Eesti Maaülikool
Estonian University of Life Sciences

IMPACT OF GASTROINTESTINAL PROTOZOAN INFECTIONS ON THE ACUTE PHASE RESPONSE IN NEONATAL RUMINANTS

SEEDETRAKTI ALGLOOMNAKKUSTE MÕJU MÄLETSEJALISTE ÄGEDA JÄRGU VASTUSELE NEONATAALPERIOODIL

TARMO NIINE

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

Väitekiri
filosoofiadoktori kraadi taotlemiseks loomaarstiteaduse erialal

Tartu 2019

Eesti Maaülikooli doktoritööd

Doctoral Theses of the Estonian University of Life Sciences

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Estonian University of Life Sciences

According to verdict Nº 3 of September 17, 2019, the Doctoral Committee of Veterinary and Food Science of Estonian University of Life Sciences has accepted the thesis for the defence of the degree of Doctor Philosophy in Veterinary Medicine and Food Science.

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Defence of the thesis: Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, room A-201, F.R. Kreutzwaldi 62, Tartu on November 21, at 10:00.

The publication of this dissertation is granted by the Graduate School in Biomedicine and Biotechnology and by the Estonian University of Life Sciences.

Language editors:
Elsevier B.V. (English)
Mrs. Urve Ansip (Estonian)

ISSN 2382-7076
ISBN 978-9949-629-97-8 (printed)
ISBN 978-9949-629-98-5 (pdf)

In memory of my mother

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following papers, which are cited in the text by Roman numerals. Under the non-commercial Creative Commons user license (CC-BY-NC-ND) 4.0 the articles from the journals Comparative Immunology, Microbiology and Infectious Diseases and Research in Veterinary Science were reproduced.

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. DOI: 10.1016/j.cimid.2017.08.001

II Niine, T., Dorbek-Kolin, E., Lassen, B., Orro, T. 2018. *Cryptosporidium* outbreak in calves on a large dairy farm: effect of treatment and the association with the inflammatory response and short-term weight gain, Res. Vet. Sci. 117, 200-208. DOI: 10.1016/j.rvsc.2017.12.015

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. DOI: 10.1016/j.rvsc.2018.10.013

The contribution of author's to the research papers

Paper	Original idea, study design	Data Collection, sample analysis	Data analysis	Manuscript preparation
I	TO, TS	TO, AO, MN, KP	**TN**, TO	All
II	TO, **TN**, BL	**TN**, TO, ED, BL	**TN**, TO	All
III	TO, **TN**, KP	**TN**, TO, AK, KP, LT	**TN**, TO	All

TN – Tarmo Niine, AK – Ants Kuks, AO – Antti Oksanen, BL – Brian Lassen, ED – Elisabeth Dorbek-Kolin, KP – Kristel Peetsalu, LT – Lea Tummeleht MN – Mauri Nieminen, TO – Toomas Orro, TS – Timo Soveri, All – all authors of the paper

ABBREVIATIONS

ADWG – average daily weight gain
ALB – albumin
APP – acute phase proteins
APR – acute phase response
AUC – area under the curve
BRD – bovine respiratory disease complex
EDTA – ethylenediaminetetraacetic acid
Fb – fibrinogen
FPT – failure of passive transfer
GGT – gamma glutamyltransferase
GLOB – globulins
Hb – hemoglobin
HL – halofuginone lactate
Hp – haptoglobin
Ig – immunoglobulin
IL – interleukin
LPS – lipopolysaccharide
M-SAA3 – mammary-associated serum amyloid A 3
SAA – serum amyloid A
TNF-α – tumour necrosis factor α
VSP – variant surface protein

1. INTRODUCTION

After birth, neonatal ruminants go through a crucial and sensitive period of rapid development and growth. In this period, they may interact with plethora of different microorganisms found in the surrounding environment. It would be beneficial for both farmers and veterinarians to have general markers that quantify the success of this adaptation process, which might help improve the prediction of future performance. These interactions with infectious agents (including parasites) can cause damage to tissues and can trigger the innate immune response indicated by the acute phase response (APR), which leads to an increase or decrease in specific acute phase proteins (APPs) in the blood serum of newly born ruminants (Ceciliani et al., 2012). Fluctuations in concentrations of APPs could serve as a proxy for assessing inflammatory processes during the adaptation period of newborn animals transitioning from a low microbe environment (uterus) to a high microbe environment (extrauterine life) (Orro et al., 2008). Additionally, there is evidence that the strength of the APR in the first weeks of life could influence future production (Orro et al., 2006; Seppä-Lassila et al., 2017, 2018; Peetsalu et al., 2019).

Protozoan parasitic infections, including *Cryptosporidium* spp. and *Eimeria* spp., might also trigger APR in neonatal ruminants (Lassen et al., 2015; Dinler et al., 2017). These intestinal parasites can cause severe diarrhoea that can occasionally result in mortality without timely intervention (Quílez et al., 2002; Bangoura and Daugschies, 2007; Lassen and Talvik, 2009; Delafosse et al., 2015). More commonly, sub-clinical symptoms, such as a setbacks in growth and production, can be observed in both young and older animals in endemically infected environments (Sartin et al., 2000; Foster and Smith, 2009; Lassen and Ostergaard, 2012).

Neonatal parasitic infections and their association with APR in ruminants are yet to be thoroughly investigated, although some indicative results have already been published (Pourjafar et al., 2011; Lassen et al., 2015; Dinler et al., 2017). There is still a general lack of knowledge in this field of research. Interactions between the immature immune system and parasites, measured through quantifiable biomarkers (e.g., APPs), could benefit both farmers and veterinarians in better understanding

the complex interactions and possibly predict outcomes more accurately (e.g., average daily weight gain (ADWG)).

This dissertation focuses on the description of the production of APPs in the first weeks of life in neonatal ruminants in relation to protozoan parasitic infections. A better understanding and more precise measurement of the host's immune system interaction with parasitic infections can benefit the more accurate prediction of future performance. All of the included studies were longitudinal, and each focused on a different ruminant species: reindeer calves (**I**), dairy calves (**II**) and lambs (**III**).

2. REVIEW OF THE LITERATURE

Unless otherwise stated, if a specific animal species is not described in the text, then it is assumed to be neonatal ruminant.

2.1 Acute phase response and proteins

The reaction of the immune system to a variety of tissue damage and physical stress through complex inflammatory processes (triggered by pathogen associated and/or damage associated molecular patterns PAMP/DAMP stimuli) is called the acute phase response (APR) (Ceciliani et al., 2012; Yun et al., 2014). Inflammation process is further amplified by three major pro-inflammatory cytokines: interleukin 1β (IL1β), tumour necrosis factor α (TNF α) and IL6 (Gauldie et al., 1987). A stress reaction and viral or bacterial infections in calves can cause an APR of varying duration and, in the case of co-infection, might even prolong the duration of APR (Gånheim et al., 2003; Burdick et al., 2011).

One of the main results of APR is the increased hepatic production of highly conserved acute phase proteins (APPs) (Table 1) (Baumann and Gauldie, 1994; Qu et al., 2014). The liver has been reported to be a principal source of positive APPs, including haptoglobin (Hp) and serum amyloid A (SAA) in ruminants (APR triggers concentration increases – thus termed as positive), but these proteins could be synthesized in other tissues, which has been confirmed by mRNA expression (Lecchi et al., 2012). Fibrinogen (Fb) can act as a positive APP marker, as it has been demonstrated that its concentration in serum can be elevated during the inflammatory processes (Tóthová et al., 2011). APP concentrations stay elevated long after the challenge, which makes them good candidates for assessing the severity of inflammatory reactions (Hinds et al., 2014). Production of these APPs should eventually lead to restoration of homeostasis (Schrödl et al., 2016).

Albumin (ALB) is also considered to be an important APP in ruminants, but it is a negative APR marker and thus its concentration decreases in serum during APR (Ceciliani et al., 2012).

Various parasitic infections have been shown to be potent inducers of APR, thus increasing APPs concentrations in domestic ruminant serum, i.e., *Sarcoptes scabiei* (Rahman et al., 2010), *Psoroptes ovis* (Wells et al., 2013), *Eimeria zuernii* (Lassen et al., 2015) and *Cryptosporidium parvum* (Dinler et al., 2017).

In this thesis, the literature review focuses mostly on SAA, Hp and ALB, as these APPs were used in all of the studies and were considered the most important in describing APR.

Table 1. Selected acute phase proteins (APPs) in ruminants. The list of APPs presented is not exhaustive, for more detailed overview please check chapter 2.3 and review by Ceciliani et al. (2012)also called acute phase reaction (APR.

Acute phase protein	Category*	Known (main) functions	Reference(s)
Serum amyloid A (SAA)	Major	Binding and uptake of cholesterol Immune cells recruitment Inhibition of neutrophil myelo-peroxidase release and migration Opsonization of Gram-negative bacteria	(Liang and Sipe, 1995; Xu et al., 1995; Gatt et al., 1998; Hari-Dass et al., 2005)the concentration of serum amyloid A (SAA
Haptoglobin (Hp)	Major	Binding of free haemoglobin (Hb) Suppressing T helper cell type 2 cytokine release Bacteriostatic (through binding iron)	(Eaton et al., 1982; Arredouani et al., 2003; Yang et al., 2003)yet its protective role(s

Table 1 (continued)

Fibrinogen (Fb)	Moderate	Marker of vascular rupture Coagulation factor (precursor to fibrin) Improves neutrophils binding to endothelial cells in early stages of inflammation	(Cruz-Topete et al., 2006; Davalos and Akassoglou, 2012)
Albumin (ALB)	Negative	Osmotic pressure of plasma Major source of amino acids (i.e. during acute phase response (APR))	(Tothova et al., 2014)

* Major – increase in serum (compared to baseline) 10- to 100-fold; Moderate – increase in serum (compared to baseline) 2- to 10-fold; Negative – decrease of concentration in serum (compared to baseline)

2.1.1 Serum amyloid A

Serum amyloid A (SAA) is primarily produced in the liver and was originally discovered from systemic amyloid deposits (Benditt and Eriksen, 1971; Levin et al., 1972). The induction of SAA synthesis in hepatocytes is mainly mediated by cytokines: interleukin-1β (IL-1β), tumour necrosis factor α (TNF-α) and IL-6 (Uhlar et al., 1997). One of the main roles of SAA is to recruit T lymphocytes, monocytes and neutrophils to the site of infection (Xu et al., 1995). It also has anti-inflammatory properties demonstrated by inhibiting neutrophil myeloperoxidase (MPO) release and their migration to tissues (Gatt et al., 1998). SAA can quickly and strongly bind to gram-negative bacteria outer membrane protein A (OmpA) family members (Hari-Dass et al., 2005). This in turn causes the opsonisation of the bacteria and increases the speed of phagocytosis by acting as a chemoattractant for monocytes (Badolato et al., 1994; Shah et al., 2006). SAA concentration starts at very low levels a few hours after birth, but reaches its peak concentration in the first to second week of life (Orro et al., 2008; Dinler et al., 2017). Extrahepatically produced mammary-associated serum amyloid A 3 (M-SAA3) has been found in abundant concentration in the colostrum of bovines and ovines (McDonald et al., 2001).

2.1.2 Haptoglobin

Bovine haptoglobin (Hp) molecular mass is 1000 to 2000 kDa, and it is composed of two α and two β chains connected by disulfide bridges (Morimatsu et al., 1991). The main function of the Hp is to bind free haemoglobin (Hb), thereby reducing oxidative stress and mitigating inflammation processes (Yang et al., 2003). The Hp-Hb complex is removed from the blood by attaching to receptor CD163 on macrophages (Kristiansen et al., 2001). Hp strongly shifts the T helper cell type 1 (Th1) and type 2 (Th2) immune response balance towards Th1, thus promoting cellular immunity (Arredouani et al., 2003).

During acute and chronic respiratory infections, Hp levels in calf serum increase significantly (Orro et al., 2011; Youssef et al., 2015). Lipopolysaccharide (LPS) challenge has been reported to trigger increase of Hp and SAA at different time intervals. In a study by Hinds et al. (2014) with 65-82 day old bulls, the Hp and SAA concentrations reached their peak concentrations 30 and 12 hours after the challenge, respectively, and both stayed elevated for at least 96 hours. In four-week-old bull calves after LPS challenge, Hp peaked at 18 hours and SAA at 24 hours after infection; Hp concentration fell to pre-challenge levels 48 hours later and SAA approximately 54 hours later (Plessers et al., 2015).

2.1.3 Albumin

Serum ALB has molecular mass of 67 kDa and is a transport molecule produced by liver. It has various ligands for carrying a wide array of different molecules (i.e., fatty acids, hormones, drugs, bilirubin and metal cations) (Bujacz, 2012). ALB is also critically important in maintaining oncotic pressure of plasma (Tothova et al., 2014).

The most common reasons for decreased ALB (hypoalbuminemia) are malnutrition and inflammation (e.g., APR) (Don and Kaysen, 2004). ALB concentration decreases in cow serum after calving and returns to normal values in a week (Trevisi et al., 2009; Osorio et al., 2013). Cows experiencing uterine inflammation due to infection have significantly lower serum ALB approximately three weeks before calving (Schneider et al., 2013). In dairy calves that were inoculated with *Chlamydia psittaci,* a week-long decrease in serum ALB concentration was seen (Prohl et al., 2015). Higher serum concentration of ALB, measured at the time

of mixing in different calf groups that were freshly arrived at the farm, was associated with an increase in the length of time when the first antimicrobial treatment became necessary (Seppä-Lassila et al., 2018). Interestingly, in the case of haemoprotozoan *Trypanosoma vivax* infection, ALB serum concentration increased in dairy cattle (Sampaio et al., 2015).

2.1.4 Globulins and gamma glutamyltransferase

Colostrum is an important source of immunity for neonatal ruminants. Dairy calves with a serum immunoglobulin (Ig) concentration that is <5-10 g/l 24-48 hours after birth are considered to have developed failure of passive transfer (FPT) (Beam et al., 2009; Furman-Fratczak et al., 2011). Calves that do not receive adequate amounts of high-quality colostrum are more likely to have higher veterinary costs associated with them and lower milk production in the long term (Faber et al., 2005). Calves with Ig serum levels below 8 g/l were at higher risk of developing bovine respiratory disease and/or diarrhoea (Furman-Fratczak et al., 2011).

Gamma glutamyltransferase (GGT) is an enzyme produced by mammary gland ductile cells and thus can be found in colostrum (Baumrucker, 1979). In the first 24 to 48 hours, protein absorption is non-selective in neonatal ruminants, and thus many proteins and macromolecules end up in the newborn's serum (Weaver et al., 2000; Britti et al., 2005). Low serum GGT activity (less than 100 IU/l) up to 48 h after birth is a very strong indicator of insufficient Ig transfer from colostrum for dairy calf and lamb (Parish et al., 1997; Britti et al., 2005; Hogan et al., 2015). Serum total proteins (STP) <52 g/l after birth could also be used to diagnose FPT, but it has been found to have poorer predictive value than GGT activity (Windeyer et al., 2014).

2.2 Acute phase proteins in young ruminants

2.2.1 Dairy calves

In cattle Hp, SAA, M-SAA3 are considered to be major APPs. Moderate APPs are α1 acid glycoprotein (AGP), lipopolysaccharide binding protein (LBP), ceruloplasmin and fibrinogen (Fb) (Eckersall and Conner, 1988; McDonald et al., 2001; Ceciliani et al., 2012; Simplício et al., 2013). Major

APP serum concentrations can increase 10- to 100-fold during APR (over a period of 24-48 hours), while moderate APP concentrations rise approximately 5- to 10-fold (2-3 days) (Ceciliani et al., 2012; Iliev and Georgieva, 2016). APPs: Hp, SAA and Fb are also influenced by the time of day, although the exact mechanism that causes this variation is unknown (Giannetto et al., 2012). The age of the animal plays a role in the level of SAA serum concentration (Orro et al., 2008; Seppä-Lassila et al., 2013). Healthy calf SAA and Hp levels are approximately 80-200 mg/l and 100-200 mg/l, respectively (Seppä-Lassila et al., 2013). SAA and Hp are more associated with aseptic inflammation than Fb and white blood cell count (Danscher et al., 2011).

Different types of pathogens and injuries can lead to the development of APR. Bacterial and viral pathogens that may lead to development of bovine respiratory disease complex (BRD) may trigger the increase of SAA and Hp (Godson et al., 1996; Gånheim et al., 2003). Additionally, gastrointestinal disorders due to tissue damage (traumatic reticuloperitonitis) can significantly increase Hp plasma levels (Hirvonen and Pyörälä, 1998). Higher levels of Hp in the first weeks of life significantly increased the odds of death before 4 months of age (Murray et al., 2014).

2.2.2 Lambs and kids

Major APPs in sheep and goat are also considered to be SAA and Hp, while AGP and Fb are moderate (Ceciliani et al., 2012). APP ranges have been published for healthy adult sheep, but not for neonatal lambs (Iliev and Georgieva, 2016). Nevertheless, there are several studies describing APP concentrations, although with considerable differences in the first weeks of life. In the first day of life, Hp concentration is approximately 0.02 to 0.2 g/l, and it peaks at approximately 5 days of age at 1.8 g/l; it then gradually starts to decrease in the second week of life (Eckersall et al., 2008; Dinler et al., 2017; Peetsalu et al., 2019). Average Hp concentration in weaned lambs is approximately 0.08 g/l (Lepherd et al., 2009), while in healthy adult sheep the Hp concentration in serum is negligible and well below 0.2 g/l (Skinner and Roberts, 1994).

After birth and before consumption of colostrum, the concentration of SAA is relatively low (approximately 2.6 mg/l), but quickly rises to 21 to 29 mg/l in the first hours (Eckersall et al., 2008; Dinler et al., 2017). SAA

stays elevated to approximately 5 days of age, when its concentration gradually starts to decrease (Peetsalu et al., 2019). In healthy weaned lambs and ewes, the average SAA concentration is 1 to 4 mg/l but has been found to increase up to 6460 mg/l during bacterial infection (Winter et al., 2003; Lepherd et al., 2009).

In the case of pneumonic pasteurellosis, SAA and Hp have been found to be more specific and sensitive than Fb, with concentrations increasing from base level up to 34 and 7 times, respectively (El-Deeb and Elmoslemany, 2016). In the study by Pfeffer et al. (1993), performed on adult sheep that were injected with yeast by the intrathoracic route, Hp had strong negative correlation to cumulative weight change, but only weakly correlated with daily weight gain. Significant changes in host APP serum concentrations have been described in different infections, including ovine caseous lymphadenitis (*Corynebacterium pseudotuberculosis*) (Eckersall et al., 2007; Bastos et al., 2011), clinical Scrapie (Meling et al., 2012), *Mannheimia haemolytica* (Ulutas and Ozpinar, 2006), experimentally induced clinical mastitis with *Staphylococcus epidermidis* (Winter et al., 2003) and sheep scab (*Psoroptes ovis*) infestation (Wells et al., 2013).

Saanen goat kid SAA and Hp concentrations reached peak values approximately 7 days after birth (Ulutas et al., 2017). Additionally, kid SAA and Hp concentrations increase significantly due to infections with coccidia (*Eimeria arlongi*) (Hashemnia et al., 2011).

2.2.3 Reindeer calves

Very few studies have been published about APPs in reindeer calves, but SAA, Hp and Fb concentrations increase shortly after birth in neonatal reindeer calves and appear to stay elevated up to 3 weeks of life (Orro et al., 2006).

2.3 *Cryptosporidium*

Cryptosporidium was in subclass *Coccidia*, but now belongs to *Apicomplexa* phylum and *Gregarinomorphea* class and was reclassified as a gregarine (Cavalier-Smith, 2014; Abeywardena et al., 2015). *Cryptosporidium* parasite has worldwide spread in dairy cattle and sheep and has also been detected in Estonian dairy cow and sheep farms (O'Handley and Olson, 2006; Lassen et al., 2009, 2013; Plutzer et al., 2018). The parasite infects new

hosts through the faecal-oral route via ingestion of oocysts. It has both asexual and sexual development phases; the infective oocysts (round shaped, 5-9 μm wide) are excreted into the environment with faeces and can be immediately infective. In the same or in a new host, oocysts develop into sporozoites that attach to epithelial cells, and further differentiate into trophozoites, which can start sexually reproducing as merozoites and produce oocysts (Widmer, 2014).

Cryptosporidium is considered to be "minimally invasive", as it parasitizes the host's gastro-intestinal tract epithelium cells intracellularly, but extracytoplasmatically in a parasitiphorus vacuole, feeding through feeder organelles (Widmer, 2014). It needs to prevent the target cell's apoptosis, as it can only complete its life cycle in a living cell (Widmer et al., 2000). Both innate and adaptive immune system branches work against the parasite infection, but the exact mechanisms are still largely unknown (Petry et al., 2010). In the process of resolving infection, $CD4^+$ T cells appear to play critical roles, with activation of the MyD88 pathway in infected cells, while B cell and antibody functions are debatable (Pantenburg et al., 2008). Cryptosporidiosis can cause considerable problems starting 3 days post infection in ileum, with inflammatory cell infiltration, villous atrophy, and crypt hyperplasia, thus leading to impairment of sodium and water absorption and accelerated intestinal transit (Argenzio et al., 1990). Although one oocyst might be enough to establish successful infection, in immunocompetent individuals the infection is usually self-limiting and lasts for 5 to 10 days, but in immunocompromised animals it can become chronic and life-threatening (Petry et al., 2010; Messner and Berger, 2016).

2.3.1 *Cryptosporidium* in dairy calves

In cattle, three *Cryptosporidium* species are most commonly known to cause infection: *C. parvum*, *C. andersoni*, and *C. bovis* (O'Handley and Olson, 2006; Abeywardena et al., 2015).

