quantities to the lamb serum, as it has been found in dairy calves that SAA isoforms differ between those 2 sources (Orro et al., 2008). Nevertheless, there was a significant correlation between the lambs' Hp and SAA serum concentrations during the first 3 weeks of life (Fig. 3), indicating that their increase was induced by common sources, which we speculate might have started with the colostrum's low-abundance proteins, including cytokines and environmental factors (for example, pathogens and commensal microbiota).

In this study, we did not directly measure Hp in the colostrum and we did not see a significant increase in serum Hp in the first days after birth. Another reason of quick increase in Hp serum concentration after birth is deprivation from colostrum and lack of calories intake (Hernández-Castellano et al., 2015).SAA and Hp concentration changes in the first weeks of life (Fig. 1) were similar to what has been reported

before (Eckersall et al., 2008).

In a previous study, we found negative association with SAA concentration at second week of life and ADWG in first months of life (Peetsalu et al., 2013). Interestingly, in current study the association was not significant (Table 2), this might be the result of addition colostrum indicators (IgG and colostrum SAA) to the model. The other APPs (Hp and ALB) had a significant positive association to ADWG, which still suggests the importance of immune system development at that specific time (second week of life). This study could have potentially benefited from directly controlling colostrum quality - especially the colostral specific gravity, but this indicator has not been found to correlate strongly IgG concentration (Morin et al., 2001) and does not have significant association with ADWG at 120 days (Karakuş and Atmaca, 2016). Still, controlling colostrum as a confounder was considered important, as ewe's nutrition has been found to influence the APPs abundance in lambs serum during first weeks of life (Eckersall et al., 2008). The variables linked to colostrum had mixed results in the model, as it was found that IgG in lambs' serum in first days of life did have significant positive effect on ADWG, while colostral SAA only had tendency of significance. This makes it difficult to conclude if discussed colostrum linked indicators should be included in potential future studies. We speculate that the animals with higher growth rates had more immune system activation at the second week, which in the long run helped them to become less susceptible to infectious agents.

Colostrum's effects on neonate APPs concentrations and factors influencing APR in neonates need further studies, as these may give clues as to how neonatal animals' immune systems adapt to the outside environment, this is especially important because it can benefit the sheep producers in more precise herd health management thus providing future strategies on how lambs could survive early infections with minimal repercussions.

4.2. Cryptosporidium and Giardia infection

Both *Cryptosporidium* and *Giardia* infection has been diagnosed in Estonian sheep herds, but the overall prevalence remains at the moment unknown (Lassen et al., 2013). According to O'Handley and Olson (2006) the true prevalence of *Giardia* infection among sheep herds could be up to 100%, which eventually leads to infection of all the animals in the herd, but there are no data available to translate this into a high number of clinical cases. The prevalence of these parasites in the current study was difficult to assess, as we were unable to obtain faecal samples from each lamb without causing excessive stress to the animals.

In the current study, GLOB levels decreased in the second and third weeks of life, which correlated with an increase in *Cryptosporidium* infections. The latter effect with increased number of oocysts has been previously observed in ruminants (O'Handley and Olson, 2006). Some authors have suggested on colostrum's protecting effect against the parasite (Martín-Gómez et al., 2006) while others have not seen any effect (Ortega-Mora et al., 1993). Colostrum has been shown to have an alleviating effect in cases of clinical cryptosporidiosis (Martín-Gómez et al., 2005). Another factor suggesting that colostrum quality (GLOB concentration) and quantity played key roles in delaying infection was the significant association of GGT activity at the first week of life and *Cryptosporidium* infection at the third week. We hypothesise that higher quality colostrum also might have delayed the formation of a proper immune response against the parasite, thus leaving these lambs more vulnerable at the third week of life. It has been demonstrated that there is a strong correlation between consumed colostrum quantity (thus a higher quantity of anti-cryptosporidium antibodies) and GGT activity in lamb serum (Britti et al., 2005).

APR response (increase of SAA, Hp and white blood cell count) to *C. parvum* has been observed in experimentally infected lambs (Dinler et al., 2017). It could be speculated, that the most likely route of the lambs' infection with *Cryptosporidium* and *Giardia* in the current study must have been via the ewes, as it has been demonstrated that shedding increases for both parasites from ewes to the environment around the time of parturition (Xiao et al., 1994).

Lack of *Giardia* detection and thus finding no associations with other parameters might be explained by study period when faecal samples were collected (0–21 days of age). While first *Giardia* infection was detected at age of 3 days it has been previously been reported that on average infection in lambs starts at 23 days and peaks around 37 days of age (Taylor et al., 1993). The humoral response to *Giardia* infection can take weeks to develop in lambs, but what makes it even more difficult to detect is that IgM levels do not rise significantly (compared to no infection) and IgG increases significantly in only 30% of animals (and even then 5 to 11 weeks after infection) (Yanke et al., 1998). In contrast, neonatal *Cryptosporidium* infection induces a significant increase in antibodies (IgG and IgM) and APPs (SAA and Hp) production, which peak around 25–30 and 2–6 days post-infection respectively (Ortega-Mora et al., 1993; Dinler et al., 2017). In the 3-week observation window, we did not see that *Giardia* would have had significant associations with APP or GLOB concentrations.

5. Conclusions

This study described serum concentrations of APPs (SAA, HP, ALB), TP and GLOB during first 3 weeks of age in organically grown lambs in relation to colostrum SAA, IgG, weight gain, GGT activity and parasitic protozoan infections. The results suggest a positive association between colostral SAA and lamb serum SAA and Hp concentrations in the first days of life. ADWG at 122 days of life was positively associated with HP and ALB concentrations at the second week of life, while controlling for colostrum SAA and lamb serum IgG concentrations as possible confounders, suggesting and emphasising the importance that immune system development in that time period has on future performance.

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IV

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Effect of colostrum on the acute-phase response in neonatal dairy calves

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ABSTRACT

The core part of the mammal innate immune system is the acute-phase response (APR), during which acute-phase proteins (APP) are synthesized. Colostrum contains immunomodulating factors such as proinflammatory cytokines and APP in large quantities. We looked at proinflammatory cytokines [IL-1β, IL-6, and tumor necrosis factor-α (TNF-α)] and APP [serum amyloid A (SAA) and haptoglobin (Hp)] in colostrum and in calves' serum. The aim of this study was to evaluate the effects of colostrum on the calves' systemic APR and the associations of the calves' serum APR with short- and long-term weight gain (at the age of 1, 3, and 9 mo). A total of 143 female dairy calves were studied during their first 3 wk of life. The calves were separated from their mothers immediately after birth and bottle-fed 3 L of quality-controlled colostrum once within 2 h after birth. Serum samples were collected once a week during the first 3 wk of life (a total of 1–3 samples per calf). Mean sampling age (±standard deviation) was 4.3 (±2.0) d in the first week, 11.0 (±2.0) d in the second week, and 18.0 (±2.0) d in the third week. Linear regression models were used to study associations of colostrum APP and cytokine concentration with serum APR markers and for studying associations of colostrum and serum APR markers with calves' average daily weight gain (ADWG). Mixed linear regression models were used to compare serum concentrations of APR markers by study weeks. The colostrum IL-6 concentrations were positively associated with serum IL-6 in the first 3 wk of life. Colostrum IL-1β was positively associated with calves' serum IL-1β during the first week of life, and colostrum TNF-α was positively associated with calves' serum TNF-α during the first 2 wk of life. Serum IL-1β concentrations differed over the 3 wk, being the highest during the first week and the lowest during the second week. For IL-6, the concentration during the first week was the highest, and for TNF-α, a steady decline in the concentration was observed. Serum SAA concentrations were elevated during the first 2 wk of life and subsequently declined during the third week. Albumin concentrations were lowest in the first week, whereas Hp concentrations were highest during the second week. Serum concentrations of SAA, Hp, IL-6, and TNF-α during the second week were negatively associated with ADWG at 9 mo of age. The SAA concentrations during the third week of age had a negative association with 9-mo ADWG. Serum Hp concentrations in the third week were negatively associated with 3-mo ADWG. The results of our study suggest that colostrum cytokines influence calf serum cytokine concentrations. Thus, they influence the newborn calves' adaptation to the environment and the development of their immune system. Factors that activate an APR during the second and third week of life have a long-term influence on calves' development.
Key words: colostrum, proinflammatory cytokines, neonatal calf, APR