Cryptosporidium parvum infection has been most extensively studied in calves, where it has been found most often; at age one to four weeks it can cause diarrhoea, weight loss and/or impaired weight gain (Olson et al., 2004; Abeywardena et al., 2015). In general, clinical cryptosporidiosis usually occurs in the first 6 weeks of life and can have a negative impact on weight gain and survival in the first month of life (Lopez et al., 1988;

Silverlås et al., 2013; Delafosse et al., 2015). Calves are more likely to be shedding and clinically affected by the *Cryptosporidium* infection than adult livestock (Featherstone et al., 2010). The highest risk of becoming infected is at one to three weeks of age (Maddox-Hyttel et al., 2006). The highest numbers of oocysts were usually shed at two to three weeks of age, during which time these actively shedding animals were 3 times more likely to be diarrhoeic than non-infected calves (Trotz-Williams et al., 2005; Santín et al., 2008; Coklin et al., 2010). Up to 60% of calves can become diarrhoeic due to infection, and with oocysts shedding peaking in the first month of life (Harp and Goff, 1998; Geurden et al., 2007). *Cryptosporidium* with *Escherichia coli*, rota- and coronavirus infections have been associated with neonatal calf diarrhoea and can have synergic effects by aggravating clinical symptoms (Bartels et al., 2010; Pourjafar et al., 2011).

Passive immunization via colostrum does not appear to be effective against *Cryptosporidium* infection in calves (Harp and Goff, 1998). Although feeding hyperimmune colostrum does not prevent infection, it still shortens the time period of being diarrhoeic and shedding oocysts (Fayer et al., 1989). The colostrum also contains long-chain fatty acids that appear to inhibit the sporozoites' attachment to host cells (Schmidt and Kuhlenschmidt, 2008). Higher absorption rates of the colostrum also have negative associations with *Cryptosporidium* oocysts shedding in the first four weeks of life (Lopez et al., 1988). Overall, clinical and experimental studies suggest that cellular immune response is more important in recovering, and the carefully orchestrated cooperation of intestinal epithelial cells, dendritic cells, monocytes and antibodies are crucial in the prevention of the infection (Mead, 2014; Laurent and Lacroix-Lamandé, 2017). Intestinal microbiota might also play critical roles in prevention of cryptosporidiosis (Mead, 2014; Laurent and Lacroix-Lamandé, 2017).

2.3.2 *Cryptosporidium* in lambs

The most common *Cryptosporidium* species found in sheep are *C. parvum*, *C. ubiquitum* and *C. xiaoi* (Widmer, 2014). In terms of clinical signs, all of these mentioned species have been associated with diarrhoea in lambs (Navarro-i-Martinez et al., 2007; Díaz et al., 2010; Cacciò et al., 2013). Approximately two weeks before parturition, ewes can start to shed oocysts in increasing numbers, thus predisposing lambs to acquiring

the infection (Santín et al., 2007). In infected herds, the prevalence of cryptosporidiosis in lambs reaches its peak at 6 to 10 weeks of age (Robertson et al., 2010).

Cryptosporidiosis, in general, can cause diarrhoea, which starts approximately 3 days after inoculation and lasts 7 to 12 days, with a possibility of being fatal (Tzipori et al., 1981). Infection causes villous atrophy and fusion in the small intestine, leading to malabsorption and cachexia (Tzipori et al., 1981; Xiao et al., 1993). Cryptosporidiosis has been found to predispose lambs to secondary umbilical infections (Giadinis et al., 2011). It has been found that age has a strong positive correlation with immunity and severity of infection; thus, older sheep (e.g., ewes) are more capable of getting infection under control and are less likely to develop clinical signs (Ortega-Mora and Wright, 1994; Santín et al., 2007).

2.3.3 Treatment and prevention of *Cryptosporidium* in calves and sheep

Many different species of *Cryptosporidium* have been recognized in ruminants (Abeywardena et al., 2015), but from the perspective of diagnosing clinical cryptosporidiosis, species determination seems to have little importance (Silverlås et al., 2013). Inoculation of irradiated *Cryptosporidium* oocysts can give limited protection against the infection (Jenkins et al., 2004). Experimental oral vaccination with lyophilized *Cryptosporidium* did not give protection against the development of diarrhoea or reduce the shedding of oocysts (Harp and Goff, 1998).

Currently, there are few metaphylactic options for domestic ruminants - halofuginone lactate (HL), paromomycin, and azithromycin (Shahiduzzaman and Daugschies, 2012). Feeding calves bovine serum concentrate has been shown to decrease cryptosporidiosis symptom severity (Hunt et al., 2002). HL does not appear to prevent the infection or development of clinical signs, but delays the peak of shedding period in calves (Silverlås et al., 2009). Some authors have also reported that treatment does not improve survival (Almawly et al., 2013; Meganck et al., 2015). HL metaphylactic treatment has also been shown to be effective in lambs by reducing the incidence of diarrhoea and improving survival (Giadinis et al., 2007). Additionally, improved hygiene levels can significantly decrease *Cryptosporidium* oocyst excretion by using straw

bedding during weaning and high pressure cleaning (Maddox-Hyttel et al., 2006).

2.4 *Giardia*

Giardia duodenalis (syn. *Giardia lamblia, Giardia intestinalis*) is a protist parasite belonging to *Metamonada* phylum (Abeywardena et al., 2015). The relationship between taxonomy and epidemiology of *Giardia* species is still somewhat complex, as it appears that some species are more host-adapted than others (e.g., Assemblage E – *G. bovis* in cattle and other hoofed animals), and potentially zoonotic species are considered to have lower host specificity (Thompson and Ash, 2016).

Giardia has a worldwide distribution and wide range of hosts; nevertheless, its role as a pathogen is still controversial (Geurden et al., 2010a). *Giardia* has been found in Estonian sheep herds that were located on western islands of Estonia, but a nation-wide cross-sectional study is still lacking (Lassen et al., 2013; Plutzer et al., 2018). In nearby countries, *Giardia* has been found to be prevalent in Sweden, where 68% of flocks tested positive (Ljungstrom et al., 2001).

Giardia is excreted into environment with faeces as cysts that are immediately infectious and develop into trophozoites in infected hosts (pear shaped, 12-15 μm long) (Lujan, 2011). Newly infected animals can start shedding cysts 3 days post-infection, but in the case of neonatal ruminants, this could be postponed up to 31 days, probably due to the protective effect of maternal antibodies (O'Handley et al., 1999, 2003; Lujan, 2011). In the trophozoite stage, the parasite is motile and attaches onto intestinal mucosal cells with a ventral disc (Schwartz et al., 2012). In the jejunum, giardiasis can lead to shortening of microvilli, loss of brush border surface area, reduced disaccharidase activity, increased crypt/villus ratios, persistent dysbiosis and reduced protease activity, resulting in malabsorption of nutrients (Seow et al., 1993; Scott et al., 2004; Beatty et al., 2017). Both live *Giardia* parasite and its excretory and secretory antigens can induce inflammatory reactions in the small and large intestine, including eosinophilic infiltration, hypercellularity and enterocytic desequamation (Jimenez et al., 2004).

An immune response does develop against *Giardia* antigens and is both cellular and humoural, but reinfection is common (Müller and

Von Allmen, 2005; Abdul-Wahid and Faubert, 2008). One of the most important aspect of controlling acute giardiasis appears to be the presence of CD4+ T-cells, while the absence of functioning B-cells had no significant effect in murine models (Singer and Nash, 2000). Additionally, the absence of mast cells and their production of IL-6 predisposes mice to uncontrollable giardiasis (Li et al., 2004). One of the essential ways in which *Giardia* evades the immune response is by changing its surface proteins through antigenic variation - VSP (variant surface protein) (Rivero et al., 2010).

2.4.1 *Giardia* in dairy calves

Experimentally infected dairy calves start shedding *Giardia* cysts approximately 7 days post-infection (Grit et al., 2014). In naturally occurring infections, the start of shedding can vary greatly (6 to 70 days from birth) (O'Handley et al., 1999). On average in neonatal dairy calves, *Giardia* cysts are detected in faeces in the third to fourth week of life, and it is not uncommon for *Giardia* infections to become chronic and last more than 120 days (O'Handley et al., 1999; Nydam et al., 2001). Population density and age can increase the risk of infection in dairy calves (Maddox-Hyttel et al., 2006; Geurden et al., 2012).

During *Giardia* infection, dairy calves produce weakly binding antibodies against the parasite, and at same time there is downregulation of inflammatory reaction related genes in jejunum, which could partly explain the lack of clinical signs and the chronic nature of the infection (O'Handley et al., 2003; Dreesen et al., 2012). High amounts of anti-*Giardia* immunoglobulin G (IgG) have been found in dairy cattle colostrum, which could be a reason why so few calves shed *Giardia* cysts before 21 days of age (O'Handley et al., 2003).

In some studies, faeces characteristics, such as soft or diarrhoeic, and *Giardia* infection have not been found to have significant association (Maddox-Hyttel et al., 2006; Winkworth et al., 2008). At the same time, in experimental inoculation it has been noted that dairy calves develop occasional spells of mild and watery diarrhoea (Grit et al., 2014). Some *Giardia* positive calves could act as "super-shedders" producing and shed massive amounts of cysts, thus infecting others (Hoar et al., 2009).

2.4.2 *Giardia* in lambs

The prepatent period of *Giardia* in experimentally infected lambs has been found to be 7-21 days and persisting approximately 5-10 weeks (Taminelli et al., 1989; Olson et al., 1995). In naturally acquired infections, lambs start to shed cysts at approximately two to three weeks of age, with shedding peaking at three to four weeks of age (Taylor et al., 1993; Xiao et al., 1994). Parturition can induce increased shedding of *Giardia* from ewes, thus being a potential source of infection for lambs (Xiao et al., 1994).

Giardia infection could negatively affect lamb growth and feeding efficiency. Giardiasis is also associated with non-pelleted faeces and mild diarrhoea in lambs (Olson et al., 1995; Sweeny et al., 2011). Very little is published about the peculiarities of the sheep immune system and *Giardia* interaction, but lambs have also been found to produce weakly binding antibodies against *Giardia* (Yanke et al., 1998).

2.4.3 Treatment and prevention of *Giardia* infection

Currently, there are no specific anti-*Giardia* drugs registered (Geurden et al., 2010a). Albendazole and fenbendazole are effective against giardiasis in cattle (Xiao et al., 1996). Unfortunately, fenbendazole has limited success in calves, as treated animals started shedding oocysts two weeks after the end of treatment (O'Handley et al., 2000; Geurden et al., 2010b). Paromomycin has been shown to be highly effective in reducing *Giardia* cysts shedding in dairy calves, but not in preventing diarrhoea or improving weight gain (Geurden et al., 2006). Vaccination with sonicated *G. duodenalis* in calves did not prevent development of giardiasis or reduce shedding of cysts (Uehlinger et al., 2007).

2.5 *Cryptosporidium* and *Giardia* co-infection

Most parasite studies focus on either *Cryptosporidium* or *Giardia,* and there are a limited number of papers about their co-infection. This might be due to their antagonistic nature against each other (Ruest et al., 1997), and also to the fact that their infection patterns do not generally overlap, as they attack cells in different ways (Xiao and Herd, 1994). In a study by Gillhuber et al. (2014) in which authors examined 1564 diarrhoeic

faecal samples from dairy calves, only 0.96% of these had co-infection, and at same time 40% were *Cryptosporidium* positive. Contrary to a previously described study, (O'Handley et al., 1999) found that 26% of diarrhoea cases in their study group calves had *Cryptosporidium* and *Giardia* co-infection.

2.6 *Cryptosporidium* and *Giardia* in other ruminants

An epidemiologic study conducted by Kemper et al. (2006) on semi-domesticated reindeer in Northern Finland and Norway was unsuccessful in detecting *Cryptosporidium* infections. *Cryptosporidium* and *Giardia* have been detected in wild reindeer faecal samples in Norway (Hamnes et al., 2006). *Cryptosporidium* has also been detected in Northern Alaska caribou (*Rangifer tarandus*), which indicates a wide spread of the parasite in wild ruminants living in colder climates and on different continents (Siefker et al., 2002).

In goats, both *Cryptosporidium* and *Giardia* infections have been recognized, and their shedding has also been found to increase around the time of parturition (Castro-Hermida et al., 2005).

2.7 *Cryptosporidium* and *Giardia* as a zoonotic risk

There have been many reports of potentially zoonotic *Cryptosporidium* or *Giardia* species from domestic ruminants (Castro-Hermida et al., 2007; Quílez et al., 2008; Plutzer et al., 2018), as well as confirmed human cases (Robertson et al., 2010; Lassen et al., 2014; Kinross et al., 2015). Both parasites are potentially zoonotic and can be easily transmitted by water and food (Karanis et al., 2007; Smith et al., 2007). In a cross-sectional study in New Zealand, potentially zoonotic *C. parvum* was found in 50.5% of dairy farms (Al Mawly et al., 2015). Potentially zoonotic *C. parvum* subtypes have also been isolated in 62.3% of Estonian dairy farms (Santoro et al., 2019). Young ruminants are considered to be potential sources of *Cryptosporidium* infection to humans, and their manure should be deposited in such a way that the runoff would not reach drinking water (Widmer, 2014). In *Giardia* spp., there is little evidence that livestock or wildlife pose significant risks to public health (Hunter and Thompson, 2005; Gillhuber et al., 2013; Thompson and Ash, 2016).

2.8 Acute phase response to different parasitic infections in neonatal domestic ruminants

Different classes of parasites have been found to induce APR in ruminants. Gastro-intestinal nematodes (*Cooperia* spp., *Haemonchus* spp., *Oesophagostomum* spp., and *Trichostrongylus* spp) and pulmonary (*Dictyocaulus viviparous*) parasite infections can significantly increase calf serum Hp concentrations while not necessarily causing obvious clinical symptoms (Table 2) (de Cezaro et al., 2016). Nematode *D. viviparous* infection in dairy calves has been demonstrated to induce APR and increase SAA, Hp and Fb concentrations (Gånheim et al., 2004). *Dictyocaulus viviparous* and *Dictyocaulus eckerti* infections in red deer (*Cervus elaphus*) have also been shown to induce Fb concentration increase in the blood (Johnson, 2002).

Experimental infection of the gastro-intestinal nematode *Trichostrongylus colubriformis* in lambs induced detectable APR, measured through increase of Hp in efferent intestinal lymph fluid (Bond et al., 2014). *Haemonchus contortus* (blood feeding parasite) infection in lambs caused increase of SAA by 10-150-fold and Hp by 10-30-fold when compared to uninfected animals (Zhong et al., 2014). *Toxocara vitulorum* (gastro-intestinal parasite) infection in calves caused significant increase of Hp in calves (Bozukluhan et al., 2017). In the study by Reck et al. (2009), cattle (age 10-12 months) were infected with cattle tick *Rhipicephalus* (*Boophilus*) *microplus,* and it caused a significant increase of Fb.

Previous studies (Enemark et al., 2003a, 2003b) have shown that *Cryptosporidium* can induce Hp increase in dairy calves, which occurs just before the onset of diarrhoea, but there were two calves each in those experiments, resulting in high individual variation. A similar effect has been observed in lambs experimentally infected with *C. parvum*, which induced significant increase of SAA and Hp concentrations (Dinler et al., 2017).

Bloody diarrhoea and increased concentrations of SAA and Hp have been associated with eimeriosis in dairy calves (Balikci and Al, 2014). During the prepatent period of *E. zuernii* infection, there was significant Hp concentration decrease, but severe clinical eimeriosis had the opposite effect and Hp concentration increased (Lassen et al., 2015). In a study by (Seppä-Lassila et al., 2015), *Cryptosporidium* and *Giardia* infection did not seem to have significant association with APP concentrations in calves.

SAA and Hp significantly increased in goat kids experimentally infected with *E. arlongi* (Hashemnia et al., 2011).

Multi-infection in calves with coronavirus or rotavirus and *Cryptosporidium* triggered stronger SAA and Hp increase than mono-infection with either of those viruses or parasites (Pourjafar et al., 2011). A similar synergic effect of multi-infection (rotavirus and *Cryptosporidium*) has also been seen in piglets (Enemark et al., 2003a, 2003b).

Table 2. Published significant APP concentration changes associated with parasitic infections in domestic ruminants (dairy calves, sheep, goat and reindeer) up to one year of age. Serum Amyloid A (SAA), Haptoglobin (Hp), Fibrinogen (Fb)

Ruminant species	Acute phase protein	Parasite	No. of animals in study	Age range	Reference
Cattle	SAA, Hp	*Cryptosporidium*	6	0 to 4 weeks	(Pourjafar et al., 2011)
Cattle	Hp	*Cryptosporidium parvum*	2	Unknown	(Enemark et al., 2003a)
Cattle	Hp	*C. parvum*	2	Unknown	(Enemark et al., 2003b)
Cattle	SAA, Hp	*Eimeria zuernii*	41	10 to 28 days	(Lassen et al., 2015)
Cattle	Hp, Fb	*Eimeria* spp.	100	15 to 60 days	(Seppä-Lassila et al., 2015)
Cattle	SAA, Hp	*Eimeria* spp.	15	2 to 48 days	(Balikci and Al, 2014)
Cattle	SAA, Hp, Fb	*Dictyocaulus viviparous*	22	2 to 3 months	(Gånheim et al., 2004)
Cattle	Hp	*D. viviparous, Cooperia* spp., *Haemonchus placei, Oesophagostomum* spp., *Trichostrongylus* spp.	86	2 to 24 months	(de Cezaro et al., 2016)
Sheep	Hp	*Trichostrongylus colubriformis*	18	6 to 7 months	(Bond et al., 2014)
Sheep	SAA, Hp	*C. parvum*	20	0 to 20 days of age	(Dinler et al., 2017)
Goat	SAA, Hp	*Eimeria arloingi*	18	15 days	(Hashemnia et al., 2011)

3. AIMS OF THE STUDY

The main aim was to investigate if gastrointestinal protozoan parasites *Cryptosporidium* and *Giardia* can affect the APR in neonatal ruminants (**I**, **II**, and **III**). Additionally, APR to *Eimeria* was investigated in dairy calves (**II**).

Specific aims of the dissertation were:

1. To investigate the effects of the APR and parasitic infections in the neonatal period to short-term production results (**I**, **II** and **III**).

2. To study and describe how metaphylaxis of cryptosporidiosis with halofuginone lactate (HL) in dairy calves affects the APR and short-term growth in dairy calves (**II**).

3. To describe changes in the APP concentration in the first weeks of life in reindeer calves (**I**), dairy calves (**II**) and lambs (**III**).

4. MATERIALS AND METHODS

A short overview of the methods is presented in this section. More detailed descriptions of the methods can be found in the corresponding sections in the articles **I**, **II** and **III**. If not stated otherwise, the same described method was used in each study.

4.1 Study population

Reindeer calves (**I**): 56 reindeer (*Rangifer tarandus*) calves (28 males and 28 female), born from 9th to 22th May 2004 in the Kaamanen experimental herd, Finnish Lapland.

Dairy calves (**II**): 144 female dairy calves born from 21st January to 16th March 2015 on a large-scale Central-Estonian dairy farm (>1800 cows).

Lambs (**III**): 269 lambs (124 female and 145 male) born from 4th April to 4th May in 2014 on a Southern-Estonian organic sheep farm (>200 ewes).

4.2 Blood, colostrum and faecal samples

In the reindeer calf study (**I**), 609 serum (Table 3), 210 EDTA and 366 faecal samples were collected. Weight was measured immediately after birth, at 3 weeks of age and at 114 to 127 days of age. Faecal samples were frozen before further analysis.

In the dairy calf study (**II**), 906 serum and 767 faecal samples were collected. Weight was measured immediately after birth and at one and three months of age.

In the lamb study (**III**), 692 serum and 141 faecal samples were collected. Additionally, 187 colostrum samples were collected one to two hours after lambing. Weight was measured in the first week of life and on 18th August 2014.

In all studies, blood samples were collected directly from the jugular vein into a sterile test tube.

In the dairy calf (**II**) and lamb (**III**) studies, faecal samples were collected directly from the rectum with a clean, disposable latex glove and placed in a clean plastic cup. In the reindeer study (**I**), the same procedure was followed, with the exception that samples were not deposited into plastic cups, but into clean latex gloves. Faecal samples were frozen before analysis and stored at −20°C until analysis in the reindeer calf (**I**) and lamb (**III**) studies, but the dairy calf (**II**) samples were analysed and stored at 4°C for up to 48 h. All of the faecal samples that were frozen went through only a single freeze-thawing cycle, and this has been found to have a negligible effect on detecting *Cryptosporidium* oocysts and *Giardia* cysts (Robertson and Gjerde, 2004).

Table 3. Samples collected for the studies (n = no. of samples/animals)

Animals	No. of animals (n)	Blood samples (n)		Faecal samples (n)
		Serum	EDTA	
Reindeer calves (**I**)	56	609	210	366
Dairy calves (**II**)	144	906	None	767
Lambs (**III**)	269	692	None	141
Total	469	2207	210	1274

4.3 Analysis of blood and colostrum samples

In the reindeer calf study (**I**), a clinical blood chemistry analyser (KONE Pro, Konelab, Thermo Clinical Labsystems Oy, Vantaa, Finland) was used to measure serum ALB using the bromocresol green method (commercial kit: Accent-200 Albumin II Gen, PZ Cormay S.A., Poland). A paragon electrophoresis system (Beckman Coulter, Inc., Fullerton, CA, USA) was used to measure γ-globulins (**I**).