INTRODUCTION

The neonatal period of calves is considered to be a crucial time for the animal to adapt to the environment, and the development of the immune system is part of that process. At the time of birth, dairy calves, like other ruminants, lack placental immunoglobulins and hence also lack adequate acquired immunity. Therefore, the intake of high-quality colostrum is critical for the survival of neonatal calves. In addition to providing immunoglobulin and essential nutrients for the calf's energy needs, cow colostrum is rich in leukocytes, growth factors, hormones, enzymes, and other immunologically bioactive molecules, meaning that colostrum also has other immunomodulatory properties (Blum and Hammon, 2000; Barrington and Parish, 2001). Colostrum contains a variety of components associated with innate immunity, such as various peptides, small proteins, and enzymes with innate immune function (Vorbach et al., 2006). Colostrum ingestion supports the functional and morphological development of calves (Blum and Hammon, 2000). Therefore, deprivation of colostrum leads to poor weight gain, morbidity, and even increased

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mortality after birth (Nocek et al., 1984). During the last decade, the importance of specific colostrum proteins has gained attention, yet the exact bioactive roles of different proteins in neonate ruminants still need clarification (Hernández-Castellano et al., 2015). The understanding of innate immunity functions in neonatal calves and their role in resistance to infections and maintaining homeostasis cannot be underestimated.

Part of the innate immune system is the acute-phase response (**APR**), a first-line defense mechanism of the organism, initiated by infection, injury, tissue damage, stress, immunological disorders, or neoplasia (Baumann and Gauldie, 1994; Gruys et al., 2005). At the site of the inflammatory stimulus, monocytes and macrophages are the predominant cells that elicit the APR by releasing proinflammatory cytokines, of which tumor necrosis factor α (**TNF-α**) and IL-1β and IL-6 are the predominant ones (Baumann and Gauldie, 1994; Ceciliani et al., 2012). One of the main functions of these proinflammatory cytokines is the activation of a large group of serum proteins, known as acute-phase proteins (**APP**), which initiate an effective innate immune response. The APP are mainly produced in the liver and in lesser quantities in local tissues to restore the homeostasis of the body (Baumann and Gauldie, 1994; Koj, 1996). As the APR process is part of the first-line antibody-independent defense mechanism, it is crucial for neonate animals who are immunologically naïve to invading pathogens.

The APP has been commonly used in medicine as a quantitative sensitive diagnostic and prognostic biomarker (Schrödl et al., 2016). In cattle, the 2 major positive APP are serum amyloid A (**SAA**) and haptoglobin (**Hp**), and their concentrations increase notably during APR (Ceciliani et al., 2012). Albumin and transferrin are negative APP in cattle, whose concentrations decrease during APR (Petersen et al., 2004).

The SAA and Hp belong to the evolutionarily conserved set of APP (Uhlar and Whitehead, 1999; Wang et al., 2001). Serum amyloid A has many roles, mainly the binding of cholesterol; immunomodulatory functions via pro- and anti-inflammatory activities, such as chemotactic recruitment of inflammatory cells to sites of inflammation; and opsonization (Liang and Sipe, 1995; Uhlar and Whitehead, 1999; Shah et al., 2006; Ceciliani et al., 2012). Haptoglobin's main function is to bind free hemoglobin from erythrocytes, providing antioxidant and antimicrobial activity by decreasing the available iron to microbes (Dobryszycka, 1997; Tóthová et al., 2014).

The mammary gland epithelium expresses SAA and Hp. In bovine colostrum, highly elevated levels of extrahepatically secreted mammary-associated SAA isoform 3 (SAA3) have been demonstrated, especially during the first few days after calving (McDonald et al., 2001; Thomas et al., 2016). Moderately elevated Hp concentrations are detectable in cow colostrum, which then decreases on the fourth day postcalving (Thomas et al., 2016). The role of elevated SAA levels in the mammary gland is associated with the cow's health state, as SAA is involved in mammary gland defense against pathogens (Molenaar et al., 2009). Colostrum whey also contains high quantities of the cytokines IL-1β, IL-6, TNF-α, and IFN-γ; their concentrations are significantly higher in colostrum than in mature milk (Hagiwara et al., 2000).

Colostrum SAA has been assumed to have a protective role in offspring by modulating gastrointestinal immunity (Molenaar et al., 2009). The SAA induces mucin 2 gene expression in the gastrointestinal tract (Shigemura et al., 2014). This is a major component of the mucus layer in the intestines that separates bacteria from the epithelium and protects the gastrointestinal tract of newborns from pathogen colonization. It has been demonstrated that locally expressed SAA in intestinal epithelial cells has a role in intestinal immune homeostasis, as SAA reduces bacterial growth in vitro (Eckhardt et al., 2010). Therefore, colostrum SAA probably has a balancing effect during microbiota colonization at and after birth. The immunostimulatory effect of orally administered IL-1β in newborn calves has been found to affect the activation of neutrophils and proliferation of T cells (Hagiwara et al., 2001). The colostrum proinflammatory cytokines (IL-1β, TNF-α, and IFN-γ) improve the mitogenic response of peripheral blood mononuclear cells (Yamanaka et al., 2003). Thus, colostrum cytokines contribute to the maturation of neonatal immune functions (Yamanaka et al., 2003).

The concentrations of different APP change during the first few weeks of ruminant life and are termed age-related changes in APP. In lambs and goat kids, SAA concentrations increase during the first week of life, start to decrease during the second week, and then stabilize at the end of the third week (Eckersall et al., 2008; Ulutas et al., 2017; Niine et al., 2018a; Peetsalu et al., 2019; Dinler et al., 2020). It has been suggested that colostrum intake influences the APP concentration in calves, as their SAA concentrations were found to be low before colostrum consumption and then increased during the first 24 h after birth (Orro et al., 2008; Tóthová et al., 2015). Nevertheless, no direct transfer of SAA isoforms in calves was found in the study of Orro et al. (2008), who measured colostrum and calf serum SAA isoforms. A proteomic study in sheep demonstrated colostrum led to higher SAA concentrations in lambs (Hernández-Castellano et al., 2014). In our previous study, we found positive associations between colostrum SAA and lamb serum SAA at 1 to 5 d of age,

which indicates the important effect of ewe's colostrum on lambs (Peetsalu et al., 2019).

In the same study, a negative association between SAA concentration during the second week of life and weight gain at 3 to 4 mo of age was evident. This suggests that the second week of life is important, as environmental factors and diseases at that time have a long-term effect on animal health, whereas the colostrum effect has already diminished. Similar negative associations between second-week SAA concentrations and future weight gain have also been found in reindeer, beef calves, and dairy calves reared for meat (Orro et al., 2006; Seppä-Lassila et al., 2017, 2018).