A clinical blood chemistry analyser (Mindray BS-200, Mindray Medical International Limited, Shenzhen, China) was used to analyse the activity of GGT (commercial kit: Accent-200 GGT, PZ Cormay S.A., Łomianki, Poland), ALB concentrations (commercial kit: Photometric Colorimetric Test for Albumin Liquicolor, Human Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany) and TP (commercial kit: Photometric Colorimetric Test for Total Proteins Liquicolor, Human Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany) (**II**, **III**). Globulin (GLOB) concentration in the lamb study (**III**) was calculated by subtracting the ALB value from TP. IgG concentration on the first and second day of life in lambs (**III**) was calculated using GGT serum activity with the formula: ln(IgG) (mg/dl) = 2.251 + 0.700 × ln[GGT] (IU/l) + 0.378 age (days), where age 1 (day) = 0 and age 2 (days) = 1 (Britti et al., 2005).

SAA serum and colostrum (only in **III**) concentrations were determined using a commercial sandwich ELISA kit (Phase BE kit, Tridelta Development Ltd., Dublin, Ireland). Hp was measured from serum using a haemoglobin binding assay method described by Makimura and Suzuki (1982), with a slight modification introduced using tetramethylbenzidine (0.06 mg/ml) as a substrate (Alsemgeest et al., 1994).

In the reindeer calf study (**I**), Fb concentration in the plasma was determined by the heat precipitation method (Millar et al., 1971).

In studies **I** and **III,** neonatal animals received colostrum from their mothers, and its quality was not controlled at the farm. In the dairy calf study (**II**), colostrum quality was controlled visually and with a hydrometer (specific gravity); also, the administered quantity was controlled by farm personnel.

4.4 Faecal sample analysis

Cryptosporidium oocysts and *Giardia* cysts in faecal samples were detected using the immunofluorescent staining method (Crypto/Giardia Cel, Cellabs Pty Ltd., Sydney, Australia).

In all of the studies, a light microscope was used to count the number of cysts and oocysts in the visual field at 200× magnification. In the reindeer study (**I**), the number of cysts or oocysts in the samples was

qualitatively ranked by a single visual field observation: none (negative sample), low (1 to 5 oocysts/cysts), medium (6–30 oocysts/cysts) and high (over 30 oocysts/cysts).

In the dairy calves and lamb studies (**II**, **III**), approximate cysts and oocyst counts were calculated as (oo)cysts per gram of faeces (OPG) (De Waele et al., 2010; Lassen and Lepik, 2014). Dairy calf faecal samples were categorized as diarrhoeic or non-diarrhoeic based on visual examination (**II**).

In the dairy calf study (**II**), all faecal samples were examined using the concentration quantitative flotation method to detect *Eimeria* and nematodes (Roepstorff and Nansen, 1998).

4.5 Metaphylactic treatment of cryptosporidiosis

In the dairy calf study (**II**), animals were retrospectively divided into different groups based on HL metaphylaxis characteristics: I) not treated (n = 34), II) incorrectly treated (n = 45) (treatment started >48 h after birth and/or lasted less than 7 days) and III) correctly treated (n = 65) (first HL given <48 h after birth and treated for at least 7 consecutive days).

In reindeer calves (**I**) and lamb (**III**) studies, animals were not treated with HL.

4.6 Statistical analysis

To summarize the changes in different APPs over time, the area under the curve (AUC) was calculated using the trapezoidal method (**I**, **II**). The formula for calculation was as follows: $AUC = \sum [(t_i - t_{i-1})f_{i-1}] + [0.5(t_i - t_{i-1})(f_i - f_{i-1})]$, where t_i = the time of observation, t_{i-1} = the previous time of observation, f_i = APP concentration at the time, and f_{i-1} = APP concentration at the previous time. Dependent variables in logistic or linear regression models were transformed, if necessary, to meet the normal distribution presumption (**I**, **II** & **III**). In all studies, the results were considered significant if $p \leq 0.05$. In the linear models, independent variables were excluded through manual backward stepwise elimination if $p > 0.05$, except when the remaining variable coefficients changed more than 10%; then, it was kept as a possible confounder (**I**, **II** & **III**).

Multiple linear regression models were used in the following cases: a) serum Hp and SAA associations with different *Cryptosporidium* OPG categories at different ages (**II**), b) GLOB association in the first weeks of life with detection of *Cryptosporidium* and *Giardia* (oo)cysts in faeces (**III**), c) SAA and Hp serum concentrations in the first week of life in association with colostrum quality (specific gravity) (**II**), and d) ADWG association with Hp or Hp AUC and different HL treatment regimens (**II**).

Mixed linear regression models were used for exploring associations in the following cases: a) colostrum SAA association with APPs in the first days of life (**III**), b) AUCs of γ-globulin, SAA, Hp, Fb and ALB associations with *Giardia* infection and other APPs (**I**), and c) ADWG association with APPs in lambs (ewes where included as random factors) (**III**).

Logistic regression models were used in the following cases: a) association between diarrhoeic faecal samples and detection of *Cryptosporidium* and/or *Giardia* (oo)cysts (**II**), and b) to retrospectively control risk factors in association with dairy calf mortality (**II**).

More detailed descriptions of the statistical models that were used can be found in the respective articles' materials and methods sections.

Statistical analysis was performed using STATA 13.1 (**I**) and 14.1 (**II**, **III**) (StataCorp LP, College Station, TX, USA). Initial data management was done using Microsoft Excel 2013 (**I**) and Microsoft Excel 2016 (**II**, **III**) (Microsoft, Redmond, WA, USA), and more advanced data management was done using Python 3.5.1 (**II**, **III**) (Anaconda 4.0.0 by Continuum Analytics, Austin, TX, USA). Regression model coefficient plots were constructed using the STATA add-on coefplot (Jann, 2014).

5. RESULTS

In this chapter, selected results from the studies are presented with additional previously unpublished results.

5.1 *Cryptosporidium* and *Giardia* infection in neonatal ruminants

In each study, faeces were examined for the presence of *Cryptosporidium* oocysts and *Giardia* cysts. The first positive samples were diagnosed at the first days of age (Table 4). The exact infection rate is unknown, but *Cryptosporidium* and *Giardia* were detected in 21.8% and 98.2% of reindeer calves (Figure 1), in 84.7% and 76.4% of dairy calves, and in 20.9% and 20.0% of lambs, respectively (Table 4).

Giardia infection appeared to spread relatively quickly in the reindeer calf study (**I**) population; at two weeks of age, approximately 60% of calves had at least one positive sample, and a week later, this measure was already at 98% (Figure 1). *Cryptosporidium* infection in dairy calves had the highest number of oocysts per gram of faeces (OPG) at approximately 16 to 19 days of age, and *Giardia* cyst counts in one gram of faeces peaked just before 40 days of age (Figure 2). SAA and Hp concentrations were significantly higher in the second week of life in dairy calves (**II**) which had more than the median level of *Cryptosporidium* OPG in their faeces (Figure 3). There was also a significant association between *Cryptosporidium*-positive faecal samples and being diarrhoeic (**II**). In lambs, there did not appear to be a distinct period of time where shedding of (oo)cysts peaked in the first three weeks of life (Figure 4), but it was correlated with a decreased GLOB serum concentration (Figure 5).

Table 4. *Cryptosporidium* and *Giardia* infection characteristics in different neonatal ruminant species (**I**, **II** and **III**).

Animal species	Total no. of faecal samples (no. of animals sampled)	No. of positive faecal samples (no. of positive animals)**	Min-max of (oo)cysts per gram of faeces (OPG) in a positive sample	Age at first positive sample (age at first sample collection) (days)
		Cryptosporidium		
Reindeer calves	312 (55)	13 (12)	*	9 (0)
Dairy calves	656 (144)	218 (122)	69; 10,602,130	1 (1)
Lambs	141 (110)	22 (23)	500; 23,021,500	4 (0)
		Giardia		
Reindeer calves	312 (55)	187 (54)	*	0 (0)
Dairy calves	656 (144)	203 (110)	69; 2,652,344	4 (1)
Lambs	141 (110)	22 (23)	500; 9,914,500	3 (0)

* In the reindeer study (**I**) OPG was measured qualitatively in scale 0 to 3
** Some animals tested positive multiple times

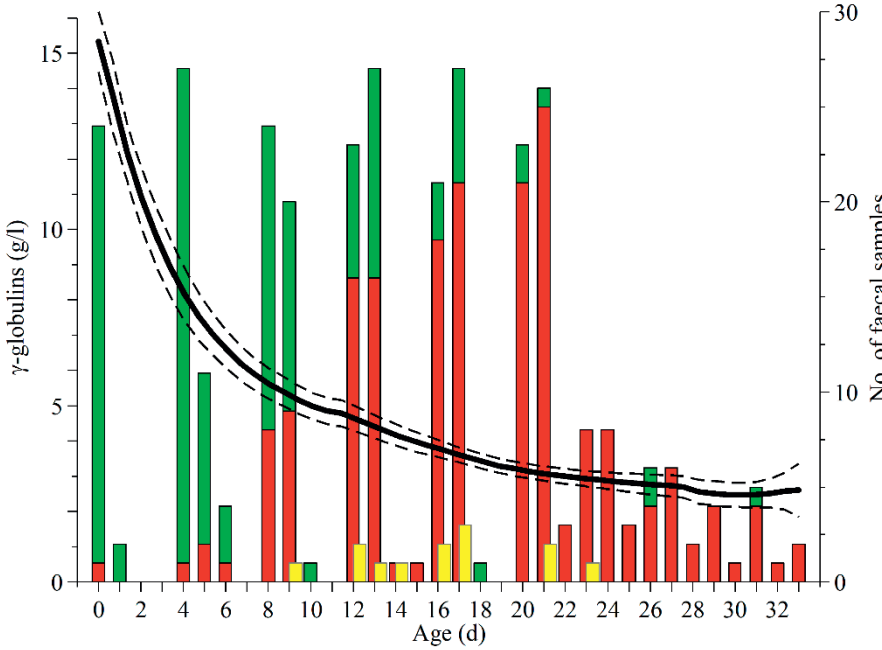

Figure 1. No. of *Giardia* and *Cryptosporidium* positive faecal samples in combination with γ-globulins concentration in the reindeer calves (**I**). The age of reindeer calf for first positive faecal sample are presented on x-axis. Left hand y-axis and black line on top layer of figure represent local polynomial smooth plot with confidence intervals (95%) (dashed lines) of γ-globulins concentration in serum from the age 0 to 33 days. The degree of the polynomial smooth was set at 3 and the Epanechnikov kernel function was used for calculating the weighted polynomial estimate. Right hand y-axis shows no. of faecal samples collected on given day of age. Green bar – *Giardia* negative faecal samples, Red bar – *Giardia* positive faecal samples. Yellow bar – *Cryptosporidium* positive faecal sample. Concentration of γ-globulins was measured in 55 reindeer calves 367 serum samples, All the animals (n = 54) that had faecal sample collected tested positive for *Giardia*: in total 312 faecal samples were collected.

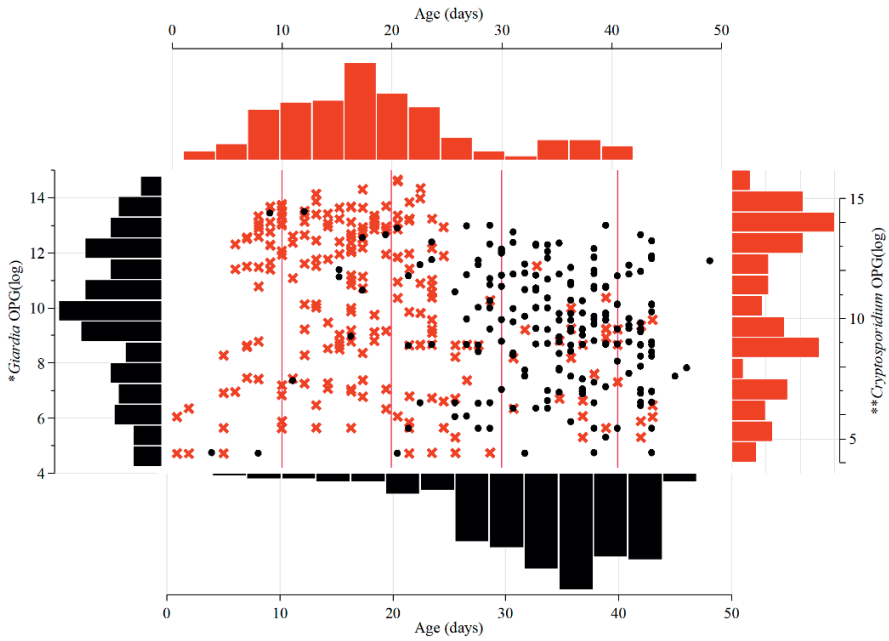

Figure 2. The positive faecal samples of *Cryptosporidium* (n = 218; marked as red crosses) and *Giardia* (n = 209; marked as black dots) illustrating infection dynamics in the dairy calves (**II**). Only the faecal samples that tested positive are represented. Distribution of the proportion of positive findings versus age are presented as histograms on the top and in the bottom of the central scatter plot (Red = *Cryptosporidium*; Black = *Giardia*). Distribution of the proportion of the number of (oo)cysts detected in faecal samples (OPG) in logarithmic (log) scales are presented on left and right of the central scatter plot. In case if 38 faecal samples from 32 dairy calves, both parasites were detected in the same faecal sample.

* *Giardia* – no. of cysts in 1 gram of faeces (OPG) on logarithmic scale
** *Cryptosporidium* – no. of oocysts in 1 gram of faeces (OPG) on logarithmic scale

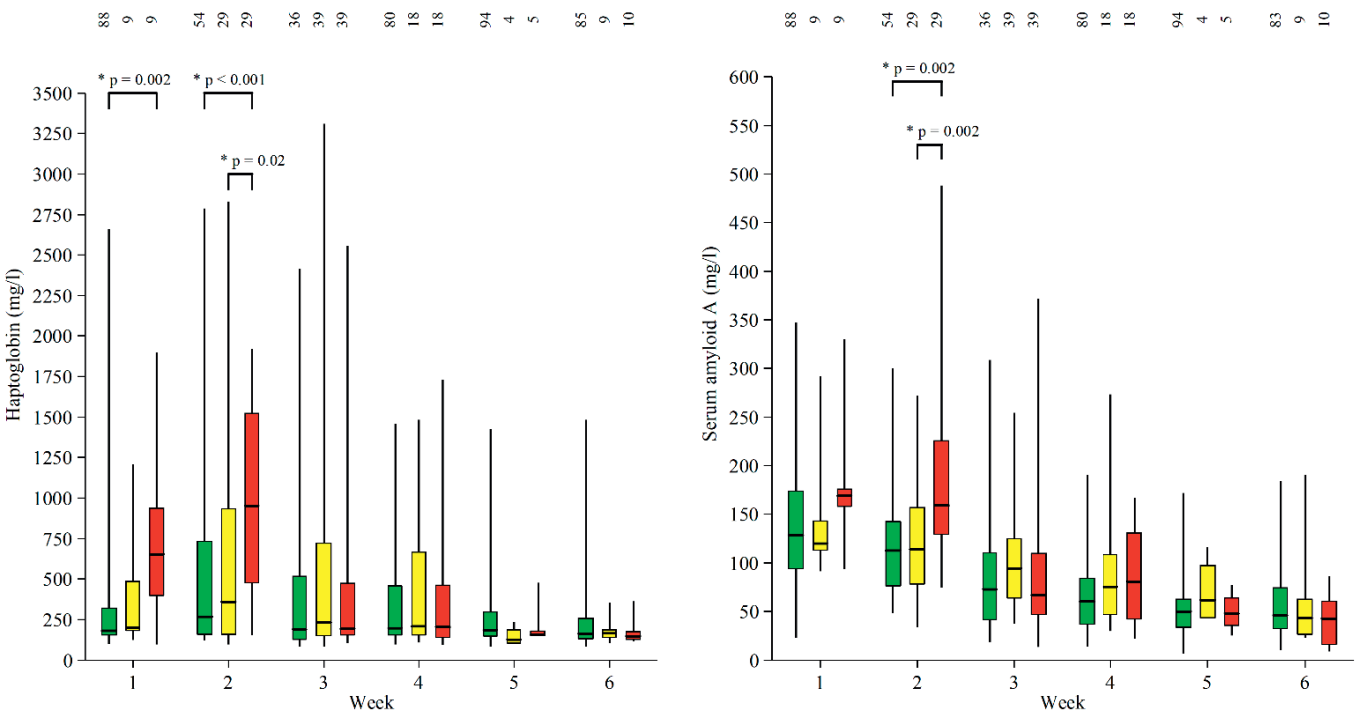

Figure 3. Haptoglobin (Hp) and serum amyloid A (SAA) concentrations of dairy calves (**II**) in serum and different categories of *Cryptosporidium* oocyst counts in faecal samples. Green = negative (no oocysts found), yellow = low (below median oocysts per gram (OPG)), red = high (more than median OPG found in a faecal sample). The number of calves in each group is marked above each bar. Results from the 3 months of age were not presented because only one dairy calf had a *Cryptosporidium* positive faecal sample.
* significant association between groups.

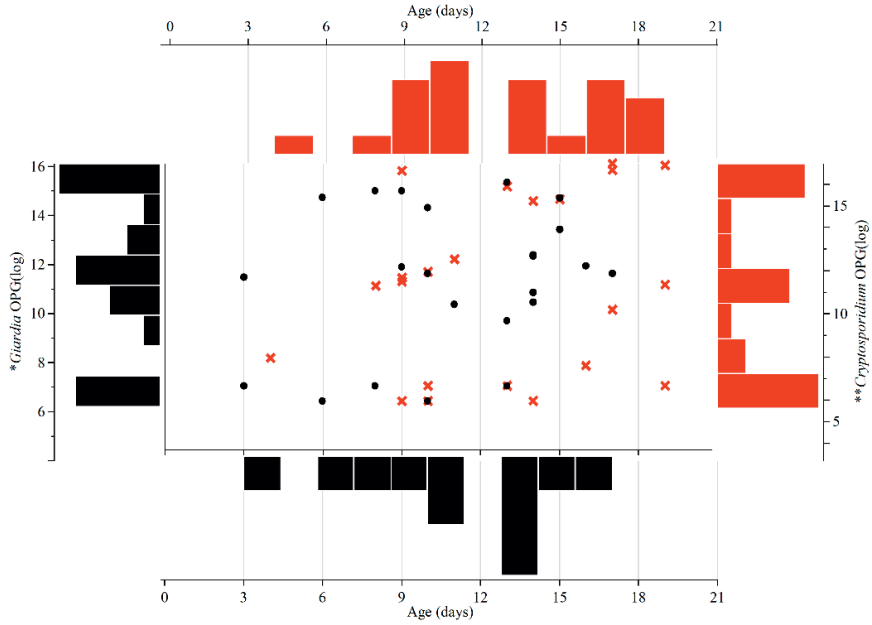

Figure 4. The positive faecal samples of *Cryptosporidium* (n = 23; marked as red crosses) and *Giardia* (n = 23; marked as black dots) illustrating infection dynamics in the lambs (**III**). Only the faecal samples that tested positive are represented. Distribution of the proportion of the positive findings versus age are presented as histograms on the top and in the bottom of the central scatter plot (Red = *Cryptosporidium*; Black = *Giardia*). Distribution of the proportion of the number of (oo)cysts detected in the faecal samples (OPG) in logarithmic (log) scale are presented on the left and right of the central scatter plot. In case of five faecal samples from five lambs, both parasites were detected in the same sample.

* *Giardia* – no. of cysts in 1 gram of faeces (OPG) on logarithmic scale
** *Cryptosporidium* – no. of oocysts in 1 gram of faeces (OPG) on logarithmic scale

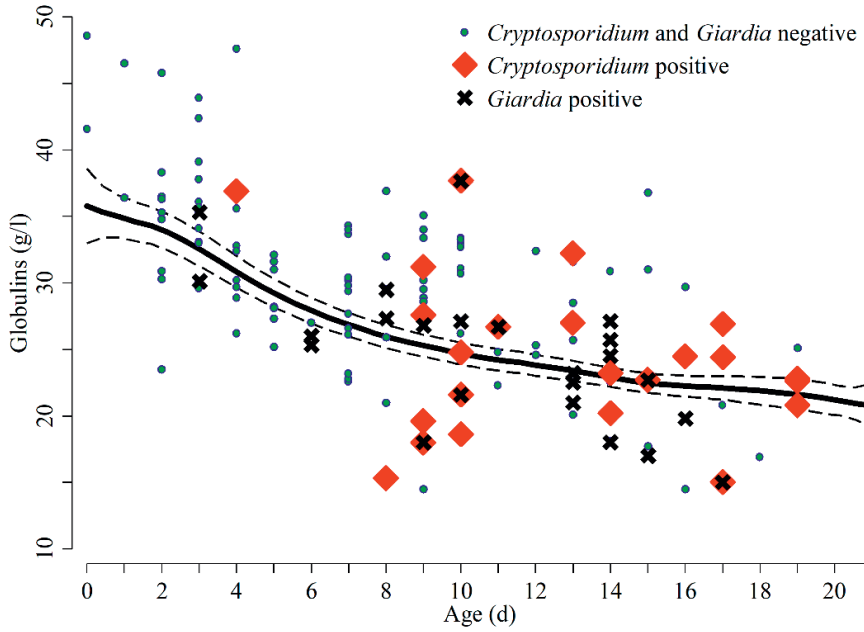

Figure 5. Scatterplot of globulins (GLOB) serum concentrations in lambs (**III**) with *Cryptosporidium* or *Giardia* positive (both n = 23) faecal samples (n = 141). The thick black line represents the relation/function of GLOB on age by local polynomial smooth. The degree of the polynomial used in 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, $p = 0.033$ and $p < 0.001$, respectively.

Significant associations were found between γ-globulin AUC (Figure 6) and SAA AUC (Figure 7) with early *Giardia* infections in the reindeer calf study (**I**). No significant associations were found between Hp, Fb and ALB AUCs and early *Giardia* infection in similarly constructed regression models (**I**).

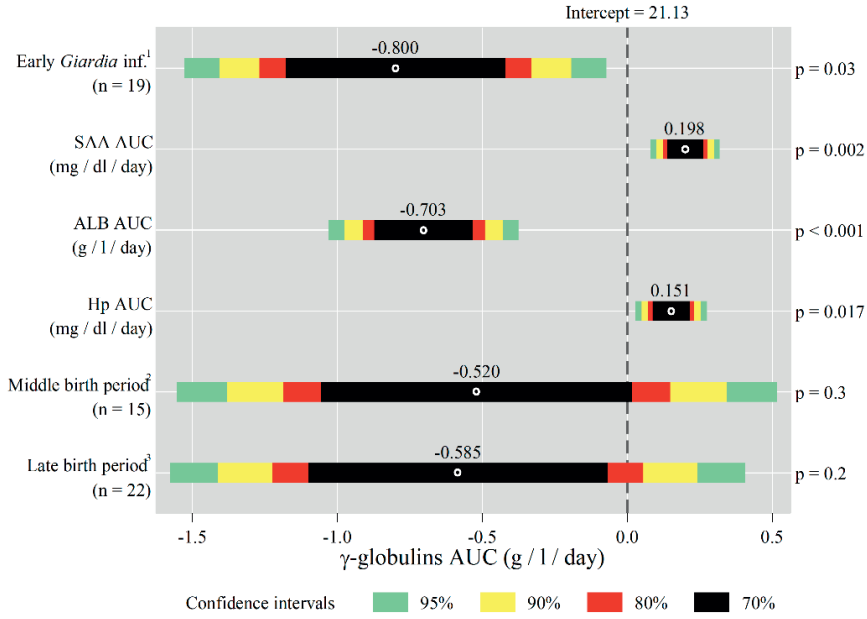

Figure 6. γ-globulin area under curve (AUC) regression model coefficient plot (age 0 to 22 days) of reindeer calves (**I**). Model confidence intervals are presented as horizontal bars. Point estimates for variables are shown on top of the bars. AUC was calculated for each animal (n = 48) using a trapezoidal method for 6 time points and averaging for number of days (20-22 days of age).

[1] First detection of *Giardia* before 12 days of age and compared to late *Giardia* infection group (n = 29)

[2] Middle birth period (15-17 May) compared to early birth period (9-14 May 2004; n = 11)

[3] Late birth period (18-22 May) compared to early birth period (9-14 May 2004; n = 11)

SAA – serum amyloid A, ALB – albumin, Hp – Haptoglobin.

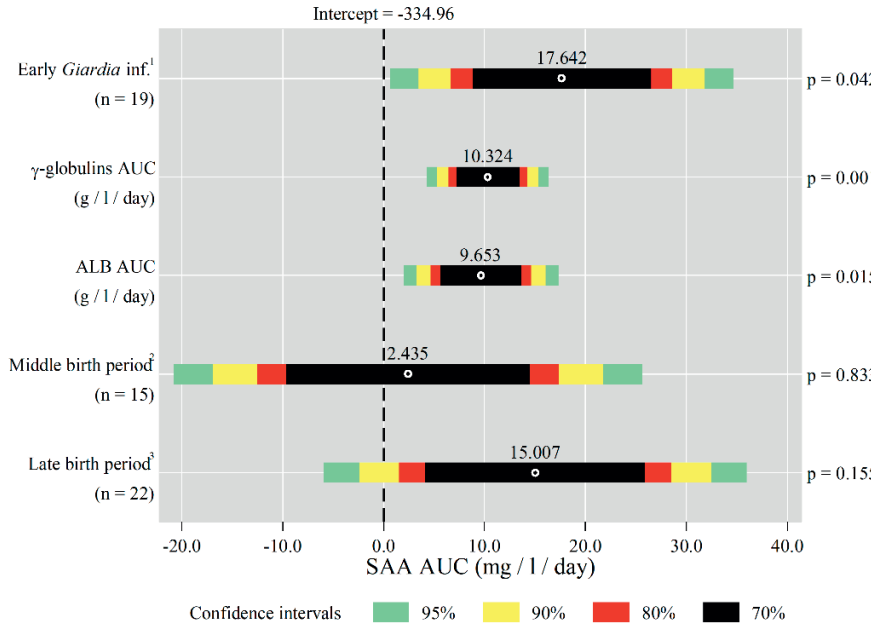

Figure 7. Serum amyloid A (SAA) area under curve (AUC) regression model coefficient plot (age 0 to 22 days) of reindeer calves (**I**). Model confidence intervals are presented as horizontal bars. Point estimates for variables are shown on top of the bars. AUC was calculated for each animal (n = 48) using a trapezoidal method for 6 time points and averaging for number of days (20-22 days of age).
[1] First detection of *Giardia* before 12 days of age and compared to late *Giardia* infection group (n = 29)
[2] Middle birth period (15-17 May) compared to early birth period (9-14 May 2004; n = 11)
[3] Late birth period (18-22 May) compared to early birth period (9-14 May 2004; n = 11)
ALB – albumin.

5.2 Other parasitic infections

In the dairy calf study (**II**), *Eimeria* infection was detected, but no helminths or nematode eggs were found, most likely because the animals were kept indoors. In 73 dairy calves (**II**) (median age 99 days), *Eimeria* parasite was detected in their faecal samples, with species: *E. bovis* (71%), *E. zuernii* (45%), *E. ellipsoidallis* (37%) and *E. auburnensis* (16%). In lamb (**III**) and reindeer (**I**) studies, the flotation method was not used.

5.3 Acute phase proteins associated with growth and performance in neonatal ruminants

In all of the studies, body mass was measured and ADWG calculated (Table 5, 6 and 7). In the lamb study (**III**), there was a significant positive association between Hp and ALB in the second week of life and later weight gain (Table 8). In the reindeer calf study (**I**), there were no significant associations found between APP concentrations during the first weeks of life and ADWG later. The dairy calves (**II**) ADWG association with APR is presented in the next chapter in conjunction with HL treatment. Dairy calves (**II**) that had higher SAA concentrations in the first week of life had higher odds of dying in the first six weeks of life (Table 9).

Only in the dairy calf study (**II**) was colostrum quality assessed by measuring its specific gravity, which on average was 1.055 (n = 139; range 1.04 to 1.075). No significant associations were found between SAA or Hp concentrations in the first week of life and colostrum quality ($p = 0.4$ and $p = 0.4$, respectively) (**II**).

Table 5. Weight and average daily weight gain (ADWG) in reindeer calves (**I**).

Variable	Average (± SD)	No. of animals
Birth weight (kg)	6.3 ± 0.6	49
Weight at 3 weeks of age (kg)	14.1 ± 1.5	54
ADWG in 3 weeks of age (g/d)	377 ± 49	48
Weight at 21 weeks of age (kg)	50.0 ± 5.2	52
ADWG in 21 weeks of age (g/d)	365 ± 35	46

Table 6. Weight and average daily weight gain (ADWG) in dairy calves (**II**).

Variable	Average (± SD)	No. of animals
Birth weight (kg)	41.3 ± 4.8	144
Weight at 1 month of age (kg)*	53.8 ± 5.9	122
ADWG at 1 month of age (g/d)*	419 ± 149	122
Weight at 14 weeks of age (kg)**	121.3 ± 17.6	120
ADWG in 14 weeks of age (g/d)**	784 ± 130	120

* Average age (± SD) 29.6 ± 4.5 (days)
** Average age (± SD) 101.5 ± 10.6 (days)

Table 7. Weight and average daily weight gain (ADWG) in lambs (**III**).

Variable	Average (± SD)	No. of animals
Weight at first measurement (kg)*	7.4 ± 2.5	255
Weight at 17 weeks of age (kg)**	30.7 ± 7.1	238
ADWG at 17 weeks of age (g/d)**	207 ± 50	238

* Average age (± SD) 11.7 ± 6.1 (days)
** Average age (± SD) 123.7 ± 6.9 (days)

Table 8. Results of lambs (**III**) 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 9. Retrospective case control logistic regression modelling of factors associated with mortality of dairy calves (**II**) up to 43 days of age.

Variable (n = no. of calves)	OR	Confidence interval 95%	p-value
SAA (mg/l)*	1.013	1.001; 1.026	0.041
GGT (IU/l)*	0.993	0.988; 0.998	0.004
Birth weight (kg)	0.762	0.607; 0.957	0.019
Multiparous (n = 23)	1.0	–	–
Primiparous (n = 40)	0.111	0.017; 0.731	0.022

n (observations) = 63 (the case group (n = 14) and the control group (n = 49)), SAA = serum amyloid A, GGT = gamma glutamyltransferase, *sample collected first week of life

5.4 Acute phase response and metaphylactic treatment of cryptosporidiosis

Dairy calves (**II**) that were correctly treated with HL against cryptosporidiosis had significantly lower ADWG when compared to animals that were not treated (Table 10). Additionally, average Hp AUC was negatively associated with ADWG at 3 months of age (Table 10) (**II**).

ADWG at 3 months of age was significantly different based on different HL treatment regiments (Figure 8).

Table 10. Association of average daily weight gain (ADWG) (g/days) of 109 dairy calves (**II**) at 3 months of age, haptoglobin (Hp) average area under the curve (AUC), halofuginone lactate (HL) treatment* and age at weight measurement.

Variable (n = no. of calves)	Estimate	Confidence Interval 95%	p-value
Hp average AUC (mg/l/ day)	-0.16	-0.27; -0.05	0.004
Not HL treated (n = 18)	0	–	–
Incorrect HL treatment (n = 42)	-55.53	-126.63; 15.58	0.125
Correct HL treatment (n = 49)	-107.22	-176.37; -38.06	0.003
Age at weight measurement (days)	2.15	-0.75; 5.05	0.145
Intercept	708.69	390.65; 1026.72	0.000

* I) not treated; II) treatment start was delayed or duration was <7 days; III) treatment was done correctly (started in the first 48 hours of life and lasted ≥7 days of treatment).

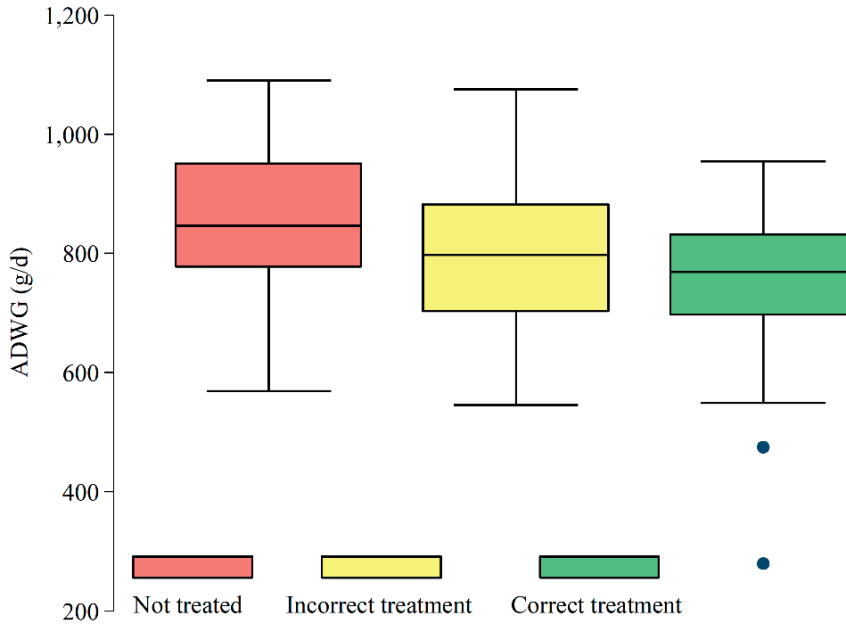

Figure 8. The medians, quartiles and 95% confidence intervals of dairy calves (**II**) average daily weight gain (ADWG) (g/d) at 3 months of age. Animals were grouped based on different halofuginone lactate (HL) treatment regiments. "Not treated" (orange) (n = 19) did not receive metaphylactic treatment for cryptosporidiosis. "Incorrect treatment" (yellow) group (n = 43) did receive HL treatment, but it was not done according to manufacturer's instructions (starting at least 48h after birth and lasting at least 7 days). "Correct treatment" (green) group (n = 58) were treated with HL according to manufacturer's instructions.

Most of the deaths in the dairy calf study (**II**) occurred in a group that was not treated with HL (Figure 9 and 10). In total, 21 dairy calves died during the first 3 months of life (Figure 10). Hp concentrations were significantly higher in the no HL treatment group at the second week of age (Figure 11).

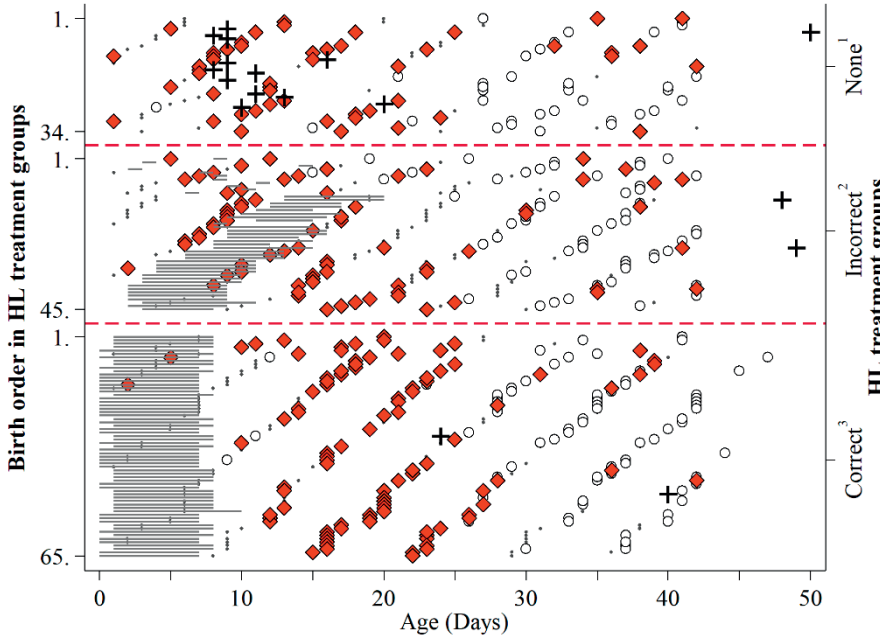

Figure 9. *Cryptosporidium* and *Giardia* infection patterns differentiated by halofuginone lactate (HL) treatments (**II**). Dairy calves were assigned into groups retrospectively based on HL treatment regiments (separated by dashed lines on figure): [1]) not treated (n = 34), [2]) treated incorrectly (treatment started >48 hours after birth, or lasted <7 days) (n = 45), and [3]) treated according to manufacturer's instructions (started <48 hours after birth, and lasted ≥7 days) (n = 65). (◆) *Cryptosporidium* positive; (○) *Giardia* positive; (·) *Giardia* and *Cryptosporidium* negative; (+) death (n = 17); horizontal lines represent HL treatment and the length represents the treatment in days. The y-axis represents the birth order of calves in different HL treatment groups, starting with the oldest and ending with the youngest; x-axis represent the age of the calf.

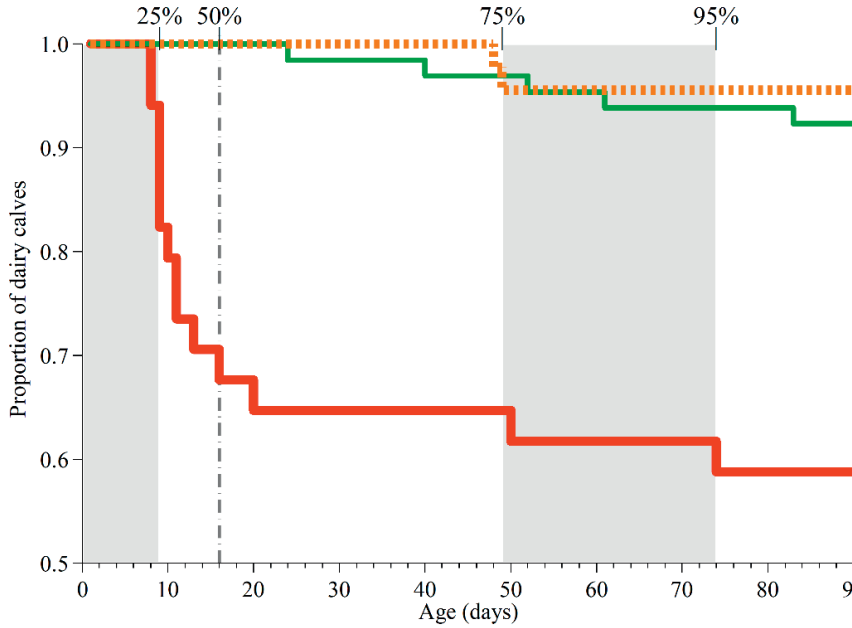

Figure 10. Survival curves of the dairy calves (**II**) based on different halofuginone lactate (HL) treatment categories: no treatment (n = 34; red line), incorrect treatment (n = 45; orange dashed line; treatment start was delayed or was <7 days long), correct treatment (n = 65; green line; started in the first 48 hours of life and had ≥7 days of treatment). Total percentage of deaths (n = 21) in all the groups is shown at the top of figure by quartiles.

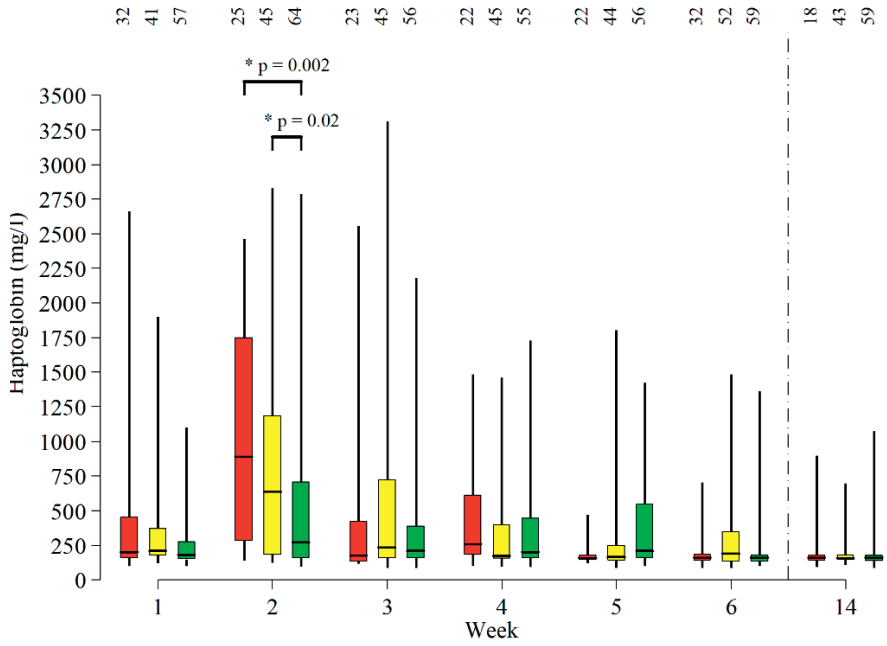

Figure 11. Haptoglobin (Hp) concentrations in serum and different halofuginone lactate (HL) treatment groups in dairy calves study (**II**). Statistically significant differences demonstrated with a horizontal bar on top of second week results. The number of animals in a group shown at the top of a bar. HL treatment groups: red = no treatment; yellow = incorrect treatment (treatment start was delayed or was <7 days long); green = correct treatment (started in the first 48 hours of life and had ≥7 days of treatment).
* significant association between groups.

5.5 Acute phase proteins profiles in neonatal ruminants

5.5.1 Serum amyloid A

In all of the studies, SAA started from a low base value. The highest average SAA concentration was seen in dairy calves (**II**) compared to reindeer calves (**I**) and lambs (**III**) in the first three weeks of life (Table 11). SAA peaked in reindeer calves at 11 to 18 days, in dairy calves at 4 to 10 days and in lambs at 3 to 5 days of age, after which the concentration started to decrease (Figure 12).

In the lamb study (**III**), colostrum SAA concentration was measured (average 20.5 mg/l; n = 181), and it had a significant association with lamb SAA and Hp concentrations at 2 to 4 days of age.

Table 11. Serum amyloid A (SAA) concentrations in serum in all the studies, summarized by weeks. All results in mg/l.

Animal species	Total sample no. (Animals sampled no.)	SAA average ± SD	SAA median	SAA 95% confidence interval
Age 0 to 7 days				
Reindeer calves	103 (55)	27.3 ± 42.4	9.1	19.0; 35.6
Dairy calves	130 (130)	143.0 ± 65.3	129.9	60.3; 296.2
Lambs	277 (251)	86.4 ± 99.7	47.1	6.4; 292.5
Age 8 to 14 days				
Reindeer calves	107 (54)	72.2 ± 44.9	60.3	63.6; 80.8
Dairy calves	134 (134)	141.0 ± 75.9	125.3	51.9; 284.1
Lambs	236 (236)	21.7 – 41.5	7.2	0.3; 104.3
Age 15 to 21 days				
Reindeer calves	109 (54)	73.7 ± 48.5	67.3	17.0; 173.8
Dairy calves	127 (124)	92.5 ± 61.6	78.5	28.3; 199.9
Lambs	179 (179)	16.0 ± 44.0	4.2	0.3; 91.8

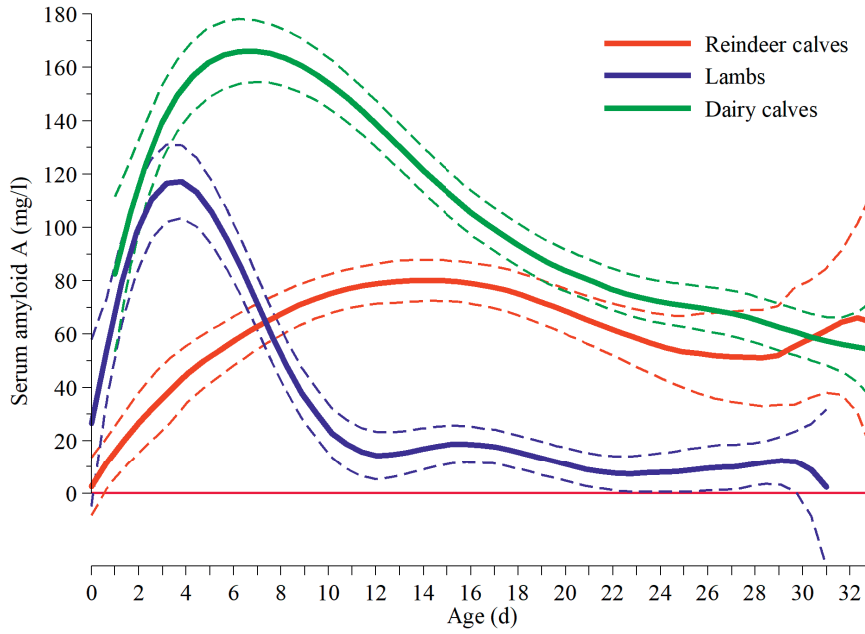

Figure 12. Local polynomial smooth plot with confidence intervals (95%) of serum amyloid A (SAA) concentration in serum from age 0 to 33 days in reindeer calves, dairy calves and lambs. The degree of the polynomial smooth was set at 3, and the Epanechnikov kernel function was used for calculating the weighted polynomial estimate. The calculation of confidence intervals may give results where SAA values are under zero, which is biologically implausible, thus additional line was added to the y-axis for clarification at zero value. Observations among different animal groups: reindeer calves n = 370, lambs n = 796 and dairy calves n = 645.