Based on our previous results, colostrum influence on APR of offspring was not proven. We hypothesized that proinflammatory cytokines and APP in colostrum are associated with the systemic APR of neonatal calves, and thus indirectly influence the offspring's immune system. In addition, we evaluated the changes in calves' APR marker concentrations during the neonatal period and their possible associations with weight gain, measured at the ages of 1, 3, and 9 mo.

MATERIALS AND METHODS

Sampling took place in 2015 on a dairy farm in Estonia, housing approximately 1,800 cows at the time, and the average milk production per cow was 10,000 kg (Eesti Põllumajandusloomade Jõudluskontrolli AS, 2015). The present study is part of a large-scale study conducted to describe the inflammatory response during an acute *Cryptosporidium parvum* outbreak in female dairy calves previously described by Niine et al. (2018b). For this study, we used fecal and serum samples from the first 3 wk, in addition to colostrum samples. Sample collection was conducted based on ethical permission issued by the Ethical Committee of Animal Experiments in the Estonian Ministry of Agriculture (no. 7.2-11/2).

Animals

All Holstein-Friesian female calves (n = 143) included in the present study were born between January 21 and March 16, 2015. The calves were separated from their mothers immediately after birth and bottle-fed 3 L of quality-controlled colostrum once within 2 h after birth (median ± SD; 61 ± 30 min). The colostrum given to the calves was collected from the dam and the quality examined visually and with a colostrum densimeter (Jørgen Kruuse A/S). In 2 cases colostrum quality was poor (specific gravity <1,035, or total protein <50 g/L), so deep-frozen colostrum from another cow was used. Obstetric aid at birth were recorded as spontaneous delivery (n = 85), aid by one person (n = 46), and aid by 2 persons (n = 12). Seventy-six mothers of the calves were primiparous (first time calving) and 67 multiparous (second time 28, third time 22, and 17 fourth time or more).

Detailed descriptions of the housing conditions, feeding, vaccinations, and prophylactic treatments of the animals are given in Niine et al. (2018b). In short, during the first 4 wk, the calves were kept in individual pens with a wooden floor and straw bedding. After that, they were moved to group pens (8–10 calves per pen) with concrete flooring, straw, and sawdust bedding. The calves were weaned at approximately 70 d of age. They were kept in a different barn after that until the age of 4 to 5 mo (on straw bedding). At the age of 6 to 7 mo, they were moved to a new barn (large stalls with concrete floors) where they remained until pregnancy.

The calves were fed 2 to 3 kg of warmed unpasteurized raw milk twice per day with free access to hay and starter feed (Prestarter, Agrovarustus OÜ) up to 15 to 17 d of age and afterward a milk powder (Josera GoldenSpezial, Josera GmbH and Co. KG) solution and free access to starter feed and hay. Around weaning time (70–80 d of age), the calves received 2 × 2 L/d of the milk powder solution. After weaning, the calves had ad libitum access to starter feed (Starter, Agrovarustus OÜ), hay, and silage.

The calves were weighed with a digital scale immediately after birth, at approximately 1 mo of age (mean ± SD; 29.6 ± 4.5 d) and at 9 mo of age (264 ± 6.5 d). At 3 mo of age (101.4 ± 10.6 d), the calves' weight was estimated using a measuring tape (ANImeter, Albert Kerbl GmbH) because the digital scale was not available. Average daily weight gain (**ADWG**, g/d) was calculated for a period from birth to 1 mo of age (n = 122), from birth to 3 mo of age (n = 120), and from birth to 9 mo of age (n = 121).

The calves were vaccinated against parainfluenza virus type 3 and bovine respiratory syncytial virus (Rispoval, Zoetis Belgium SA) on their second day of life and against bovine herpesvirus-1 (Hiprabovis, Laboratorios HIPRA, S.A.) at 3 mo of age. Toltrazuril (Cevazuril, Ceva Santé Animale) was used once at the age of 25 to 65 d as prophylactic treatment against *Eimeria* spp. infection. During the acute outbreak of cryptosporidiosis, halofuginone lactate (**HL**; Halocur, Intervet International B.V.) was used. The prophylactic HL mass treatment for controlling the outbreak of diarrhea caused by *Cryptosporidium* spp. was started on February 17 and ended on March 22, 2015 (the study period was from January 21 to March 16, 2015). All calves younger than 14 d were treated. In all, 110 calves were treated an average of 6 times (range 1–9 d).

Table 1. *Cryptosporidium* oocyst count (oocysts per gram of feces; OPG) of fecal samples in 143 dairy calves sampled during the first 3 wk of life (1–3 samples per calf)

Variable	Statistics	Fecal sample (d of age)		
		1–7 (n = 103)	8–14 (n = 112)	15–22 (n = 114)
Cryptosporidium (OPG)	Positive samples, n	17	59	78
	Median (Minimum–maximum)	1,871 (69–866,119)	475,293 (208–5,764,653)	418,237 (69–10,602,130)
Cryptosporidium group[1]	Negative, n	86	53	36
	Low level, n	8	29	40
	High level, n	9	30	38

[1]Positive samples were divided into low and high oocyst count categories using median values in the same week.

Based on the HL treatment regimen, the calves were retrospectively divided into 3 groups: (1) no treatment, (2) incorrect treatment (daily treatment started >48 h after birth and lasted <7 d), and (3) correct treatment (according to the manufacturer's instructions; daily treatment started <48 h after birth and lasted ≥7 d). Detailed descriptions of HL treatments and *Cryptosporidium* spp. infection by individual calves are published in the paper by Niine et al. (2018b). No other clinical diseases were diagnosed or treated during the study period.

Sample Collection

Serum and fecal samples collected from 143 female calves once a week during the first 3 wk of life (1–3 samples per calf) were used in the present study. Calves that did not have fecal matter in the rectum at the time of sampling were not included in the models of that study week (their serum sample of that day was also excluded). To avoid further stress, the calves were not caught and restrained a second time on that day. Thus, not every calf included in the study has samples from all 3 wk available. In total, 103 samples were available from the first week, 112 from the second, and 114 from the third, making for an overall sample size of 329. Mean sampling age (±SD) was 4.3 (±2.0) d in the first week, 11.0 (±2.0) d in the second week, and 18.0 (±2.0) d in the third week.

Blood samples were collected into sterile evacuated test tubes with an 18-G sterile needle from the jugular vein. Samples were centrifuged (1,800 × *g*, 10 min) and the serum was separated and stored in aliquots at −20°C until further analysis. A sample of the colostrum was collected before it was provided to the newborn. These were collected into sterile 10-mL vials and stored in aliquots at −20°C until further analysis. Before the laboratory analyses, the colostrum samples were skimmed by centrifugation (7,840 × *g*, 10 min, 4°C) followed by removal of the fat layer.

Laboratory Analysis

Fecal samples were prepared and analyzed for the detection of *Cryptosporidium* and *Giardia* approximate oocysts or cyst counts (oocysts per gram of feces, **OPG**; and cysts per gram of feces, **CPG**) as modified by Niine et al. (2018b) using an immunofluorescence method. For staining, fluorescein isothiocyanate (**FITC**)-conjugated anti-*Cryptosporidium* and anti-*Giardia* monoclonal antibodies (Crypto/Giardia Cel, Cellabs Pty Ltd.) were used. For statistical analysis, the calves were divided into 3 groups based on the *Cryptosporidium* oocyst count each week (negative, no oocysts; low oocyst level, oocyst count below the median value; and high oocyst level, oocyst count above the median value; Table 1). Because only 16 fecal samples were positive for *Giardia* and *Giardia* infection did not have an association with the APR (Niine et al., 2018b), *Giardia* data were not used in the present study (data not shown).