5.5.2 Haptoglobin

The highest average Hp results were observed in the first week of life in lambs compared to the other studies (**I** & **II**) (Table 12). The Hp serum concentration started from a relatively low concentration and peaked in reindeer at 14 to 18 days, in dairy calves at 7 to 11 days and in lambs at 4 to 7 days, before starting to decrease (Figure 13).

Table 12. Haptoglobin (Hp) results at in all the studies, summarized by weeks. All results in mg/l.

Animal species	Total sample no. (Animals sampled no.)	Hp average ± SD	Hp median	Hp 95% confidence interval
Age 0 to 7 days				
Reindeer calves	103 (55)	423.0 ± 84.8	421.0	292.0; 545.0
Dairy calves	130 (130)	370.8 ± 431.4	194.5	122.0; 1209
Lambs	277 (251)	518.8 ± 941.1	247.4	95.3; 2317.9
Age 8 to 14 days				
Reindeer calves	107 (54)	550.4 ± 65.4	551.0	441.0; 657.0
Dairy calves	134 (134)	699.4 ± 660.2	407.5	126.0; 1962
Lambs	236 (236)	407.0 ± 974.3	201.7	107.6; 851.7
Age 15 to 21 days				
Reindeer calves	109 (54)	572.4 ± 64.6	566.0	486.0; 675.0
Dairy calves	127 (124)	439.0 ± 539.1	201.0	106.0; 1565.0
Lambs	179 (179)	283.2 ± 591.6	192.6	97.6; 388.8

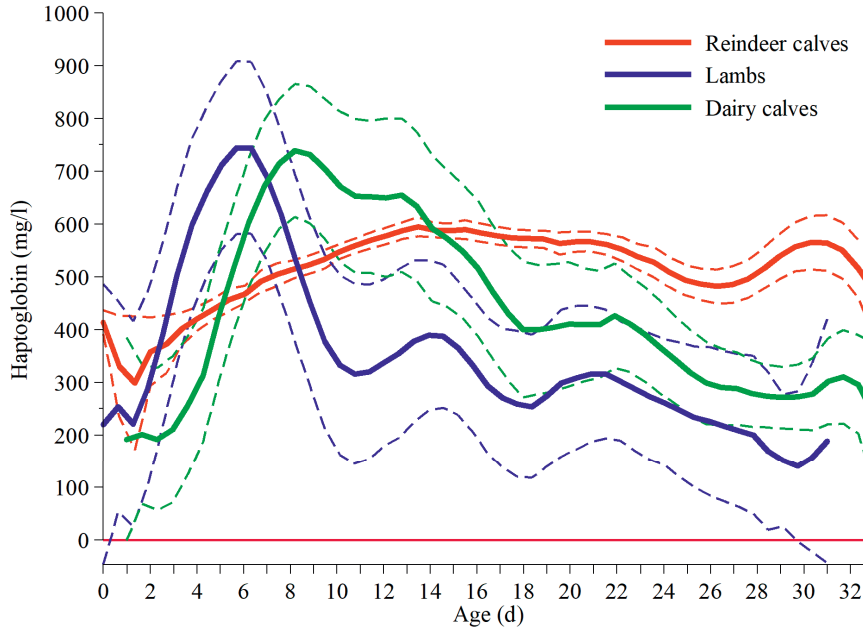

Figure 13. Local polynomial smooth plot with confidence intervals (95%) of haptoglobin (Hp) concentration in serum from age 0 to 33 days. The degree of the polynomial smooth was set at 3, and the Epanechnikov kernel function was used for calculating the weighted polynomial estimate. Calculation of confidence intervals may give results which are not biologically plausible, e.g., Hp values under zero; thus additional red line was added to y-axis for clarification. Observations among different animal groups: reindeer calves n = 370, lambs n = 796 and dairy calves n = 645.

5.5.3 Albumin

ALB concentrations started relatively close to each other in all of the studies (Table 13). In the reindeer and lamb studies, ALB concentration increased throughout the observation period, while in calves the concentration initially increased and then gradually started to decrease (Figure 14).

Table 13. Albumin (ALB) results at in all the studies, summarized by weeks. All results presented in g/l.

Animal species	Total sample no. (Animals sampled no.)	ALB average ± SD	ALB median	ALB 95% confidence interval
Age 0 to 7 days				
Reindeer calves	103 (55)	27.9 ± 3.4	28.5	23.5; 32.7
Dairy calves	128 (128)	29.2 ± 5.6	30.5	17.8; 36.5
Lambs	277 (251)	24.5 ± 3.6	24.8	18.6; 30.0
Age 8 to 14 days				
Reindeer calves	107 (54)	34.1 ± 1.7	34.1	30.9; 36.8
Dairy calves	134 (134)	31.5 ± 6.2	32.7	18.6; 37.5
Lambs	236 (236)	28.0 ± 3.3	28.4	21.7; 32.5
Age 15 to 21 days				
Reindeer calves	109 (54)	36.1 ± 3.2	36.9	29.9; 39.1
Dairy calves	126 (123)	31.8 ± 6.3	33.1	18.6; 38.5
Lambs	179 (179)	29.1 ± 2.9	29.4	23.8; 32.7

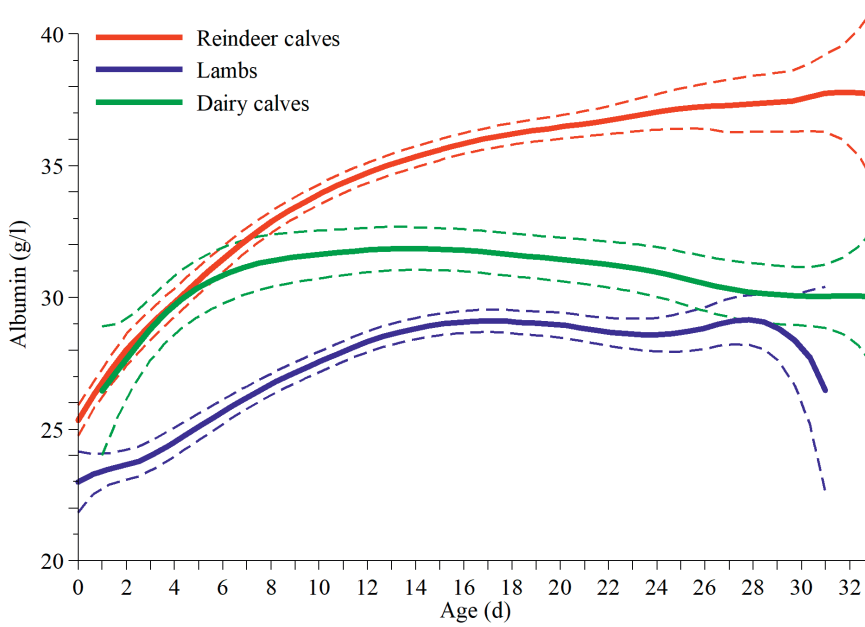

Figure 14. Local polynomial smooth plot with confidence intervals (95%) of albumin (ALB) concentration in serum from age 0 to 33 days. The degree of the polynomial smooth was set at 3, and the Epanechnikov kernel function was used for calculating the weighted polynomial estimate. Observations among different animal groups: reindeer calves n = 370, lambs n = 796 and dairy calves n = 640.

6. DISCUSSION

The results discussed in this chapter are presented in more depth in the articles (**I**), (**II**) and (**III**) with additional previously unpublished results.

6.1 Parasitic infections in neonatal ruminants

Cryptosporidium and *Giardia* infection prevalence differed considerably in the current studies. In the reindeer study (**I**), all of the animals were infected with *Giardia*, while in dairy calves (**II**), most of the animals were also infected with *Cryptosporidium*. Similar *Giardia* infection rates in the reindeer and lamb studies (**III**) might be related to extensive management systems, wherein mothers and neonates were not strictly separated. It is unlikely that both parasites could have survived the winter in soil due to physical damage causing numerous freeze-thaw cycles (Robertson and Gjerde, 2004). A single freeze-thaw cycle does not significantly reduce recognizable *Cryptosporidium* oocysts and *Giardia* cysts, but multiple cycles can have significant negative effects on them (Robertson and Gjerde, 2004). In the dairy calves, the animals were kept indoors and the temperature never dropped below the freezing point, thus providing a very favourable environment for parasites to survive outside of the host (Li et al., 2010). In both reindeer calves (**I**) and lamb (**III**) studies, faecal samples were frozen before analysis and thus went through a single freezing-thawing cycle, but as described earlier, this most likely had a negligible effect on detecting *Cryptosporidium* oocysts or *Giardia* cysts.

The first positive *Giardia* sample was found at a very early age in the reindeer study (**I**) (at the day of parturition), while in lambs and dairy calves, the first positive samples were detected at the third and fourth day of life, respectively. The gradual decrease in ɣ-globulins concentration in the first weeks of life in reindeer calves coincided with *Giardia* infection spreading through the study population. This finding suggests at least partial protection from infection by maternal antibodies, but there was no significant association between being infected and ɣ-globulins concentration in the first week of life. What was also interesting is that reindeer calves that started shedding *Giardia* in the first week of life, did not have significantly reduced growth rates later. This suggests that *Giardia* infection by itself did not mobilize host defences in the form of

an immune response sufficient to cause a decrease in the growth rate. This is in accordance with what (Yanke et al., 1998) suggested before, where weak interactions between *Giardia* and the host immune system result in a barely detectable immune response, measured as changes in antibody levels. In lambs (**III**), the time between infection and shedding *Giardia* cysts has been reported to be 10 to 21 days (Taminelli et al., 1989; Olson et al., 2004); this is similar to our study, wherein most *Giardia* infections were detected between ages 6 to 17 days. In the calf study, the first positive *Giardia* sample was detected at 4 days of age, which is comparable to the findings of (O'Handley et al., 1999), where the first positive sample was detected at age 6 days.

In recent studies, it has been suggested that *Giardia* can cause significant and persistent dysbiosis in human gut microbiota (Beatty et al., 2017). It would have been beneficial to study faecal sample microbiotas in these dissertation studies to investigate if *Giardia* and *Cryptosporidium* infections caused significant changes in microbiota composition. Dysbiosis itself has been found to cause significant stress in intestinal cells, indicated by increased production of IL-1β, which is one of the cytokines that can trigger increased production of SAA (Uhlar et al., 1997; Suez et al., 2018). It might be that in our studies, a similar effect occurred where protozoan parasite infections triggered dysbiosis and activated APR.

The patterns of *Cryptosporidium* infection differed between studies. In reindeer calves (**I**), the first positive sample was detected at 9 days. While *Cryptosporidium* has been detected in wild reindeer faecal samples (Hamnes et al., 2006), to the best of our knowledge no longitudinal studies have been conducted about shedding of oocyst patterns in this species. In lambs (**III**), *Cryptosporidium* oocyst shedding started at day 4, which suggests that animals were infected right after birth, as the time from infection to shedding has been found to be approximately 4 to 5 days (Bukhari and Smith, 1997). In the dairy calf study (**II**), *Cryptosporidium* infection was already detected on the first day after parturition, while in a study by O'Handley et al., (1999), in which infection also occurred naturally, the first positive sample was retrieved at age 6 days. We speculate that the initiation of *Cryptosporidium* shedding in reindeer calves could have started later than in our other studies. This might be because of the protective effect of antibodies received through colostrum, an effect that has been described in dairy calves (Lopez et al., 1988) and ovines (Martín-Gómez et al., 2006).

Metaphylactic treatment of cryptosporidiosis with HL in the dairy calf study (**II**) did seem to reduce mortality during acute outbreaks of this disease. While there have been mixed results in cases of therapeutic use of HL, it can (if used correctly) delay onset of oocyst shedding (Jarvie et al., 2005; Klein, 2008; Silverlås et al., 2009). This delay of oocysts shedding because of metaphylactic treatment also interestingly shifted the Hp peak concentration from the first week to the second week of life (**II**). This suggests that Hp concentration could be a good indicator for assessing the severity of *Cryptosporidium* infection in dairy cattle.

In previous studies, the within herd prevalence of *Cryptosporidium* and *Giardia* in pre-weaned lambs was reported to be 13 to 77% and 4 to 32%, respectively (Santín et al., 2007; Geurden et al., 2008; Yang et al., 2009; Ye et al., 2013; Paz e Silva et al., 2014; Kaupke et al., 2017). Unfortunately, in our lamb study (**III**), it was difficult to determine the prevalence of both parasite infections in the study farm, as we were only able to retrieve faecal samples from a non-representative subset of the population.

In the weeks before and after lambing, ewes infected with *Cryptosporidium* and/or *Giardia* can start to shed (oo)cysts at increased rates, which could be one reason why lambs are infected in the first days after birth (Xiao et al., 1993, 1994; Ortega-Mora et al., 1999; Ye et al., 2013). We speculate that in the reindeer calves (**I**) and lamb (**III**) studies, animals most likely acquired *Cryptosporidium* and *Giardia* infection from their mothers, as they were not separated from them. In the dairy calf study (**II**), neonates were immediately separated from their mothers, and thus, they most likely acquired infection from their previously infected neighbouring calves.

It would have been interesting to measure *Cryptosporidium* and *Giardia* antibody titres in the colostrum to see if they would have had significant effects on delaying infection. In a study where ewes were hyper-immunized against *C. parvum*, lambs experienced fewer clinical signs and achieved higher weight gains (Martín-Gómez et al., 2005). In some cases, feeding regular colostrum has been shown to delay and shorten the time period of oocyst shedding (La Ragione et al., 2006), but in another study, even complete colostrum deprivation did not significantly affect shedding patterns (Ortega-Mora et al., 1993). The quality and quantity of administered colostrum was measured directly in the dairy calf study

(**II**) and indirectly in the reindeer calves (**I**) and lamb (**III**) studies, but no significant associations were found with *Cryptosporidium* and *Giardia* infections.

6.2 Acute phase response and parasitic infections

As described in the literature overview (chapter 2.9 and Table 2), there is limited information available about APR associated with parasitic infections in young ruminants. In this dissertation, animals were all infected naturally; thus, without a proper control group, it was difficult to estimate by how much the diagnosed parasitic infections increased the APP concentrations. Nevertheless, in the dairy calf study (**II**), thanks to HL metaphylaxis, the onset of acute cryptosporidiosis was delayed in correctly treated animals when compared to nontreated ones. The most pronounced difference was seen in the second week of life, when the nontreated animal group's average Hp concentration was 1030 mg/l versus the correctly treated animals 503 mg/l; in comparison, the reference value for healthy animal is <196 mg/l (Seppä-Lassila et al., 2013).

There have been suggestions that colostrum does not provide protection against *Cryptosporidium* infection in lambs (Ortega-Mora et al., 1993). Nevertheless, we saw that levels of GLOB concentration and *Cryptosporidium* infection in lambs were negatively correlated, indicating that colostrum might still have had some protective effect. This beneficial effect of colostrum has been previously observed in other studies (Martín-Gómez et al., 2005, 2006). The downside of consuming colostrum containing anti-*Cryptosporidium* antibodies might be that it prevents the lamb immune system from coming into contact with parasite antigens and developing protective immunity, which was indicated by detecting increased numbers of infections at the second and third weeks of age.

In *Cryptosporidium* infection, the APR reaction strength was dose-dependent, but this would have to be tested with an experiment. Dairy calves that had no oocysts detected in the faeces had an average Hp = 537 mg/l, while shedding animals had on average an approximately 900 mg/l Hp concentration. At the same time, SAA values remained close to the healthy animal reference value <178 mg/l. This finding suggests that heavy *Cryptosporidium* infection, which could cause damage to small

intestine villi, can trigger a more pronounced Hp increase compared to SAA. Different APP concentration profile changes were also previously observed during various bacterial infections in ruminants (Eckersall et al., 2007; El-Deeb and Elmoslemany, 2016). Additionally, it would have been interesting to take into consideration possible co-infections, such as rotavirus and coronavirus, which had been previously diagnosed on the farm and which amplify APR when combined with cryptosporidiosis (Pourjafar et al., 2011).

6.3 Acute phase proteins and acute phase response in neonatal ruminants

The mean SAA and Hp concentrations at the day of parturition in the reindeer study (**I**) was similar to the results previously reported by Orro et al. (2006): SAA = 3 mg/l vs. 5 mg/l and Hp 0.41 g/l vs 0.49 g/l. At the second week of life, the mean SAA was at a higher level than what Orro et al. (2006) described, at 7.2 mg/l vs. 6.2 mg/l, but Hp was lower, at 5.5 g/l vs. 6.1 g/l.

In the lamb study (**III**), it was not possible to determine if serum samples were collected before the consumption of first colostrum; thus, the average SAA concentration (21.1 mg/l) appeared to be significantly higher than what was reported in a study by Dinler et al. (2017), who reported it to be approximately 2.6 mg/l. Nevertheless, the Hp concentrations were similar between the two studies (0.2 and 0.2 g/l) and considerably higher than in the study by Eckersall et al. (2008), 0.2 vs. 0.02 g/l. Despite this substantial difference in Hp, it is interesting to note that results for SAA were more similar, at 18 vs. 21 mg/l (Eckersall et al., 2008). This gives us hope that in lambs, reference values for healthy SAA and Hp can be obtained. The exact source of SAA in colostrum is still debatable, as it has been found in both ewes whose udder is healthy and in those experiencing mastitis (Miglio et al., 2013; Scumaci et al., 2015). Nevertheless, colostrum's SAA concentration had a significant association with lamb APPs (SAA and Hp) in the first days of life (**III**), which appears to indicate that this low abundance protein might have some influence on APR development in this period. The magnitude of APR regulation by colostrum remains to be studied.

The mean SAA levels in the dairy calf study (**II**) peaked at approximately 7 days of age at 189 mg/l, which was similar to the results of the Orro

et al. (2008) study in which healthy calves' SAA also peaked at 7 days but at a lower level (112 mg/l). Negative associations of second week of life APP concentrations and short-term growth rates have already been seen in lambs (Peetsalu et al., 2019), dairy calves (Seppä-Lassila et al., 2018) and beef calves (Seppä-Lassila et al., 2017); we saw a similar effect in dairy calves (**II**), but not in reindeer calves (**I**). In lambs, a positive association was found between ADWG and Hp (**III**). Even in light of these mixed results, the adaptation process of the immune system at the second week of life still appeared to have a significant impact on future production as indicated through ADWG. We propose this to be a critical period in neonatal ruminants' immune system development, where it adapts to microflora present in the surrounding environment, reflected through APR. It was also observed that SAA and Hp peaked at different times across the studies, which might also reflect various stages of immune system adaptation to the surrounding microflora (Kanther et al., 2014).

ALB concentrations in different studies did not appear to have clearly and strongly definable peaking periods, as seen with SAA and Hp. Thus, measuring and using ALB in future studies related to neonatal APR appears to have limited value.

6.4 Acute phase response and future performance

When investigating the association between parasitic infection in combination with APR and using ADWG as an outcome, we did not find significant associations in any of the studies with the infections. This result might have occurred because all of the studies were observational and the infections occurred naturally. If one would experimentally infect neonatal ruminants with *Cryptosporidium* and/or *Giardia*, then in case of a successful infection, detectable APR should follow (Enemark et al., 2003a; Pourjafar et al., 2011; Dinler et al., 2017).

In the dairy calf (**II**) and lamb (**III**) studies, Hp had a significant association with ADWG, but interestingly the relationship was in opposite directions, negative and positive, respectively. No significant associations between short-term growth and early life SAA concentrations were seen in the reindeer (**I**) or lamb (**III**) studies, which is opposite to what has been reported in the case of SAA concentrations in reindeer (Orro et al., 2006), beef calves (Seppä-Lassila et al., 2017), dairy calves (Seppä-

Lassila et al., 2018) and lambs (Peetsalu et al., 2019). Positive associations with ADWG have been reported in beef calves with ALB concentration (Seppä-Lassila et al., 2017) and in dairy calves with Hp levels (Seppä-Lassila et al., 2018), but no significant association was found in lambs with Hp concentrations (Peetsalu et al., 2019). These contradicting results in our studies (**II** & **III**) might be partially due to different infection pressures, as dairy calves experienced strong *Cryptosporidium* outbreaks. Dose-dependent APR was observed in dairy calves that were inoculated with *E. zuernii* oocysts (Lassen et al., 2015), which might also have been the case in our dairy calf study (**II**). Infections are not the only factor that can modulate the strength of APR in the neonatal period, and alternatively these might also be related to the mothers' nutrition during pregnancy (Eckersall et al., 2008).

Hp concentration was used in the ADWG model in the form of AUC in the dairy calf study (**II**), which is a reflection of a cumulative concentration curve over a period of time. High Hp AUC suggests long-lasting and strong APR, which could have had negative effects on feed intake and triggered increases in catabolism, resulting in a lower ADWG (Gabay and Kushner, 1999). In the lamb study (**III**), Hp was used in the ADWG model as a single time point measurement at two weeks of age. This single time point might have indicated that the innate immune system was activated in this period, and that the outcome of this reaction was beneficial for the host, reflected later by a higher ADWG.

Considering the previously presented findings, it should be possible to measure APPs for assessing the magnitude of infection pressure, including neonatal parasitic infections and their impact on future performance. For example, improper management of *Eimeria* infections in dairy calves at the herd level has been demonstrated to have significant negative economic effects (Lassen and Ostergaard, 2012). Thus, assessing the magnitude of unwanted inflammatory responses and knowing its impact on future performance could be important for better herd management. The best candidate for measuring APR appears to be Hp, measured at the second week of life (**II** & **III**). For best results, several consecutive tests should be performed to calculate the AUC, which reflects APR over a period of time (Lassen et al., 2015). If this task were quick and easy, or even automated, allowing veterinarians or farmers to apply it to a group of animals, and if the results were significantly above reference values (Seppä-Lassila et al., 2013), then it would be a very good indication

that further corrective actions would be needed or future performance would degrade otherwise. In addition, the timing of Hp testing appears to be very important; it should be done at two weeks of age or calculated as AUC from several consecutive tests from the first weeks of life (**II** & **III**). Measuring serum Hp in older beef calves that have a body mass of approximately 250 kg after long periods of transport and sorting them based on test results did not give a significant beneficial result in terms of future performance (Holland et al., 2011).

6.5 Perspectives of future research

In the dairy calf study, we saw that *Cryptosporidium* infection triggered a more pronounced increase in Hp but not SAA. This phenomenon should be studied in more detail and possibly tested in experimental settings.

The transfer of immunity with APPs from mother to offspring by colostrum requires further studies. In our lamb study (**III**), we did see a positive association of colostral SAA and lamb serum SAA. The transfer of APPs has been suggested in lambs by Hernández-Castellano et al. (2014), while a study in dairy calves was unable to find an association (Orro et al., 2008). Nevertheless, it remains to be investigated how much the APPs in colostrum influence the development of neonatal ruminants' immune systems.

An important question is how much does the microbiota influence the neonatal ruminant APR? Research done by Kanther et al. (2014) in zebrafish showed that microbiota directly influenced neutrophil activity by inducing increased production of SAA. Future research on similar interactions in ruminants could elucidate this possible connection.

Current results indicate the importance of immune system development/adaptation during the second week of life, as APP concentration changes at that time had a significant association with future performance measured through the growth rate. It could be that the decrease of maternal immunity and increase of infection pressure from the surrounding environment reached their critical point at this time period (the "window of susceptibility"). In this case, it would beneficial to develop a quantitative indicator to assess the success of this critical adaptation period.

7. CONCLUSIONS

A higher GLOB concentration in lamb serum in the first week of life is positively associated with *Cryptosporidium* infection in the third week of life, thus indicating that colostrum might delay the natural onset of the immune response against the parasite, leaving lambs more susceptible to infection later (**III**).

Early *Giardia* infection in reindeer calves (before 12 days of life) is positively associated with higher SAA and lower ɣ-globulins concentrations. This association suggests *Giardia* infection triggering the calf immune reaction (**I**).

In reindeer calves, there is a negative association between higher concentrations of ɣ-globulins and early life *Giardia* infection early in life, which suggests a protective effect from passive immunity against parasitic infection (**I**).

Correctly performed HL metaphylaxis against *Cryptosporidium* infection delays the onset of shedding oocysts (**II**). Even incorrectly performed treatment of HL appears to lower overall mortality when compared to non-treated animals, during acute outbreaks (**II**).

Dairy calves that did not receive treatment or that received incorrect treatment against *Cryptosporidium* with HL had significantly stronger APRs (indicated by an increase in Hp) than correctly treated animals. This result suggests that Hp could be a good indicator of the severity of the infection (**II**).

Colostral SAA concentration is positively associated with lamb serum SAA and Hp in the first days of life. This association suggests that the colostrum contains components that could affect the APR in neonatal lambs in the first days of life (**III**).

Higher concentrations of Hp in the second week of life are positively associated with short-term average daily weight gain in lambs (**III**). ADWG is negatively associated with Hp AUC in dairy calves (**II**). These findings suggest that APR in the early period of life can have a significant impact on future performance in two ways.

The current results add confidence that in future, if measuring of APPs (SAA and Hp) is performed routinely and is automated, then both veterinarians and livestock managers can more precisely assess the health of animals.

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SUMMARY IN ESTONIAN

Seedetrakti algloomanakkuste mõju mäletsejaliste ägeda järgu vastusele neonataalperioodil

Sissejuhatus

Pärast sündi läbivad vastsündinud mäletsejalised väga tähtsa ja tundliku ajajärgu. Sellest perioodist alates puutuvad nad kokku ümbritsevas keskkonnas leiduvate erinevate mikroorganismidega. Nii loomaarstile kui ka loomakasvatajale on kasulik, kui neil oleksid hindamise skaala ja/või astmik, mille põhjal saaks kohanemisprotsessi edukust täpsemalt mõõta ja hinnata.

Haigustekitajad, näiteks parasiidid, võivad ägeda nakkuse ajal kahjustada peremeesorganismi kudesid, mis omakorda kutsub esile mittespetsiifilist immuunsüsteemi reaktsiooni, mille ühe osana võib käivituda ägeda järgu vastus (*acute phase response*, APR). Selle tulemusena suureneb või väheneb teatud valkude kogus vereseerumis – neid nimetatakse ägeda järgu valkudeks (*acute phase proteins*, APP). Nende valkude koguse muutust seerumis saab kasutada haigustekitaja poolt vallandatud põletikureaktsiooni tugevuse mõõtmiseks. Samuti on leitud, et esimestel elunädalatel toimunud APP-de kontsentratsiooni suuremal muutusel on märkimisväärne seos hilisema massi-iibega.

Parasitaarsete algloomade *Cryptosporidium spp*. ja *Eimeria spp*. nakkuste puhul on leitud, et need võivad neonataalsetel mäletsejalistel kutsuda esile APR-i. Nakkused võivad põhjustada tugevat kõhulahtisust, mis äärmisel juhul võib lõppeda surmaga. Sagedamini kulgevad need soolestikku kahjustada võivad nugitõved nii noor- kui ka täiskasvanud loomadel märkimisväärsete kliiniliste tunnusteta, avaldudes väiksemas massi-iibes.

Mäletsejaliste neonataalperioodil aset leidvate parasitaarnakkuste seost APR-iga ei ole siiani põhjalikult uuritud, kuigi mõningaid indikatiivseid viiteid on leitud. Seos noorlooma mittetäielikult väljaarenenud immuunsüsteemi ja nugitõve vahel mõõdetuna kvantifitseeritavate valkude sisalduse astmiku kaudu (näiteks kasutades APP-d) aitaks nii loomaarstil kui ka loomakasvatajal seda keerulist suhtlust paremini

hinnata. Nii saaks täpsemalt ennustada, kui palju see võib edaspidi toodangut (näiteks massi-iivet) mõjutada.

APP-sid on suuremal määral uuritud piimaveisevasikatel, võrreldes lambatallede või põhjapõdravasikatega. Viimaste puhul on APP-de kohta avaldatud ainult üksikuid uuringuid. Loetletud mäletsejaliste puhul on leitud, et tähtsamateks ja tugevamateks APP-deks on seerumi amüloid A (SAA) ja haptoglobiin (Hp). Nende valkude kontsentratsioon vereseerumis võib suureneda APR-i käigus 10–100 korda, võrreldes algtasemega. Järgneval astmel on mõõduka tugevusega APP-d: fibrinogeen (Fb), α1-happe glükoproteiin (AGP), lipopolüsahhariidi siduv valk (LBP) ja tseruloplasmiin. Nende valkude kontsentratsioon võib APR-i käigus kasvada väga madalalt algtasemelt 2–10 korda. Täiendavalt on määratletud ka negatiivsed APP-d, millest mäletsejalistel on kõige tähtsam albumiin (ALB) ja selle kogus APR-i käigus hoopiski väheneb.

Neonataalperioodil on immuunsuse väljakujunemisel äärmiselt tähtis osa ternespiimal e kolostrumil. Mäletsejalistel on sündesmokoriaalne platsenta, mis ei võimalda tiinuse ajal emaslooma toodetud antikehadel läbi emakoogi lootesse liikuda. Seega on väga oluline, et vastsündinu saaks õigel ajal kolostrumit ja tarbiks seda piisavas koguses. Nendel piimaveisevasikatel, kellel immunoglobuliinide (Ig) sisaldus veres on 24–48 tundi pärast sündi madalam kui 5–10 g/l, loetakse passiivse immuunsuse ülekanne ebaõnnestunuks (*failure of passive transfer*, FPT). Üks võimalus FPT diagnoosimiseks on ensüüm gammaglutamüültransferaasi (GGT) aktiivsuse määramine noorlooma seerumis esimestel elupäevadel. GGT ensüüm eritatakse kolostrumisse udaras, kust ta imendub ternespiimast esimestel päevadel suures osas vastsündinu vereringesse, kellel antud aine aktiivsus on esimestel elupäevadel väga madal. Erinevates uuringutes on leitud, et FPT diagnoosimiseks on võrreldes seerumi koguvalgu mõõtmisega GGT aktiivsuse määramine täpsem ja tundlikum, sest seda ei mõjuta märkimisväärselt organismi veetustumine, mida võib tekitada näiteks tugev kõhulahtisus.

Krüptosporiid on ainurakne nugiline, mis kuulub parasiitide süstemaatikas *Cryptosporidium*'i perekonda. Parasiit elab sooleepiteeli rakkudel parasitofoorses vakuoolis, mis asub tsütoplasmaväliselt. Krüptosporiidide elutsükkel kulgeb peamiselt soolestikus, kus ta võib paljuneda nii sugulisel kui ka mittesugulisel teel. Väliskeskkonda levivad

krüptosporiidid füüsikalistele ja keemilistele desinfektsioonimeetoditele vastupidavate ootsüstidena (ümarakujulised, läbimõõt 5–9 μm). Uue peremeesorganismi nakatamiseks piisab kümnest ootsüstist.

Giardia duodenalis (sünonüümid *Giardia lamblia*, *Giardia intestinalis*) on samuti ainurakne parasiit. Ta tõvestab soolestiku epiteelirakke, millele parasiit kinnitub ventraalkettaga. Ümbritsevasse keskkonda levib *Giardia duodenalis* nakatunud looma roojas pirnja kuju ja 12−15 μm läbimõõduga tsüstidena. Uue looma nakatamiseks piisab üksikutest tsüstidest.

Nii krüptosporiidi kui ka giardiat peetakse zoonootilisteks haigustekitajateks, kuigi viimase puhul on märgatud tugevamat peremeesliigi spetsiifilisust. Kummagi parasiidi puhul ei ole Eestis praegu saada otseselt mäletsejalistele ette nähtud nakkusvastaseid ravimeid. Krüptosporiidide puhul on võimalik teha küll metafülaktsiat halofuginoonlaktaadiga (HL), aga sellega saab ägedat nakkust edasi lükata, kuid mitte seda vältida. Erinevates uuringutes kasutatud *Giardia*-vastased ravimid on ainult ajutiselt suutnud nakatunud loomade parasiidi tsüstide väljutamist roojaga keskkonda vähendada.

Väitekirja raames koostatud artiklid on tähistatud rooma numbritega vastavalt loomaliigile: põhjapõdravasikad (**I**), piimaveisevasikad (**II**) ja lambatalled (**III**). Kõik doktoritöö kolm publikatsiooni olid vaatlusuuringud. Igas uuringus oli peamiselt vaatluse all neonataalne periood, mille jooksul koguti ja analüüsiti loomadelt vere- ja roojaproove ning jälgiti massi-iivet. Vaadeldava neonataalse perioodi pikkus oli uuringutes erinev, olles lambatalledel (**III**) esimesed kolm elunädalat, piimaveisevasikatel (**II**) kuus nädalat ja põhjapõdravasikatel (**I**) ligikaudu neli nädalat.

Töö eesmärgid

Käesoleva väitekirja peamine eesmärk oli selgitada seedekulglat tõvestavate algloomtõvede peamiselt − krüptosporidioosi ja giardioosi mõju mäletsejaliste APR-ile neonataalsel perioodil (**I**, **II** ja **III**). Täiendavalt selgitati eimerioosi mõju APR-ile piimaveisevasikatel (**II**).

Käesoleva väitekirja kitsamad eesmärgid olid alljärgnevad:

1) Selgitada, kuidas mõjutab krüptosporidioosi metafülaksia esimestel elukuudel piimaveisevasikate APR-i ja massi-iivet (**II**).

2) Selgitada neonataalsel perioodil toimunud APR-i ja parasitaarnakkuste võimalikku mõju massi-iibele esimestel elukuudel (**I**, **II**, ja **III**).

3) Kirjeldada põhjapõdravasikate (**I**), piimaveisevasikate (**II**) ja lambatallede (**III**) vereseerumi APP kontsentratsiooni muutusi esimestel elunädalatel.

Uurimistulemused

Väitekirja esimene uuring (**I**) hõlmas põhjapõdravasikaid (*Rangifer tarandus tarandus*) (n = 56) perioodil esimesest kuni 33. elupäevani ja neilt koguti ning analüüsiti seerumi- (n = 609) ja roojaproove (n = 366). Uuriti loomade nakatumist giardiate ja krüptosprodiididega ning erinevate APP-de ja gammaglobuliinide kontsentratsiooni seerumis. Lineaarregressiooni mudelite abil uuriti võimalikke seoseid varajase *Giardia* nakkuse, APP-de kontsentratsiooni ja passiivse immuunsuse omandamise vahel.

Giardioos tuvastati kõikide ja krüptosporidioos 23% põhjapõdravasikate roojaproovides. Statistiliselt oluline negatiivne seos ($p = 0,032$) leiti varajase giardioosi (enne 12. elupäeva) ja gammaglobuliinide kontsentratsiooni vahel. Oluline positiivne seos oli sama parasiidi varajase nakkuse ja SAA kontsentratsiooni vahel ($p = 0,042$). Antud uuringu tulemused viitasid kolostrumi võimalikule kaitsvale toimele giardioosi vastu. Samuti näis, et varane giardioos kutsus APR-i tugevamini esile kui hiljem tuvastatud nakkus.

Väitekirja teine uuring (**II**) hõlmas piimaveisevasikaid (n = 144), kellelt koguti ning analüüsiti esimese kolme elukuu jooksul seerumi- (n = 901) ja roojaproove (n = 767). Kõik kõnealused vasikad sündisid kahe kuu jooksul samas farmis. Igalt loomalt koguti proovid kuuel järjestikusel nädalal ühenädalase ajavahemikuga, lisaks võeti täiendav proov kolme kuu vanuselt. Lähtuvalt metafülaksiast HL-iga krüptosporidioosi vastu, jagati vasikad kolme rühma: a) metafülaksiata (n = 34), b) ebapiisav metafülaksia (manustamine algas > 48 tundi pärast sündi või

kestis vähem kui 7 päeva; n = 45) ja c) õigesti teostatud metafülaksia (manustamine algas < 48 tundi pärast sündi ja kestis ≥ 7 päeva; n = 65). APR-i, parasitooside ja massi-iibe vaheliste seoste uurimiseks kasutati lineaarregressioonimudeleid, milles vasikas oli juhuslik muutuja. Vasikate suremuse riski uurimiseks koostati logistiline retrospektiivne juhtumikontrollmudel.

Kolmekuulise vaatlusperioodi jooksul hukkus 21 vasikat (15%), kellest enamik (67%) kuulus rühma, kellele ei manustatud HL-i. Õigesti läbi viidud metafülaksia HL-iga lükkas märkimisväärselt ($p < 0,001$) edasi tuvastatava krüptosporiidi ootsüstide väljutamise roojaga. Hp ja SAA kontsentratsioonid olid teisel elunädalal oluliselt väiksemad nendel loomadel, kes olid saanud korrektselt HL-i võrreldes nendega, kes ei olnud saanud metafülaksiat. Kolme kuu massi-iive oli seotud nii teise elunädala Hp kontsentratsiooniga kui ka HL metafülaksia tüübiga. Huvitaval kombel oli kolme kuu vanuste metafülaksiata loomade keskmine massi-iive suurem kui metafülaksiat saanud vasikatel, seda võis põhjustada asjaolu, et metafülaksiata jäänud rühmast jäid ellu peamiselt kõige elujõulisemad ja ka kõrgeima massii-iibega loomad. Roojaproovides, kus tuvastati *Giardia* ja *Eimeria* nugilised, ei esinenud oluliselt sagedamini kõhulahtisusele viitavaid tunnuseid, kuid *Cryptosporidium*-positiivsed roojaproovid olid oluliselt sagedamini kõhulahtisuse tunnustega ($p < 0,001$).

Õigesti läbi viidud metafülaksia HL-ga kahandas oluliselt krüptosporiidi ootsüstide väljutamist esimesel elunädalal ja vähendas hukkumise riski. Ebaõigel metafülaksial näis olevat positiivne mõju elumusele, aga see ei vähendanud märkimisväärselt ootsüstide väljutamist. Teise elunädala tugevam APR oli positiivselt seotud suurema hulga krüptosporiidi ootsüstide väljutamisega roojas.

Väitekirja kolmas uuring (**III**) hõlmas mahefarmi lambatallesid (n = 269), kellelt koguti ning analüüsiti seerumi- (n = 692) ja roojaproove (n = 141) esimese kolme elunädala jooksul. Samade lambatallede uttedelt koguti ka kolostrumiproove (n = 181). Uuriti võimalikke seoseid APR-i ja hilisema massi-iibe (mõõdetuna 122 elupäeva vanuselt) ning krüptosporidioosi ja giardioosi vahel.

Hp ja ALB kontsentratsioon teisel elunädalal oli positiivselt seotud 122 päeva vanuste lambatallede massi-iibega. Uttede kolostrumi

SAA kontsentratsioon oli positiivselt seotud lambatallede SAA ja Hp kontsentratsiooniga 2.–4. elupäeval. Lambatallede globuliinide kontsentratsioon 2. ja 3. elunädalal oli negatiivselt seotud krüptosporiidide positiivsete roojaproovide leidmisega.

Järeldused

Varajane *Giardia*-nakkus põhjapõdravasikatel on positiivselt seotud suurema SAA ja väiksema gammaglobuliinide kontsentratsiooniga. See tulemus viitab olulisele vasika immuunsüsteemi ja giardioosi vahelisele vastastikmõjule (**I**).

Märkimisväärne negatiivne seos gammaglobuliinide ja varajase *Giardia*-nakkuse vahel viitab, et passiivne immuunsus võib aktiivset tsüstide väljutamise algust edasi lükata (**I**).

Õige metafülaksia HL-iga krüptosporidioosi vastu lükkab edasi perioodi, millal nakatunud piimaveisevasikad hakkavad aktiivselt ootsüste roojaga väljutama. Isegi kui metafülaksia HL-iga ei teostatud korrektselt, näis see avaldavat elumusele positiivset mõju, aktiivse haiguspuhangu perioodil, võrreldes metafülaksiata loomadega (**II**).

Piimaveisevasikad, kellel ei teosta metafülaksiat HL-iga või on metafülaksia ebakorrektne, kujuneb välja märkimisväärselt tugevam APR võrreldes korrektselt metafülaksiat saanud loomadega. Võimalik, et äge krüptosporidioos põhjustab suurema Hp kontsentratsiooni tõusu (**II**).

Uttede kolostrumis olev SAA ning lambatallede esimestel elupäevadel kogutud vereseerumist leitav SAA ja Hp kontsentratsioonide vahel on märkimisväärne seos. See viitab, et kolostrum sisaldab aineid, mis võivad esimestel elupäevadel mõjutada lambatallede APR-i (**III**).

Lambatalledel, kellel on esimesel elunädalal globuliinide suurem kontsentratsioon, diagnoositakse kolmandal elunädalal palju sagedamini krüptosporidioos. See viitab, et ternespiimaga saadav passiivne immuunsus võib aidata edasi lükata varajast aktiivset ootsüstide väljutamist, kuid samas takistada loomuliku immuunsuse väljakujunemist selle parasiidi vastu (**III**).

Tugevam APR-i reaktsioon teisel elunädalal on positiivselt seotud lambatallede massi-iibega (**III**). Piimaveisevasikate (**II**) Hp kontsentratsioon on negatiivselt seotud hilisema massi-iibega. Tulemused viitavad, et esimeste elunädalate APR-il on märkimisväärne seos hilisema toodanguga, kuid kahesuunaline.

Antud töö lisab kindlust, et kui tulevikus muutub APP-de mõõtmine loomadel rutiinsemaks ja automatiseerikse, siis SAA ja Hp, näol oleks nii loomaarstil kui loomakasvatajal võimalik täpsemalt hinnata hetke tervisliku seisundit.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Professor Toomas Orro and Doctor Brian Lassen. I would like to thank Toomas for providing me the opportunity to become a doctoral student and for introducing me to the world of science. I would like to thank Brian for giving invaluable help with parasites and excellent feedback on manuscripts.

I would like to thank the farms and their personnel for providing us with help in sample and data collection. I would especially like to thank veterinarians Kärt Kalvet and Kadri Kaugerand.