The concentrations of SAA in the serum and colostrum samples were measured using a commercial ELISA kit (Phase BE kit, Tridelta Development Ltd.), and Hp was measured using a method defined by Makimura and Suzuki (1982), with minor modifications, namely using tetramethylbenzine (60.0 mg/L) as the substrate and using microtitration plates (Alsemgeest et al., 1994). The detection limits for SAA and Hp were 0.3 and 60.0 mg/L, respectively. Cytokine concentrations in the serum and colostrum samples were determined using bovine IL-1β, IL-6, and TNF-α ELISA kits (Cusabio Biotech) according to the manufacturer's instructions. The detection limits for IL-1β, IL-6, and TNF-α were 15.6, 2.5, and 50.0 ng/L, respectively. The IgG concentrations were measured using a commercial ELISA kit (BIO K 165/2 kit, Bio-X Diagnostics S.A.). The albumin concentration in the serum was determined by using a commercial photometric colorimetric method based on bromocresol green dye binding (Accent-200 Albumin II Gen, PZ Cormay S.A.).

Statistical Analysis

Pearson correlation with Sidak-corrected *P*-values for pairwise comparisons was used to study the correlations between the measured variables in the colostrum and serum samples.

To compare the serum inflammatory markers (APP and cytokines) and IgG concentration differences by study week, linear mixed models (for SAA, Hp, albumin, IgG, Il-6, IL-1β, and TNF-α) and a mixed Tobit model (for IL-1β) were used. The Tobit regression model was chosen because >60% of the sample IL-1β concentrations were under the detection limit of the assay (15.6 ng/L), violating the regression model assumption of a normal distribution of the response variable. In the Tobit regression, all cases falling above (or below) a specified threshold value are censored, although these cases remain in the analysis (Long, 1997). Histograms of response variables were used to evaluate normal distribution. Except for albumin, all response variables had to be logarithmically transformed to achieve normal distribution.

The calf was included as a random intercept and an isotropic spatial exponential covariance structure was used to model the correlation between repeated samples from the same calf because it had the best fit to the data (lowest Akaike information criterion of the models). Sample week was included as a categorical variable (1, 2, or 3) and maternal parity as a binary variable (primiparous or multiparous). Because *Cryptosporidium* infection influences inflammatory markers, *Cryptosporidium* oocyst level group and HL treatment group were included in these models along with obstetric aid (spontaneous delivery, help by 1 person, or help by 2 persons) because dystocia can trigger APR in calves. A total of 103 samples were available from the first week, 112 from the second, and 114 from the third, making for an overall sample size of 329.

To investigate associations between the colostrum and serum variables by study week, multiple linear regression models were used. In mixed linear models that included all samples, interaction terms for all predictor variables by study week needed to be included as well, resulting in very complicated models. Thus, separate models for each study week were used for every response variable. The APR markers (SAA, Hp, albumin, IL-6, and TNF-α) and IgG serum concentrations by study week were used as response variables in those models. Tobit regression models were used for IL-1β. Colostrum APP, cytokine, and IgG concentrations were included as covariates. All models initially included age at sampling (d) and the time from birth to colostrum ingestion (min) as covariates, and maternal parity as the categorical explanatory variable. The final models were produced by backward elimination of the variables from the initial models. The linear relationship between response variable and covariates was checked and confounders were controlled (change of the coefficient by more than 10% after variable elimination). Possible cofounders (maternal parity) were identified by use of a causal diagram (Figure 1). Colostrum variables with a high correlation (IL-6 and TNF-α) were included separately to avoid collinearity.

To investigate associations between the calves' ADWG and the concentrations of IgG, SAA, Hp, IL-6, TNF-α, and IL-1β in the colostrum, 3 separate linear regression models were used. The ADWG at 1 (n = 121), 3 (n = 119), and 9 mo (n = 120) of age was used as a response variable in each of the models. Colostrum concentrations of SAA, Hp, albumin, IgG, IL-6, TNF-α, and IL-1β, age at weighing, and birth weight were used as covariates. Because HL treatment

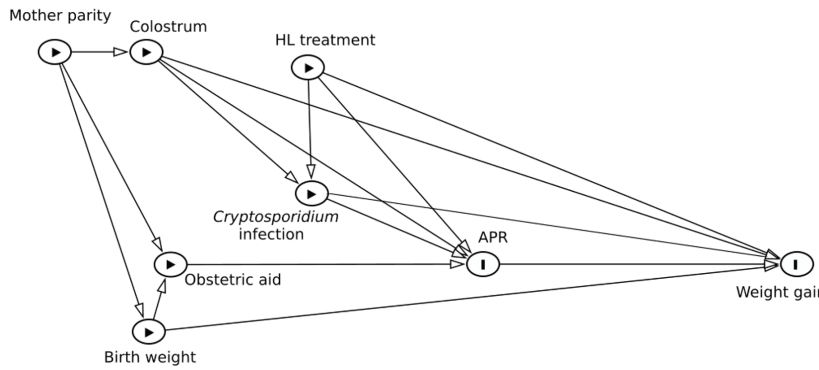

Figure 1. Causal diagram describing the studied variables relationship with calves' neonatal acute-phase response (APR) and future weight gain. HL = halofuginone lactate; obstetric aid = spontaneous, aid by 1 or 2 persons.

group was associated with weight gain, it was included in these models as an independent variable. Maternal parity was included as the binary categorical variable. Colostrum variables with a high correlation (IL-6 and TNF-α) were included separately to avoid collinearity.

Similar models were used to investigate associations between ADWG at 1, 3, and 9 mo of age and the calves' serum concentrations of SAA, Hp, albumin, IgG, IL-6, TNF-α, and IL-1β by study week. Sample sizes by week for ADWG models at 1 mo were n = 86, n = 102, and n = 108, respectively. Sample sizes by week for ADWG models at 3 mo were n = 83, n = 98, and n = 104, respectively. Sample sizes by week for ADWG models at 9 mo were n = 86, n = 100, and n = 105, respectively. Serum concentrations of the markers, age at sampling, age at weighing, and birth weight were used as covariates. The HL treatment group, *Cryptosporidium* oocyst level group at the time of sampling, and maternal parity were included as categorical explanatory variables. A stepwise backward elimination procedure was used for the final models. The linear relationships between response variable and covariates were checked, and interactions and possible confounders according to the causal diagram (Figure 1) were controlled (change of the coefficient by more than 10% after variable elimination). Highly correlated variables in the serum (SAA and Hp; IL-6 and TNF-α) were included separately in these models to avoid collinearity.

The fit of all models was controlled using normality and scatter plots of the model residuals, and results were considered as statistically significant when $P \leq 0.05$. Analyses were performed using Stata/IC 14.0 statistical software (StataCorp LP). Least squares means (**LSM**) by age at sampling were derived using the *margins* command in Stata and the delta method was used to calculate LSM standard errors. DAGitty 3.0 (http://www.dagitty.net) was used to create the causal diagram describing the relationships between studied variables.