I would like to thank everyone who helped collect and analyse the extensive number of samples that were collected: Pille Paats, Külli Must, Azzurra Santoro, Niina Sidarova, Elisabeth Dorbek-Kolin, Kristel Peetsalu, Leena Seppä-Lassila, Katri Pentjärv, Ants Kuks and veterinary medicine students.

I would like to thank Professor Toomas Orro, Professor Arvo Viltrop and Professor Ülle Jaakama for giving me an opportunity to teach veterinary immunology and physiology, thus giving me a lifeline to successfully complete my doctoral studies.

I would like to thank all of the co-authors, who all provided invaluable help in accomplishing this work.

I would like to thank the Estonian University of Life Sciences doctoral school, the Estonian Research Council project IUT8-1, the ASTRA program and NOVA University Network for providing an opportunity to participate in very good workshops, courses and conferences.

Last, but not least, I would like to thank my family and friends for their love and support on this doctoral studies journey, which was rather long and sometimes quite difficult.

I

ORIGINAL PUBLICATIONS

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. DOI: 10.1016/j.cimid.2017.08.001

Comparative Immunology, Microbiology and Infectious Diseases 54 (2017) 45–50

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Full length article

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

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ARTICLE INFO

Keywords:
Semi-domesticated reindeer
Giardia infection
Acute phase response
Serum amyloid A

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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http://dx.doi.org/10.1016/j.cimid.2017.08.001
Received 20 April 2017; Received in revised form 8 August 2017; Accepted 16 August 2017

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

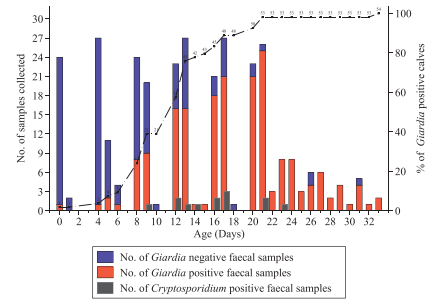

Fig. 1. Giardia positive faecal samples. The age of calf for first positive faecal sample and number samples collected in a day. Left hand y-axis shows no. of faecal samples collected on given day. Right hand y-axis shows% of calves that had at least one positive Giardia sample collected by that age.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

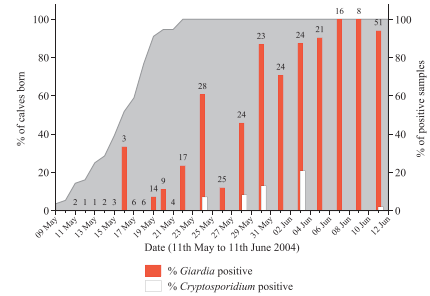

Fig. 2. *Giardia* and *Cryptosporidium* positive faecal samples (%) at given date. Left hand y-axis shows how many of the calves were born by a given date. All the calves were born between 9th May and 22nd May 2004. Right hand y-axis shows the% of how many of the collected faecal samples tested positive 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

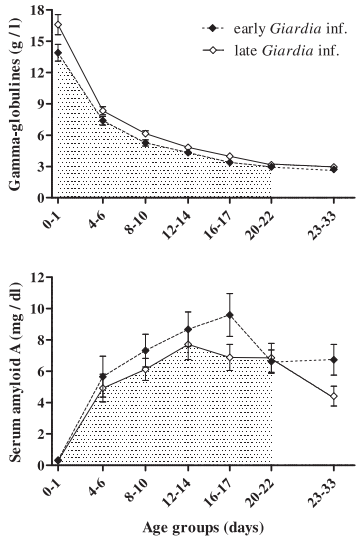

Fig. 3. Mean (± SEM) γ-globulin and serum amyloid-A (SAA) concentrations in serum of reindeer calves in early and late Giardia infection groups (from 0 to 22 days of age n = 19 and n = 29 respectively, at 23–33 age of age n = 19 and n = 26 respectively) during study period (0–33 days of age). Filled 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.

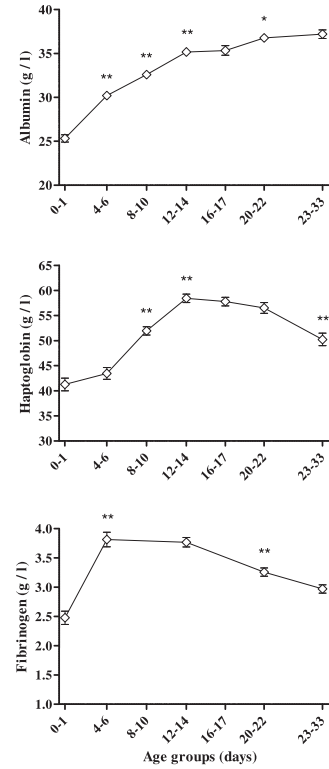

Fig. 4. Mean (± SEM) albumin (ALB), haptoglobin (HP) and fibrinogen (FIB) concentrations in serum of reindeer calves during study period (0–33 days of age). Sample size for ALB and HP at 0–22 days of age and at 23–33 days of age n = 48 and n = 45 respectively. For FIB at 0–1 days, 4–22 days and 23–33 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

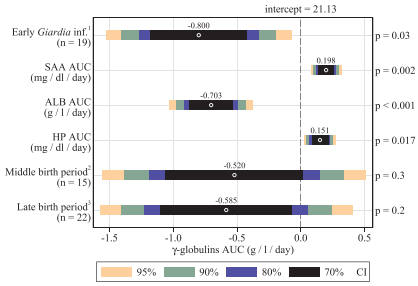

Fig. 5. γ-globulin AUC regression model coefficient plot (age 0–22 days). Model confidence intervals (CI) are presented as horizontal bars. Point estimates for variables are shown on top of the bars. AUC was calculated for each animal (n = 48) using a trapezoidal method for 6 time points and averaging 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)

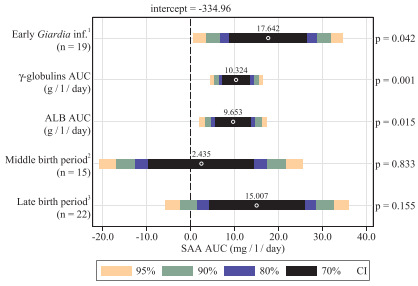

Fig. 6. Serum amyloid A (SAA) AUC regression model coefficient plot (age 0–22 days). Model confidence intervals (CI) are presented as horizontal bars. Point estimates for variables are shown on top of the bars. AUC was calculated for each animal (n = 48) using a trapezoidal method for 6 time points and 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.

Acknowledgements

We would like to thank Brian Lassen for advice and help with the manuscript. The project was partially funded by the Estonian Research Council project IUT8-1.

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Niine, T., Dorbek-Kolin, E., Lassen, B., Orro, T. 2018. *Cryptosporidium* outbreak in calves on a large dairy farm: effect of treatment and the association with the inflammatory response and short-term weight gain, Res. Vet. Sci. 117, 200-208. DOI: 10.1016/j.rvsc.2017.12.015

Research in Veterinary Science 117 (2018) 200–208

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Cryptosporidium outbreak in calves on a large dairy farm: Effect of treatment and the association with the inflammatory response and short-term weight gain

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ARTICLE INFO

Keywords:
Calf morbidity
Antiparasitic treatment
Neonatal
Gastrointestinal disorder
Innate immunity response

ABSTRACT

Cryptosporidium spp. infections in neonatal dairy calves can cause diarrhoea and, in rare cases, death. The infection is usually self-limiting, but halofuginone lactate (HL) can be used prophylactically. Calves (n = 144) in the study were born during a 2-month period on one farm. A total of 901 serum and 767 faecal samples were collected. Based on HL treatment, the calves were divided into 3 groups: I) not treated, II) treated incorrectly (treatment started > 48 h after birth, or lasted < 7 days), and III) treated correctly (started < 48 h after birth, and lasted ≥ 7 days). Over the 3-month observation period, 14.6% (n = 21) of the calves died, of which most (67%) had not been treated with HL. Correctly performed treatment of cryptosporidiosis significantly delayed the onset of oocysts shedding (P < 0.001) and reduced haptoglobin (HP) and serum amyloid A (SAA) concentrations in the second week of life. HP concentration and HL treatment were negatively associated with weight gain at 3 months of age. *Cryptosporidium* positive faecal samples were significantly (P < 0.001) more likely to be diarrhoeic but *Giardia* or *Eimeria* positive samples were not. Correct prophylactic treatment with HL delayed the shedding of *Cryptosporidium* oocysts and improved survival, but was negatively associated with weight gain. Incorrect treatment had a low impact on mortality and resembled no treatment regarding the proportion of calves shedding oocysts. Acute phase response (APR) in the second week of life seemed to be positively associated with shedding high amounts of *Cryptosporidium* oocysts.

1. Introduction

Cryptosporidium can be found in cattle herds worldwide (O'Handley and Olson, 2006) and has also been found in Estonian dairy farms (Lassen et al., 2009). *Cryptosporidium* infection in dairy calves can lead to villous atrophy in the small intestine mucosa and increase intestinal permeability (Wyatt et al., 2010). Consequently, these pathologies can lead to diarrhoea and increased risk of mortality (Delafosse et al., 2015). Neonatal calves have a higher risk of being negatively affected and shed *Cryptosporidium* oocysts more frequently than adult livestock (Maddox-Hyttel et al., 2006; Featherstone et al., 2010). The incubation period of cryptosporidiosis varies on average from 5 to 7 days, but symptoms can start as early as 2 days post-infection (Abeywardena et al., 2015). Infected calves typically excrete oocysts with faeces for about 2 weeks (Fayer et al., 1998; O'Handley et al., 1999). Under experimental conditions, the *Cryptosporidium* oocysts count in faeces rises a day before the onset of diarrhoea, peaks, and drops 2 days before the diarrhoea becomes less severe (Operario et al., 2015). In previous longitudinal studies, the highest number of *Cryptosporidium* oocysts were found during the second or third weeks of the calves' lives (Santín et al., 2008; Coklin et al., 2010).

Giardia's role as a pathogen in production animals is debated (Geurden et al., 2010). *Giardia* infections can be chronic and last for months (Grit et al., 2014). *Giardia* and *Eimeria* have multifactorial pathogenesis that leads to microvilli alteration, diarrhoea, and weight loss in production animals (Olson et al., 1995; Geurden et al., 2010; Lassen et al., 2015). In a recent study, *Giardia* infection was also associated with haemorrhagic diarrhoea in calves (Lee et al., 2016). In cases of experimental inoculation with the parasite, dairy calves usually survive the infection with only minor repercussions (Grit et al., 2014). Calves who are infected after birth start shedding oocysts around the third week of life (O'Handley et al., 1999). The *Giardia* infection rate is related to the age and has been found to peak 6 weeks after birth (Winkworth et al., 2008; Coklin et al., 2010).

If *Cryptosporidium* and *Giardia* infections are concurrent, it could cause morphological damage to the jejunum to a lesser extent; this

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https://doi.org/10.1016/j.rvsc.2017.12.015
Received 21 April 2017; Received in revised form 23 November 2017; Accepted 26 December 2017

could be because of the antagonistic nature of the coinfection (Ruest et al., 1997). Very low doses of both parasites (around 10 oocysts/cysts) are required to mount an successful infection (Rendtorff, 1954; Okhuysen et al., 1999).

For the prophylactic treatment of *Cryptosporidium*, halofuginone lactate (HL) can be used; it has cryptosporidiostatic effect that in most cases has been proven to be effective in reducing the excretion of oocysts (Joachim et al., 2003; Silverlås et al., 2009).

The acute phase response (APR) is a series of complex physiological events occurring after tissue injuries or infections (Cray et al., 2009). In response to APR, the concentrations of specific acute phase proteins (APP), serum amyloid A (SAA) and haptoglobin (HP), can increase in ruminant serum > 1000-fold (Ceciliani et al., 2012; Eklund et al., 2012). In cases of viral (foot-and-mouth disease and bovine respiratory syncytial virus) or parasitic (*Eimeria* and *Cryptosporidium*) infections, APP concentrations can increase in domestic ruminants' blood serum (Orro et al., 2011; Pourjafar et al., 2011; Stenfeldt et al., 2011; Lassen et al., 2015). In neonatal calves, APPs go through significant changes during first 2 to 3 weeks of life (Orro et al., 2008; Tóthová et al., 2015), suggesting that APPs have a role in the adaptation of neonate calves to the new environment. In reindeer calves, lambs, and beef calves, high concentrations of SAA measured in the second week of life have been associated with lower weight gain recorded many months later (Orro et al., 2006; Peetsalu et al., 2013; Seppä-Lassila et al., 2015, 2017).

Cryptosporidium infection has been shown to increase the APP concentration in dairy calves (Pourjafar et al., 2011). However, the effect of *Cryptosporidium* infection combined with prophylactic treatment on the immune system and growth remains unknown. In this study, we examined the effects of untreated, incorrect treatment, and correct treatment with HL in an outbreak of cryptosporidiosis in neonatal calves.

2. Materials and methods

2.1. Ethics statement

This study 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).

2.2. The farm

This study took place on a large dairy farm in Järvamaa County, Central-Estonia. The average milk production per cow in 2015 was 10,000 kg (Estonian Livestock Performance Recording Ltd., 2015). During the study, there were about 1800 dairy cows in the farm.

2.3. Animals

Inclusion criteria: all of the female calves born from January 21 to March 16, 2015, were included in the study (n = 145). Exclusion criteria: twins (1 pair of twins born) and male calves. One animal was dropped from the study because she died before any samples were collected.

The calves were separated from their mothers immediately after birth. In the first 4 weeks, the calves were kept in individual pens with wooden floors and straw bedding. After that, they were moved to group pens with concrete flooring and straw and sawdust bedding. Group pens were composed of 8–10 calves. Both individual and group pens were housed in the same building until the animals were 2 months old. Immediately after birth, the calves were weighed with a digital scale (MS4 PW, Excell Precision Co., Ltd, Vilnius, Lithuania). Additional weight measurements were taken around 1 and 3 months of age with a digital scale (KERN EOS 150K100NXL, Kern & Sohn GmbH, Balingen, Germany) and measuring tape (ANImeter, Albert Kerbl GmbH, Buchbach, Germany), respectively.

2.4. Feeding

The calves were fed 3 l of unpasteurised colostrum in the first 2 h of life. The colostrum given to the calves was collected from the dam and the quality examined visually and with a hydrometer (Kruuse colostrum densimeter, Jørgen Kruuse A/S, Langeskov, Denmark). If the colostrum was of unsatisfactory quality (n = 2), deep frozen colostrum from another dam was provided. The calves were fed 2–3 kg of 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. Then their feed was switched to milk powder (Josera GoldenSpezial, Josera GmbH & Co. KG, Kleinheubach, Germany) solution (1 l of warm water + 140 g of milk powder) of 2 × 3 l/day for 1 week with free access to starter feed (Prestarter, Agrovarustus OÜ) and hay. At 1 month of age, the milk powder product was changed (Josera IgluStart, Josera GmbH & Co. KG) and decreased each week with 0.5 l per feeding. Around weaning time (70–80 days of age), the calves received 2 × 2 l/day. After weaning, the calves had free access to starter feed (Starter, Agrovarustus OÜ), hay, and silage. No significant changes were made to the feeding regiments or feed itself during the study period.

2.5. Treatments

All of the calves were vaccinated on the second day after birth against parainfluenza virus type 3 (PI3V) and bovine respiratory syncytial virus (BRSV) (Rispoval, Zoetis Belgium SA, Louvain-la-Neuve, Belgium). At 3 months of age, all of the calves were vaccinated against bovine herpesvirus-1 (BoHV-1) (Hiprabovis, Laboratorios HIPRA, S.A., Girona, Spain). Prophylactic treatment against *Eimeria* infection was done once by administrating toltrazuril (Cevazuril, Ceva Santé Animale, Libourne, France) to every calf between 29 and 65 days of age. Prophylactic treatment of the *Cryptosporidium* infection was done using HL (Halocur, Intervet International B.V., Boxmeer, Netherlands).

The study was designed as observational cohort study. Based on the HL treatment regime, the calves were divided retrospectively into 3 groups: I) not treated (n = 34), II) treated incorrectly (treatment started > 48 h after birth, or lasted < 7 days) (n = 45), and III) treated according to manufacturer's instructions (started < 48 h after birth, and lasted ≥ 7 days) (n = 65).

All animals in the study requiring medical treatment received it from the farm's veterinarian. Diarrhoea was treated by administering electrolyte solutions, and if needed, antibiotics were also given. Antibiotics were also used to treat respiratory infections.

No necropsies were performed on the study animals that died. The farm's veterinarian noted the most likely cause of death based on symptoms, such as diarrhoea, respiratory distress, or lameness.

2.6. Sample collection

Once per week, up to 6 weeks of age, serum and faecal samples were collected from each calf. Follow-up sample collection was done at around 3 months of age.

Faecal samples were collected with a clean disposable latex glove directly from the rectum and placed into clean sealable plastic cups and marked with the last 5 numbers of the animal's ear tag. If the rectum was found empty and the calf could not be stimulated to defecate using finger, the sample collection was abandoned (n = 158). In addition, 83 faecal samples were not collected due to the unexpected death of 21 calves. Faecal samples were stored in an insulated container with cooling elements for 2 h and then kept at 4 °C for a maximum of 48 h until analysis. In total, 767 faecal samples were collected from 144 calves.

Serum samples (n = 901) were collected from the jugular vein in sterile evacuated test tubes using an 18-G sterile needle. Blood samples were transported to the laboratory and centrifuged (3000 RCF for

10 min). All of the serum samples were then stored at − 20 °C until further analysis.

In order to avoid dehorning affecting the APP serum concentrations, all of the blood samples were collected immediately before the procedure. For technical reasons, 8 calves were not sampled before dehorning and were marked as compromised.

2.7. Parasites

Faecal samples were prepared for *Cryptosporidium* and *Giardia* detection in a similar method described for *Eimeria* detection by Lassen et al., 2009, but with slight modifications. In detail, sample preparation followed the same steps: weighing, mixing, diluting, and centrifuging, but after the supernatant was removed and before saturated sugar and solution (ρ = 1.26 g/cm^3) was added, a 20 µl subsample of the 1 ml suspended pellet was fixed on glass slides well (14 mm diameter latex wells). Staining was done using fluorescein isothiocyanate (FITC) conjugated anti-*Cryptosporidium* and anti-*Giardia* monoclonal antibodies (Crypto/Giardia Cel, Cellabs Pty Ltd., Sydney, Australia). The slides were examined using an epifluorescence Nikon Eclipse 80i microscope using 200–400 × magnification. *Cryptosporidium* and *Giardia* oocysts were differentiated visually based on morphology, and considered positive if at least 1 oocyst or cyst was found. All of the oocysts and cysts on the slide were counted and the approximate number of oocysts per gram of faeces (OPG) was calculated, corrected to the total area of the well and to the dilution of the sample (De Waele et al., 2010). In case there were too many oocysts to count, 3 random visual fields on the slide were picked and all of the oocysts were counted in the field of view (Lassen and Lepik, 2014). The counts of each visual field were averaged and multiplied with the fraction of the visual field surface area divided by the total slide surface area to calculate the total number of oocysts on a slide.

DNA was extracted from 12 FITC *Cryptosporidium*-positive faecal samples collected at 3th March from calves borned between 21st January-20th February 2015 (mean age 21 days) using the PSP® Spin Stool DNA Kit (STRATEC Biomedical AG, Birkenfeld, Germany). The DNA was submitted to PCR amplification targeting the 18S rRNA gene of *Cryptosporidum* spp. as described by Zintl et al. (2007), and the 60 kDa glycoprotein (gp60) gene as described by Peng et al. (2001). The PCR products were run on a 2% ethidium bromide stained agarose gel and visualized under an UV transilluminator. Products of approximately 825 bp from the 18S rRNA and approximately 490 bp of the gp60 amplifications were cleaned and submitted to sequencing in two directions using Applied Biosystems® 3130xl Genetic Analyzer. Forward and reverse sequences were aligned using the BioEdit v7.2.5 software (Hall, 1999) to generate consensus sequences and correct potential mismatches. The GenBank BLASTn (Altschul et al., 1990) tool was used to find similarities between the sequences of our PCR products and with deposited nucleotide sequences in the library. Sequences of the 18S rRNA products were used to determine the species of *Cryptosporidium*, and gp60 was used to determine the subtype.

The faecal samples were classified as diarrhoeic or non-diarrhoeic based on visual examination. The remaining 1 ml of concentrated faecal sample from above was examined with a light microscope using the flotation method (Roepstorff and Nansen, 1998) for possible parasites (*Eimeria* spp. and intestinal nematodes). *Eimeria* spp. were differentiated visually based on morphology (Levine, 1985).

2.8. Acute phase proteins and gamma-glutamyltransferase

The concentration of SAA was measured by commercial ELISA kit (Phase BE kit, Tridelta Development Ltd., Dublin, Ireland). The HP concentration was assessed via the method defined by Makimura and Suzuki, 1982), with an alteration using tetramethylbenzidine (0.06 mg/ml) as a substrate and using microtitration plates (Alsemgeest et al., 1994). Bovine acute phase serum (pooled and lyophilised) were used to generate standard curves. Standard provided by the European Commission Concerted Action Project (number QLK5-CT-1999-0153) was used to standardise the assay of bovine plasma sample with a known HP concentration. The range of the standard curve was 75–1160 mg/l.

The intra-assay and inter-assay coefficients of variations for SAA were ˂11% and ˂13% and for HP were ˂13% and ˂10%, respectively.

Analysis of GGT activity was measured using the kinetic method with L-γ-glutamyl-3-carboxy-4-nitroanilide (Persijn and van der Slik, 1976) in a clinical chemistry analyzer (Accent-200 GGT, PZ Cormay S.A., Łomianki, Poland).