RESULTS

Colostrum and Serum Concentrations

Concentrations of APP (SAA, Hp, albumin), cytokines (IL-6, TNF-α, IL-1β), and IgG in the colostrum and serum (by weeks of age) are presented in Table 2. Model-based LSM of the APP and cytokines are presented in Figures 2 and 3 by day of age. The colostrum concentrations of IL-6 and TNF-α were correlated (r = 0.69, $P < 0.001$; n = 143). Other inflammatory markers and IgG in the colostrum were not correlated with each other (r < 0.2). Calves' serum concentrations of IL-6 and TNF-α up to 3 wk of age were positively correlated (r = 0.79, r = 0.92, and r = 0.64, all $P < 0.001$). Serum SAA and Hp concentrations were positively correlated during the second and third weeks of life (r = 0.45 and r = 0.36, both $P < 0.001$). All other pairwise correlations between serum protein concentrations during the first 3 wk of age were nonsignificant (r < 0.3).

There were different changes in the calves' serum APP concentrations during the first 3 wk of life (Table 2 and Figure 2). *Cryptosporidium* infection and HL treatment were included in all models to control the possible influence on the proteins changing patterns. Sample size in these mixed linear regression models was 329 (102, 112, and 114 by study weeks). The SAA concentrations were elevated during the first 2 wk and then declined in the third week. The Hp concentrations were highest in the second week compared with the first and third weeks. Albumin concentrations were lowest in the first week and then increased and stabilized during the second and third weeks. Serum IgG was highest in the first week and declined constantly over the 3-wk period investigated. Age-dependent changes in the serum concentrations of the cytokines are presented in Table 2 and Figure 3. Serum concentrations of IL-6 in the first week were higher than in the second and third weeks. The TNF-α concentrations were highest during the first week of life and then declined. The IL-1β concentrations were lower during the second week than during the first and third weeks.

Effect of Colostrum and Cryptosporidium Infection

Colostrum marker concentrations had some associations with all measured protein concentrations in the calves' serum (Figures 2 and 3). Colostrum SAA had a negative association with calves' SAA serum concentration in the first week of life (coefficient for log mg/L ± SEM: −0.158 ± 0.066 log mg/L; $P = 0.018$; Figure 4). Colostrum IL-6 concentration was positively associated with serum IL-6 concentrations during all 3 wk (coefficients for ng/L ± SEM: 0.008 ± 0.002 log ng/L, 0.006 ± 0.001 log ng/L, and 0.006 ± 0.002 log ng/L; all $P < 0.001$). Colostrum TNF-α concentration was positively associated with the same cytokine serum concentrations during the first 2 wk (coefficients for ng/L ± SEM: 0.134 ± 0.025 log ng/L and 0.118 ± 0.021 log ng/L; both $P < 0.001$), and colostrum IL-1β concentration was associated with serum IL-1β during the first week (coefficient for ng/L ± SEM: 0.001 ± 0.0002 log ng/L; $P < 0.001$). Colostrum IgG concentration was positively associated with calves' IL-6 (coefficient for g/L ± SEM: 0.014 ± 0.006 log ng/L; $P = 0.031$) and TNF-α (coefficient for g/L ± SEM: 0.013 ± 0.005 log ng/L; $P = 0.008$) concentrations in the second week, and negatively with serum albumin concentration during the first week (coefficient for g/L ± SEM: −0.100

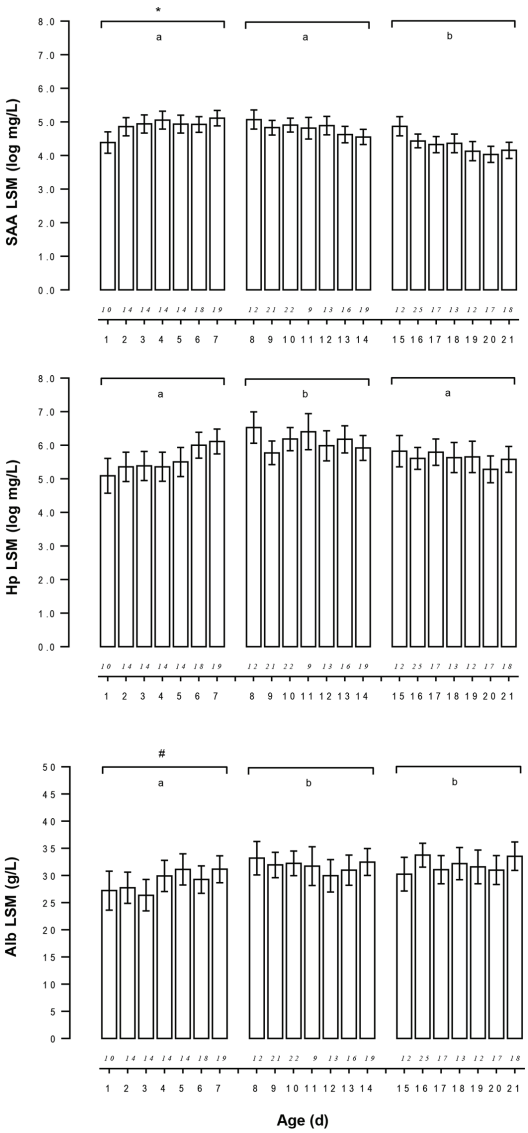

Figure 2. Least squares means (LSM) concentrations (95% CI) of calves' (n = 141; sampled 1 to 3 times during the first 3 wk after birth) blood serum amyloid A (SAA), haptoglobin (Hp), and albumin (Alb). Numbers under the columns represent the sample size for every day of age. *Negative association of colostrum SAA concentrations (P = 0.038) evaluated by linear regression models. #Negative association with colostrum IgG concentrations (P = 0.021) evaluated by linear regression models. Different letters (a,b) indicate significantly ($P <$ 0.001) different concentrations by week evaluated with mixed linear regression models.

Table 2. Colostrum and serum sample concentrations of proinflammatory cytokine and acute-phase protein concentrations and IgG in 143 dairy calves sampled once a week during the first 3 wk of life (1–3 samples per calf)

		Colostrum	Serum sample (d of age)		
Variable[1]	Statistics	(n = 143)	1–7 (n = 103)	8–14 (n = 112)	15–22 (n = 114)
SAA (mg/L)	Mean (± SD)	65.7 (47.1)	146.6 (66.5)[a]	142.3 (78.8)[a]	93.0 (61.6)[b]
	Median (minimum–maximum)	52.1 (7.5–277.0)	128.9 (22.4–347.7)	125.3 (34.0–487.9)	79.0 (13.2–371.9)
Hp (mg/L)	Mean (± SD)	190 (62)	376 (430)[a]	701 (658)[b]	451 (553)[a]
	Median (minimum–maximum)	175 (104–480)	186 (97–2,662)	408 (95–2,830)	201 (85–3,310)
Alb (g/L)	Mean (± SD)	NA	29.1 (5.8)[a]	31.7 (5.4)[b]	32.1 (5.4)[b]
IgG (g/L)	Mean (± SD)	55.1 (12.0)	16.8 (8.7)[a]	12.2 (5.7)[b]	10.7 (4.5)[c]
IL-6 (ng/L)	Mean (± SD)	55.3 (53.5)	16.5 (19.5)[a]	10.5 (14.8)[b]	11.0 (8.4)[b]
	Median (minimum–maximum)	43.2 (10.2–501.4)	9.8 (2.5–117.0)	5.9 (2.5–130.7)	10.0 (2.5–47.5)
TNF-α (ng/L)	Mean (± SD)	5,126 (3,681)	578 (486)[a]	410 (445)[b]	215 (197)[c]
	Median (minimum–maximum)	4,040 (1,290–28,620)	450 (70–2,820)	300 (50–4,200)	160 (50–1,550)
IL-1β (ng/L)	Mean (± SD)	551.0 (1,142.1)	102.8 (227.6)[a]	24.2 (33.3)[b]	49.8 (56.4)[a]
	Median (minimum–maximum)	115.9 (15.6–5,445.1)	15.6 (15.6–1,321.5)	15.6 (15.6–207.4)	34.3 (15.6–444.8)

[a–c]Different letters indicate significantly ($P < 0.001$) different concentrations by week evaluated with mixed linear regression and mixed Tobit (for IL-1β) models.

[1]SAA = serum amyloid A; Hp = haptoglobin; Alb = albumin; TNF-α = tumor necrosis factor-α; NA = not analyzed.

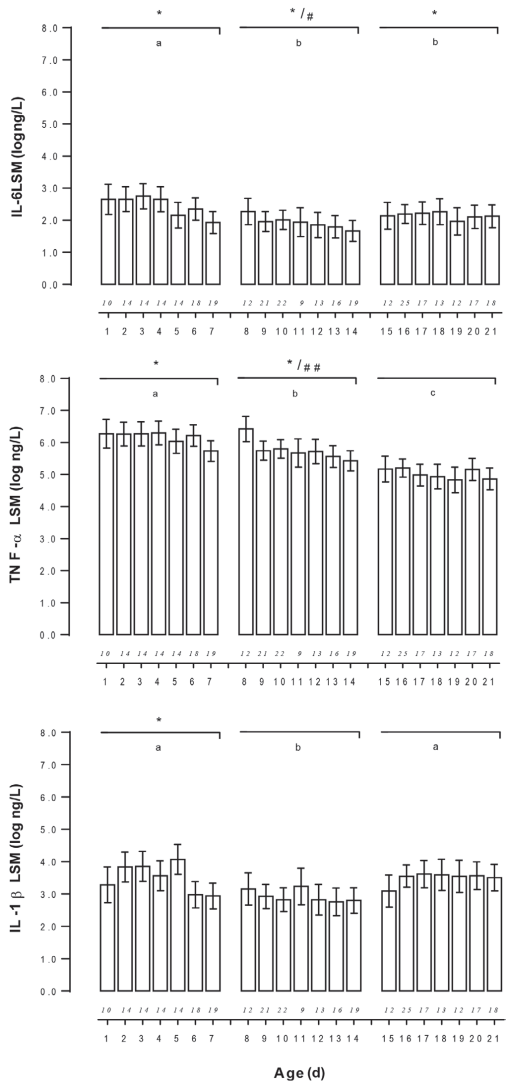

Figure 3. Least squares means (LSM) concentrations (95% CI) of calves' (n = 141; sampled 1 to 3 times during the first 3 wk after birth) blood IL-6, tumor necrosis factor (TNF)-α, and IL-1β. The numbers under the columns represent the sample size for every day of age. *Positive association with the same cytokine colostrum concentrations ($P < 0.001$) evaluated by regression (IL-6 and TNF-α) and Tobit regression (IL-1β) models. #Positive association with colostrum IgG concentrations ($P = 0.031$) evaluated by regression model. ##Positive association with colostrum IgG concentrations ($P = 0.008$) evaluated by regression model. Different letters (a–c) indicate significantly ($P < 0.001$) different concentrations by week evaluated with mixed linear regression (IL-6 and TNF-α) and mixed Tobit regression (IL-1β) models.

± 0.043 g/L; $P = 0.021$). Colostrum IgG concentration was positively associated with serum IgG concentrations during all weeks ($P < 0.001$; Table 2).

Serum SAA was higher in the high *Cryptosporidium* oocyst level group than in the *Cryptosporidium* negative group during the second week ($P = 0.002$), whereas Hp was higher in the first 2 wk of life ($P = 0.007$ and $P < 0.001$, respectively). The same association existed between high *Cryptosporidium* oocyst levels and serum IL-6 concentrations in the second and third weeks ($P = 0.038$ and $P = 0.004$) and serum TNF-α in the third week ($P = 0.005$).

The differences of the marker concentrations between the 3 study weeks could be influenced by the calves' systemic immune response to the *Cryptosporidium* infection; thus, *Cryptosporidium* oocyst level group and HL treatment group had to be controlled in all the statistical models.

Effect of Colostrum and Serum Concentrations on Weight Gain

The average birth weight of the calves was 41.2 ± 5.8 kg (ranging from 27 to 52 kg). At the age of 1 mo, ADWG (g/d) was 419.5 ± 149.2 (n = 121), at the age of 3 mo 783.8 ± 130.8 (n = 119), and at 9 mo 688.8 ± 166.9 (n = 120).

Colostrum protein concentrations did not have statistically significant associations with any age period ADWG (g/d), and the serum protein concentrations did not have associations with the calves' ADWG at 1 mo of age. Serum Hp concentration in the second week was borderline significantly negatively associated with 3-mo ADWG (coefficient for mg/L ± SEM: −0.03 ± 0.02 g/d; $P = 0.067$; n = 98) and in the third week significantly negatively associated (coefficient for mg/L ± SEM: −0.06 ± 0.03 g/d; $P = 0.025$; n = 104, Table 3). Age at the blood sampling time, HL treatment group, *Cryptosporidium* oocyst level group, and parity of the cow were included in these multiple linear regression models as possible confounders (Table 3).

Serum concentrations of SAA and IL-6 during the second week of age were negatively associated with ADWG at 9 mo of age (Table 4). A significant positive correlation was found between serum SAA and Hp (r = 0.36) and IL-6 and TNF-α (r = 0.92); thus, the associations between Hp and TNF-α of the second week of age and 9-mo ADWG (g/d) were evaluated separately from SAA and IL-6 in different models. In these separate models, serum Hp concentration (coefficient for mg/L ± SEM: −0.035 ± 0.015 g/d; $P = 0.023$) and serum TNF-α concentration (coefficient for ng/L ± SEM: −0.04 ± 0.02 g/d; $P = 0.041$) were negatively associated with 9-mo ADWG. Serum SAA concentra-

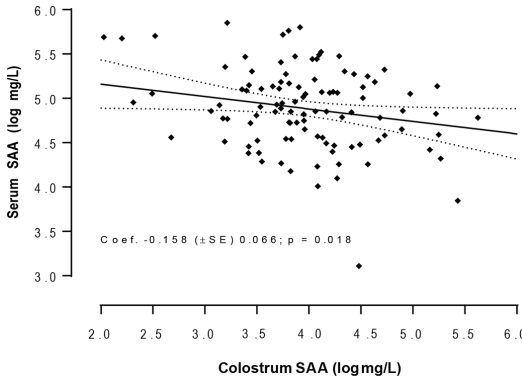

Figure 4. Negative association of calves' (n = 103) serum amyloid A (SAA) and colostrum SAA concentrations during the first week of life. The solid line represents the regression line (with 95% CI; dotted lines) evaluated with a multivariable regression model, in which calves' age at sampling and maternal parity (primiparous or multiparous) were included. Coef. = coefficient.

tion in the third week of age was negatively associated with 9-mo ADWG (coefficient for mg/L ± SEM: −0.45 ± 0.17 g/d; $P = 0.010$; n = 105). Age at the time of sampling, age at weighing, maternal parity, HL treatment group, and *Cryptosporidium* oocyst level group were included in these multiple linear regression models as confounders (Table 4).

DISCUSSION

This study found that colostrum cytokines have a direct association on the calves' immune response mainly during the first week of life and even after that (e.g., IL-6), whereas colostrum APP do not directly affect the calves' systemic innate immune response, from which we suggest that they may instead have a local protective effect in the gastrointestinal tract of young calves after ingestion.

High levels of proinflammatory cytokines (IL-6, IL-1β, and TNF-α) and APP (SAA and Hp) were measured in colostrum in this study. Higher concentrations of cytokines in colostrum compared with mature milk have been found in bovines (Hagiwara et al., 2000). The levels of the cytokines IL-6 and TNF-α were correlated in the colostrum, suggesting that they are both influenced by the same factors. These cytokines have also been found to potentiate immunological functions (Yamanaka et al., 2003). Higher concentrations of SAA and Hp in colostrum compared with mature milk have also been demonstrated before (McDonald et al., 2001; Thomas et al., 2016).

A proteomics study in sheep demonstrated the effect of colostrum on SAA concentrations in lambs' serum (Hernández-Castellano et al., 2014). In calves, it has been shown that colostrum SAA isoforms do not directly cross calf intestines (Orro et al., 2008). Our previous studies demonstrated that ewe colostrum SAA and lamb serum SAA were positively associated during the first 5 d of life, and we hypothesized that colostrum has a direct influence on the systemic innate immune response of lambs (Niine et al., 2018a; Peetsalu et al., 2019). The present study did not confirm this hypothesis, but because lambs, in contrast to calves, have ad libitum access to colostrum, the colostrum effect may be different in these 2 species. It

Table 3. Results of multivariable linear regression model for detecting association of calves' (n = 104) serum proinflammatory cytokine and acute-phase protein concentrations during the third week of life (15–21 d of age) with average daily weight gain (g/d), measured at approximately 3 mo of age (mean ± SD; 101.4 ± 10.6 d)

Variable[1]	n	Coefficient	SEM	*P*-value	Wald test *P*-value
Age at sampling (d)		−0.39	5.77	0.946[2]	
Hp serum concentration (mg/L)		−0.06	0.03	0.025	
HL treatment group[3]					0.032
No treatment	16	0			
Incorrect treatment	41	−43.17	35.76	0.230	
Correct treatment	47	−95.16	37.03	0.012	
Cryptosporidium group[4]					0.583[2]
No oocysts found	35	0			
Low oocyst level (below the median)	36	−18.16	29.31	0.537	
High oocyst level (above the median)	33	−35.45	34.14	0.302	
Parity of the cow					
Multiparous	54	0			
Primiparous	50	−44.47	23.59	0.062	
Intercept		914.06	111.81	<0.001	

[1]Hp = haptoglobin; HL = halofuginone lactate.

[2]Nonsignificant variable was retained from the model because of confounder effect.

[3]Incorrect treatment: daily treatment started >48 h after birth and lasted <7 d. Correct treatment: daily treatment started <48 h after birth and lasted ≥7 d.

[4]Positive samples were divided into low and high oocyst count categories using median values of the same week.

is also possible that in sheep, colostrum SAA directly transfers to the lambs.

In neonatal pigs, it has been shown that colostrum Hp is transferred to piglets and the endogenous Hp synthesis is also stimulated by colostrum (Hiss-Pesch et al., 2011). Studies on the relationship between colostrum consumption of calves and their serum Hp concentrations are inconclusive. Dairy calves that received milk-based formula had higher Hp serum concentrations than colostrum-fed calves (Sadri et al., 2020), a tendency that was also observed in colostrum-deprived lambs (Hernández-Castellano et al., 2015). Possible explanations include a lack of immunoglobulin and a stress reaction resulting in a lack of energy intake or higher inflammatory stimulus because of weaker passive immunity transfer. In contrast, in another study, higher plasma concentrations of Hp were found in the group that received colostrum than in the group that received milk-based formula (Liermann et al., 2020). However, the present study showed no direct positive associations of colostrum SAA or Hp with the calves' serum levels of the same parameters. We hypothesize that colostrum APP have local effects on the gastrointestinal tract or systemic effects through their proinflammatory properties. Because SAA has direct opsonizing properties, it also acts locally by stabilizing the intestinal environment (Reigstad et al., 2009). Serum amyloid A is assumed to respond to microbial colonization with the suppression of systemic neutrophil activation and bactericidal activity (Murdoch et al., 2019). A study of piglet diarrhea indicated that colostrum SAA has a beneficial effect on newborns, as litters from sows with higher colostrum SAA concentrations showed less diarrhea in the first week of life (Hasan et al., 2019). In humans, colostrum contains SAA1, which is suggested to have a role in the development of newborn intestinal defense maturation and immune responses (Sack et al., 2018). We found that colostrum SAA and calf serum SAA had a negative association during the first week of age, suggesting that the calves' own inflammatory response was sooner activated when less protection from colostrum SAA was available. This further emphasizes the protective role of colostrum SAA shortly after birth (approximate 1-wk period).

In this study, positive associations between the colostrum cytokines IL-1β, TNF-α, and IL-6 and the same cytokines in calves' serum [IL-1β (first week), TNF-α (first and second week), and IL-6 (during all 3 wk)] were found. In a study by Yamanaka et al. (2003), among the examined cytokines (IL-1β, IL-6, TNF-α, and IFN-γ), only IL-1β was detected before colostrum consumption. We suggest that colostrum proinflammatory cytokines (IL-6, IL-1β, and TNF-α) may have a direct influence on the calves' immune system during the first week of life.

We conclude that colostrum may have a systemic effect on neonatal calves' APR through the impact on the calves' cytokine milieu.

Concentrations of serum SAA and Hp fluctuate in neonate ruminants before they stabilize at adult levels. In our study, the SAA concentrations of dairy calves increased during the first 2 wk before stabilization, whereas Hp concentrations peaked in the second week of life but decreased thereafter. Studies of clini-

Table 4. Results of multivariable linear regression model for detecting association of calves' (n = 100) serum proinflammatory cytokine and acute-phase protein concentrations during the second week of life (8–14 d of age) with average daily weight gain (g/d) measured at approximately 9 mo of age (mean ± SD; 264 ± 6.5 d)

Variable[1]	n	Coefficient	SEM	*P*-value	Wald test *P*-value
Birth weight (kg)		−5.06	2.02	0.014	
Age at sampling (d)		−9.19	4.84	0.061	
Age at weighing (d)		4.48	1.49	0.003	
SAA serum concentration (mg/L)		−0.36	0.12	0.004	
IL-6 serum concentration (ng/L)		−1.48	0.63	0.021	
HL treatment group[2]					<0.001
No treatment	16	0			
Incorrect treatment	37	−96.61	27.47	0.001	
Correct treatment	47	−152.41	30.47	<0.001	
Cryptosporidium group[3]					0.158[4]
Negative	50	0			
Low oocyst level (below the median)	21	−19.12	25.04	0.447	
High oocyst level (above the median)	29	33.69	26.19	0.201	
Intercept		−9.57	387.22	0.980	

[1]Hp = haptoglobin; SAA = serum amyloid A; HL = halofuginone lactate.

[2]Incorrect treatment: daily treatment started >48 h after birth and lasted <7 d. Correct treatment: daily treatment started <48 h after birth and lasted ≥7 d.

[3]Positive samples were divided into low and high oocyst count categories using median values of the same week.

[4]Nonsignificant variable was retained from the model because of confounder effect.

cally healthy calves (dairy and cross-breed) showed the same tendency (Orro et al., 2008; Tóthová et al., 2015). This leads to the conclusion that some of the age-related changes in SAA concentration occur independently from clinical disease and are most probably caused by some physiological process or subclinical infections.

At birth, albumin is the most prominent protein fraction of total serum proteins (Tóthová et al., 2016). Its relative concentrations have been shown to decrease significantly 1 d after colostrum intake but then increase gradually over the first month (Tóthová et al., 2015). Other studies have confirmed a progressive increase in albumin after birth in calves (Bertoni et al., 2009; Piccione et al., 2009). It has been previously shown that IL-6 inhibits the production of albumin (Tanaka et al., 2014). In our study, the albumin concentration increased during the first 3 wk of life, and a negative association between colostrum IgG and albumin during the first week of life was evident. This emphasizes the inhibiting effect of colostrum globulins on neonatal albumin production.

Several explanations for the changes in APP concentrations after birth have been considered. One possibility could be the birth process itself, which is traumatic and could elicit APR (Marchini et al., 2000). Another hypothesis considers colostrum to be the trigger for APP changes (Tóthová et al., 2015), but we did not find any associations of colostrum APP and cytokines after the first week of life.

As changes in APP concentrations after birth seem to be physiological, we can assume that they have beneficial effects. Nevertheless, prolonged elevated levels of neonatal APP may negatively reflect the animal's performance (e.g., weight gain; Orro et al., 2006; Seppä-Lassila et al., 2017, 2018; Peetsalu et al., 2019).

Postnatal growth is also controlled by growth hormone and IGF-1 (Strle et al., 2004), which in turn are inhibited by the proinflammatory cytokines IL-1β and TNF-α (O'Connor et al., 2008). In this study, there were no associations between high serum cytokine levels and short-term weight gain during the first week of life, but a negative association was evident between the second week cytokine levels and long-term weight gain, similar to the associations of SAA and Hp. After the first week of life, the protection of colostrum starts to fade, and environmental factors, possibly leading to subclinical infections, may cause long-lasting immunomodulatory effects and through that negatively affect long-term weight gain. There were no associations between colostrum components and weight gain, meaning that especially the second and third week, when the colostrum effect has ceased, represents an important period for neonatal adaptation.

High shedding of *Cryptosporidium* had a considerable effect on the APR of neonatal calves in this study, so it has to be considered in field studies during the time in which shedding of *Cryptosporidium* spp. occurs. *Cryptosporidium* spp. is very common on cattle farms worldwide (O'Handley and Olson, 2006). In Estonia, *Cryptosporidium* spp. shedding in neonatal calves was found in 66% of investigated farms (Santoro et al., 2019). Halofuginone lactate is used as standard care for prophylaxis of *Cryptosporidium* spp. by reducing the excretion of oocysts (Silverlås et al., 2009), and it needs to be considered as a possible factor influencing the outcomes of our study (Figure 1). Because cryptosporidiosis is very prevalent in young calves and prophylactic treatment is widely used, our results represent a common situation in dairy farms.

One of the shortcomings of this study is the possibly high level of noise due to other factors activating the calves' APR. The calves in this study did not present symptoms of any other clinical disease (e.g., respiratory, umbilical, or joint disease) during the first 3 wk of life. The *Cryptosporidium* spp. outbreak or HL treatment (or both) may have masked the clinical signs of other infections. Before the *Cryptosporidium* spp. outbreak, veterinarians on the study farm had diagnosed several infections causing diarrhea (coronavirus, rotavirus, and *Escherichia coli*). In addition, the herd had tested positive for bovine viral diarrhea virus at the time of the study (Niine at al., 2018b). This suggests that other infections may have been involved. In addition, the results of the present study may not be representative of large populations because the study took place on only one farm, and there was an acute outbreak of cryptosporidiosis. However, the negative association between APR markers in the second week of life with weight gain has been reported in different ruminant species under different management conditions. This suggests that the results of this study are not only specific to this one farm but are also applicable more generally.

This study was an observational field cohort study describing the real-life situation in many dairy farms. However, these kinds of studies can raise more questions because they are influenced by different uncontrollable factors. These studies can eventually lead to results that can be directly transferable to farm management practices.

CONCLUSIONS

The associations between colostrum cytokines and calves' systemic immunity, predominantly during the first week of life, show that there may be an effect of colostrum beyond maternal antibodies. However, colostrum cytokines may not influence calves' serum

APP. Concentrations of inflammatory markers, such as APP and proinflammatory cytokines, in calves' serum go through time-related changes during the neonatal period, and after the first week, have a negative association with long-term weight gain. Future studies should examine environmental factors (e.g., pathogen exposure) and microbial colonization during the neonatal period more closely because they may have a long-term impact on animal health and production.

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Supervised dissertations

Piia Reija Ramona Pekkola, Degree in Veterinary Medicine, 2010, (sup) Merle Valdmann, **Kristel Peetsalu**. Transtüretiini ja türoksiini siduva globuliini kontsentratsioonide muutuste semikvantitatiivne hindamine raske mittetüreoidse haigusega koertel, (Semi-quantitative evaluation of changes in transthyretin and thyroxine binding globulin concentrations in a dog with severe non-thyroid disease), Estonian University of Life Sciences.

Aleksandr Dedjulja, Degree in Veterinary Medicine, 2013, (sup) Toomas Orro, **Kristel Peetsalu**. Koerte akuutse faasi proteiinid põletiku markeritena: tervete ja püometraga koerte võrdlus, (Canine acute phase proteins as a marker of inflammation: intact and pyometric dogs comparison), Estonian University of Life Sciences.

Anna Josefina Kilpi, Degree in Veterinary Medicine, 2015, (sup) Toomas Orro, **Kristel Peetsalu**. Serum concentrations of globulins, albumin and serum amyloid A of neonatal lambs associations with weight gain during summer rearing period, Estonian University of Life Sciences.

Susanna Katriina Mäki, Degree in Veterinary Medicine, 2015, (sup) Toomas Orro, **Kristel Peetsalu**. Concentration of serum amyloid A in serum and synovial fluid from dogs with cranial cruciate ligament rupture, Estonian University of Life Sciences.

Mervi Irene Sulin, Degree in Veterinary Medicine, 2021, (sup) **Kristel Peetsalu**, Toomas Orro. Veterinarians approach to feline lower urinary tract disease, Estonian University of Life Sciences.

Julia Eeva-Maria Sulonen, Degree in Veterinary Medicine, 2021, (sup) **Kristel Peetsalu**, Toomas Orro. Xylitol toxicosis in dogs: case series study. Estonian University of Life Sciences.

Anna-Leena Honkala, 2021, Degree in Veterinary Medicine, (sup) Toomas Orro, **Kristel Peetsalu**. Cow colostrum acute phase proteins (SAA, Hp) and cytokines (IL-1β, IL-6): associations with inflammatory response in neonatal calves, Estonian University of Life Sciences.

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Aleksandr Dedjulja, loomaarstikraad, 2013, (juh) Toomas Orro, **Kristel Peetsalu**. Koerte akuutse faasi proteiinid põletiku markeritena: tervete ja püometraga koerte võrdlus, Eesti Maaülikool.

Anna Josefina Kilpi, loomaarstikraad, 2015, (juh) Toomas Orro, **Kristel Peetsalu**. Serum concentrations of globulins, albumin and serum amyloid A of neonatal lambs associations with weight gain during summer rearing period (Tallede seerumi globuliinide, albumiini ja seerumi amüloid A sisaldus neonataalperioodil ja seos tallede kaaluiibega suvisel kasvuperioodil), Eesti Maaülikool.

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