2.9. Statistical analysis

Linear regression models were used to check if HL treatments were associated with changes in the HP or SAA concentrations in the first 6 weeks of life. HP or SAA were the dependent variables and both were logarithmically transformed in order to meet the presumption of normal distribution. The explanatory variables were the age (days) at sample collection and HL treatment as a categorical variable.

A random-effects logistic regression model was constructed to investigate if *Cryptosporidium*- or *Giardia*-positive faecal samples in the first 6 weeks of life were more likely to be diarrhoeic. *Eimeria* was excluded from these models, as all the faecal samples from the first 6 weeks of life were negative. The sample being diarrhoeic was added as a binary dependent variable. Explanatory variables were *Cryptosporidium*-positive faecal samples, *Giardia*-positive samples, and age (days). Parasite-positive samples were categorised as follows: 0 = no oocysts or cysts found; 1 = the oocyst or cyst count in the sample below the median count; and 2 = the oocyst or cyst count in the sample above the median count. The calves were added to the model as random intercepts.

A logistic regression model was used to examine if *Eimeria*-positive calves were diarrhoeic at 3 months of age. The dependent variable was diarrhoea (binary) and the explanatory variables were the total number of *Eimeria* oocysts in 1 g of sample (OPG) and the age (days) at sample collection.

For assessing the odds of death within the first 6 weeks of age, a retrospective case-control logistic regression model was constructed. The case group (n = 14) consisted of animals who died before 43 days of age. The control group (n = 49) consisted of animals born ± 3 days to a matching case group of animals that did not die before 43 days of age and whose dams were also either primiparous or multiparous. The dependent variable was death; the explanatory continuous variables were *Cryptosporidium* oocyst count in faecal samples, birth weight, GGT, and APPs (SAA and HP) at the first week of life; and the independent variable was the dam being primiparous or multiparous. Backward step-wise elimination procedure was used for final model.

Linear regression models were used to evaluate if HP and SAA concentrations differed on weekly bases over the first 6 weeks of life based on the *Cryptosporidium* oocyst count found in the faecal samples. The dependent variables were HP or SAA, and both were logarithmically transformed in order to meet the presumption of normal distribution. The explanatory variables were age at sample collection (days) and *Cryptosporidium* infection intensity as a categorical variable (0 = no oocysts found in faecal sample; 1 = low; sample containing less than the median number of oocysts (OPG) when compared to other same weeks' positive results; and 2 = high; sample containing more than the median number of oocysts when compared to the other same weeks' positive results). Bonferroni's multiple comparison correction procedure was used to control Type I errors.

The average area under the curve (AUC) was calculated using the trapezoidal method for different APPs and the parasite oocyst count over 6-week periods as:

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

where t_i = the time of observation, t_{i-1} = the previous time of observation, f_i = APP concentration at the time, and f_{i-1} = APP concentration at the previous time. AUCs were used as summary measures for concentrations of APPs and the oocyst counts over time. The AUC value was divided with the calves' age in order to be comparable between different animals. $AUC_{average}$ = AUC/age at sample collection. The AUC calculation was performed if the calf had 4 observations or more and was not compromised (had serum sample collected prior to dehorning).

Multiple linear regression models were used to determine the association between APPs-AUC results and *Cryptosporidium* and *Giardia* infection. The SAA- and HP-AUC results were used as dependent variables. The independent variables of AUCs for both parasites were: the oocyst or cyst count in the faecal samples, the age and GGT concentration at the first sample collection, and HL treatment as categorical variable. The dependent variables SAA- and HP-AUC results were logarithmically transformed to meet the presumption of normal distribution.

Multiple linear regression models were used to describe the APPs and the *Cryptosporidium* and *Giardia* infections possible association with average daily weight gain (ADWG). The dependent variable was ADWG at the age of 1 month or at the age of 3 months. The independent variables were SAA, HP average-AUCs, *Cryptosporidium* and *Giardia* oocysts-AUCs, age (days) at the first collection of the first sample, age (days) at weight measurement, proportion of diarrhoeic faecal samples, HL treatment categories, and primiparous or multiparous dam's offspring as a categorical variable.

In the linear and logistic regression models, independent variables were selected according to their *P* values using backward stepwise elimination. Independent variables were eliminated from a model if $P > 0.05$. Variables that changed the coefficient of the remaining variables with > 10% were kept as confounders.

Statistical data analysis was done using STATA 14.1 (StataCorp LP, College Station, TX, USA). Basic data management was done using Excel 2013 (Microsoft, Redmond, WA, USA) and Python 3.5.1 (Anaconda 4.0.0 by Continuum Analytics, Austin, TX, USA). The level of a significant result was $P \leq 0.05$.

3. Results

3.1. Parasite infection, diarrhoea, and halofuginone lactate treatment

The treatment initiated to control the outbreak of diarrhoea in the calves with HL was started on February 17 and ended on March 22, 2015. In total, 110 calves were treated an average of 6 times (range 1 to 9). On average, the earliest treatment started on day 3 of life (range 0 to 14) (Fig. 1).

In first six weeks of life, a total of 655 faecal samples (by HL treatment groups: I) no treatment: $n = 131$, II) incorrect treatment: $n = 228$, III) correct treatment: $n = 296$) and 774 serum samples (by HL treatment groups: I) no treatment: $n = 156$, II) incorrect treatment: $n = 272$, III) correct treatment: $n = 346$) were collected. Additionally, 112 faecal and 130 serum samples were collected at 3 months of age.

Cryptosporidium oocysts were found in 33.3% (218/655) of faecal samples and 84.7% (122/144) of calves. *Giardia* cysts were found in 30.8% (202/655) of faecal samples and 76.4% (110/144) calves. Protozoan infections detection and average age of first detection according to different HL treatment groups are presented in Table 2. Mixed protozoan infections were found in 5.8% (38/655) of faecal samples in 22.2% (32/144) of calves.

The median OPG in a positive *Cryptosporidium* and *Giardia* faecal sample in the first six weeks of life, by HL treatment groups was: I) no treatment: 242,844 and 14,035 with range of 70–2,755,554 and 69–469,367, II) incorrect treatment: 333,027 and 79,112 with range of 70–3,646,621 and 70–1,401,208, III) correct treatment: 363,436 and 29,386 with range of 69–10,145,426 and 71–2,652,344.

Out of the 12 FITC *Cryptosporidium*-positive faecal samples the 18S rRNA gene was successfully amplified in six samples and sequence analysis identified these as *C. parvum*. Seven (7/12) samples successfully amplified the gp60 gene; five of which were positive and two that were negative in the amplification of the 18S rRNA gene. Sequence analysis of the gp60-positive samples identified them as *C. parvum* subtype IIaA18G1R1.

Diarrhoea was diagnosed in 53% (344/655) of samples and in 92% (132/144) of calves during the first 6 weeks of age and in 29% (32/112) of samples and in 29% (32/112) of calves at 3 months of age. For the full observation period, 92.4% (133/144) of calves had at least 1 sample that was considered diarrhoeic. The model indicated that in the first 6 weeks of the calves' lives, *Cryptosporidium*-positive samples were associated with diarrhoea but not *Giardia* (Table 1). The age of the calf was negatively associated with diarrhoea (OR = 0.98; $P = 0.007$). Calves shedding *Eimeria* oocysts did not have increased odds of being diarrhoeic ($P = 0.2$).

In general, the highest proportion of *Cryptosporidium*-positive samples was found in calves that were 16–18 days old. When looking at the proportion of animals shedding oocysts by treatment category, calves that got no treatment or were treated incorrectly peaked around 10–12 days of age, while correctly treated animals peaked around 19–21 days of age (Fig. 2).

Eimeria oocysts were detected in 9.5% (73/767) of all the faecal samples collected. *Eimeria* was only detected in faecal samples collected at approximately three months of age (median age: 99 days). Four species of *Eimeria* were detected in a total of 73 faecal samples: *E. bovis* (71.2%, 52/73), *E. zuernii* (45.2%, 33/73), *E. ellipsoidalis* (37.0%, 27/73) and *E. auburnensis* (16.4%, 12/73). Additional results of *Eimeria* infection, grouped by different HL treatments can be found in Table 1S. No helminth eggs or nematode larvae were found.

3.2. Survival

In first 3 months of life, 21 calves (14.6%) died or were euthanised (Fig. 1 and Fig. 1S). The average age of death was 29 ± 24 days (median 16, range 8 to 83). The reasons listed by the farm veterinarian for mortalities were diarrhoea ($n = 12$), respiratory infection ($n = 6$), euthanised because of massive inflammation of the carpal joint or septic umbiliculitis ($n = 2$), and unknown cause ($n = 1$).

In total, 66.7% (14/21) of the calves in the group that got no HL treatment died. In the groups of calves that were treated incorrectly or correctly, 2 and 5 died, respectively (Fig. 1S). Based on the veterinarian's diagnosis, 71% of the deaths in the no treatment group were caused by diarrhoea, and in the other groups, 1 animal succumbed to diarrhoea.

In the retrospective case-control logistic regression model, the odds of a calf dying within the first 6 weeks of life increased with higher SAA concentrations (OR = 1.01; $P = 0.041$). Factors that decreased the odds of a calf dying were: higher GGT activity (OR = 0.99; $P = 0.004$), larger birth weight (OR = 0.76; $P = 0.019$), and having a primiparous mother (OR = 0.11; $P = 0.022$) (Table 3).

3.3. Acute phase proteins

In the linear regression models, no associations were found between HP, SAA, or HL treatment in the parasitic infection category (average AUC) ($P > 0.05$) during the first 6 weeks (average AUC).

The average SAA concentration increased during the first 2 weeks of life and then decreased. The average HP concentration peaked in the second week of life. In the first 2 weeks of life, calves with a high number of *Cryptosporidium* oocysts in their faecal samples also had elevated serum concentrations of HP compared to calves in the groups with fewer oocysts in their faeces (Fig. 3). Similarly, in the second week of life, calves with a high number of *Cryptosporidium* oocysts in their faeces also had higher SAA serum concentrations in their serum

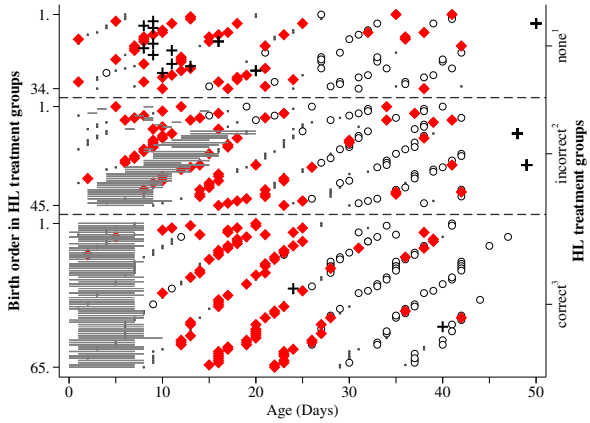

Fig. 1. *Cryptosporidium* and *Giardia* infection patterns differentiated by halofuginone lactate (HL) treatments. Calves were assigned into groups retrospectively based on HL treatment regiments (separated by dashed lines on figure): [1]) not treated (n = 34), [2]) treated incorrectly (treatment started > 48 h after birth, or lasted < 7 days) (n = 45), and [3]) treated according to manufacturer's instructions (started < 48 h after birth, and lasted ≥ 7 days) (n = 65). (◆) *Cryptosporidium* positive; (○) *Giardia* positive; (·) *Giardia* and *Cryptosporidium* negative; (+) death (n = 17); horizontal lines represent HL treatment and the length represents the treatment in days. The y-axis represents the birth order of calves in different HL treatment groups, starting with the oldest and ending with the youngest; x-axis represent the age of the calf.

Table 1
Logistic regression model examining the association between diarrhoea in 144 calves during the first 6 weeks of life and the concentration of *Cryptosporidium*, the concentration of *Giardia* oocysts in faecal samples, and age at sample collection. Calves were added as random intercepts. Final model is presented.

Variable (n = no. of samples)	OR	Confidence interval 95%	*P*-value
Cryptosporidium negative (n = 437)	1.0	–	–
Cryptosporidium low (n = 109)	1.93	1.23; 3.03	0.004
Cryptosporidium high (n = 109)	2.22	1.39; 3.56	0.001
Giardia negative (n = 453)	1.0	–	–
Giardia low (n = 101)	1.45	0.83; 2.55	0.192
Giardia high (n = 101)	1.58	0.91; 2.74	0.102
Age at sample collection (days)	0.98	0.96; 0.99	0.007

n (observations) = 655, average n (observations) per calf = 4.5.

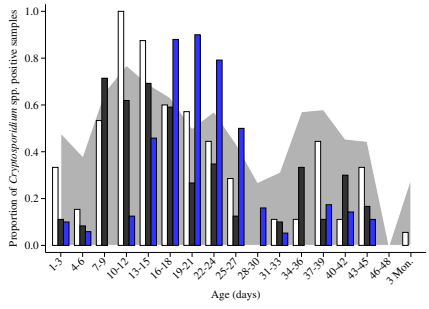

Fig. 2. Proportion of positive *Cryptosporidium* spp. faecal samples with different halofuginone lactate (HL) treatment category: (white) not treated (n = 34); (black) treatment start was delayed or duration was < 7 days (n = 45); (blue) treatment was done correctly (started in the first 48 h of life and lasted ≥ 7 days of treatment) (n = 65). Total proportion of diarrhoeic faecal samples presented as grey area in the background.

compared to the other groups (Fig. 3).

The HP concentration during the second week of life was higher in the HL untreated group compared to the incorrectly (P = 0.001) or correctly treated groups (P = 0.001) (Fig. 4). HL treatment in the second week of life was not associated with SAA (P > 0.05).

3.4. Weight gain

The average birth weight was 41.19 ± 5.1 kg (range 27 to 52 kg). Linear regression predicted a negative association (P = 0.004) between HP-AUC and ADWG at 3 months of age. Correct treatment had a negative effect (P = 0.003) on ADWG after a 3-month period when compared to the group that did not receive treatment (Table 4). Information on weight and ADWG by different HL treatment groups is presented in Table 5.

4. Discussion

Several pathogens potentially fit the differential diagnosis of diarrhoea in calves, including coronavirus, rotavirus, *E. coli*, and *Salmonella*. Before the start of the study, veterinarians on the farm had performed rapid pen-side tests with positive results for coronavirus, rotavirus, and *Cryptosporidium*. In addition, the herd had tested positive for bovine viral diarrhoea virus at the time of the study (personal communication from farm veterinarian). The significant increase in SAA and HP concentrations and presence of diarrhoea in calves has been observed in calves during rotavirus, coronavirus and *E. coli* infections (Balikci and Al, 2014). In case of naturally occurring rotavirus or coronavirus co-infection with *Cryptosporidium* versus mono-infection, significantly higher SAA and HP concentration increase has been previously reported (Pourjafar et al., 2011). Nevertheless, best to our knowledge there are no experimental studies where potential co-pathogenic synergism between rotavirus and *Cryptosporidium* infection has been demonstrated in calves. Our results indicated that *Cryptosporidium* infections effect to the production of APP was dose-dependent and associated clinical signs could be attributed to the *Cryptosporidium* infection (Fig. 4). This study reflects the conditions of a farm, and though the outbreak fits the picture of cryptosporidiosis, it is not possible to exclude the co-existence of other pathogens.

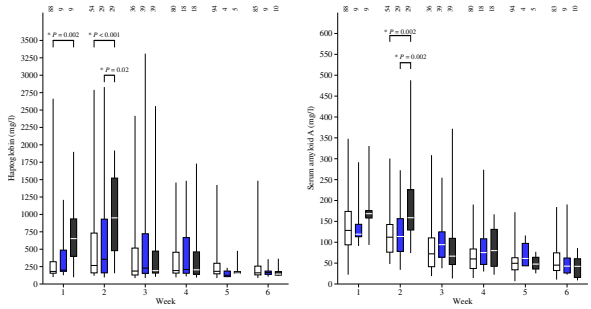

Fig. 3. Haptoglobin (HP) and serum amyloid A (SAA) concentrations in serum and different categories of *Cryptosporidium* oocyst counts in faecal samples. White = negative (no oocysts found), blue = low (below median oocysts per gram (OPG)), black = high (more than median OPG found in a faecal sample). The number of calves in each group is marked above each bar. Results from the 3 months of age were not presented because only one calf had a *Cryptosporidium* positive faecal sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

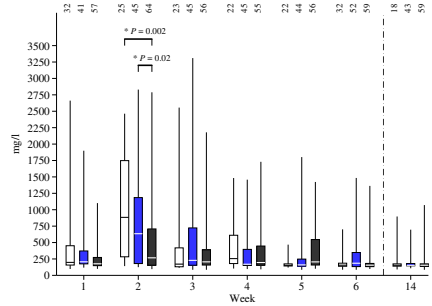

Fig. 4. Haptoglobin (HP) concentrations in serum and different halofuginone lactate (HL) treatment groups. Statistically significant differences demonstrated with a horizontal bar on top of second week results. The number of animals in a group shown at the top of a bar. HL treatment groups: white = no treatment; blue = incorrect treatment (treatment start was delayed or was < 7 days long); black = correct treatment (started in the first 48 h of life and had ≥ 7 days of treatment). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.1. Parasite infection, diarrhoea, and halofuginone lactate treatment

This study investigated the dynamics and treatment of what the farm veterinarians considered an outbreak of cryptosporidiosis. Only *C. parvum* isotype IIaA18G1R1 was found and which has been previously detected in calves faeces (Misic and Abe, 2007; Plutzer and Karanis, 2007; Brook et al., 2009). We suspect that this subtype was the main cause of cryptosporidiosis in current study, but due to relatively small PCR sample size, which was collected in single time point during the study, it was difficult to say whether there were other subtypes present. Almost all of the calves (84%) were shedding *Cryptosporidium* spp. oocysts or *Giardia* spp. cysts in their faeces, but both parasites were found in only 6% of the faecal samples, and thus did not indicate an antagonistic effect. This may be explained by the differences in the parasites infection patterns (Xiao and Herd, 1994; Santín et al., 2008, 2009). However, studies of morphological changes of the jejunum have suggested the possibility of an antagonistic effect of the two parasites (Ruest et al., 1997). Most of *Cryptosporidium* infections happened before 1 month of age, similar to what has been reported previously in longitudinal studies (Harp and Goff, 1998; O'Handley et al., 1999; Geurden et al., 2007). Calves that were shedding high amounts of *Cryptosporidium* oocysts had higher odds of being diarrhoeic than calves shedding *Giardia* or *Eimeria*, supporting *Cryptosporidium* as the causative agent of the symptoms. Some authors have suggested that *Giardia* infection itself does not cause diarrhoea (Maddox-Hyttel et al., 2006; O'Handley and Olson, 2006). A previous study in dairy cattle on several Estonian farms found a negative correlation between diarrhoea and the presence of *Eimeria* spp. in faeces, but a positive correlation between diarrhoea and higher amounts of *Cryptosporidium* spp. oocysts (Lassen et al., 2009). In Finnish calves, the opposite was observed; *Eimeria* spp. was associated with diarrhoea, while *Cryptosporidium* and *Giardia* were not (Seppä-Lassila et al., 2015). This illustrates that intestinal parasites, including *Cryptosporidium*, are important agents of disease in calves in the area, but the general clinical picture varies.

The initiation of the *Cryptosporidium* infection's prophylactic treatment with HL exemplified the connection between APR and the parasitic infection under natural conditions. The HL treatment did not seem

Table 2
Calves tested for presence of *Cryptosporidium* oocysts and *Giardia* cysts in faeces and average age of first positive sample grouped by halofuginone lactate (HL) treatment regiments[a] in the first six weeks of life.

(*n* = no. of calves)	*Cryptosporidium*		*Giardia*	
	No. of animals tested positive	Average (± SD) age of first positive sample (days)	No. of animals tested positive	Average (± SD) age of first positive sample (days)
Not treated (*n* = 34)	26 (77%)	11 ± 7	23 (68%)	28 ± 10
Incorrectly treated (*n* = 45)	41 (91%)	12 ± 7	41 (91%)	31 ± 7
Correctly treated (*n* = 65)	55 (84%)	16 ± 5	46 (71%)	31 ± 8
Total (*n* = 144)	122 (85%)	14 ± 6	110 (76%)	30 ± 8

[a] I) not treated; II) treatment start was delayed or duration was < 7 days; III) treatment was done correctly (started in the first 48 h of life and lasted ≥ 7 days of treatment).

Table 3
Retrospective case control logistic regression modelling of factors associated with mortality of calves up to 43 days of age. Final model is presented.

Variable (n = no. of calves)	OR	Confidence interval 95%	P-value
SAA (mg/l)[a]	1.013	1.001; 1.026	0.041
GGT (IU/l)[a]	0.993	0.988; 0.998	0.004
Birth weight (kg)	0.762	0.607; 0.957	0.019
Multiparous (n = 23)	1.0	–	–
Primiparous (n = 40)	0.111	0.017; 0.731	0.022

n (observations) = 63 (the case group (n = 14) and the control group (n = 49)), SAA = Serum amyloid A, GGT = gamma glutamyltransferase.
[a] Sample collected first week of life.

Table 4
Association of average daily weight gain (g/days) of 109 calves at 3 months of age, haptoglobin (HP) average area under the curve (AUC), halofuginone lactate (HL) treatment[a] and age at weight measurement. Final model is presented.

Variable (n = no. of calves)	Estimate	Confidence interval 95%	P-value
HP average AUC (mg/g/day)	− 0.16	− 0.27; − 0.05	0.004
Not HL treated (n = 18)	0	–	–
Incorrect HL treatment (n = 42)	− 55.53	− 126.63; 15.58	0.125
Correct HL treatment (n = 49)	− 107.22	− 176.37; − 38.06	0.003
Age at weight measurement (days)	2.15	− 0.75; 5.05	0.145
Intercept	708.69	390.65; 1026.72	0.000
n (observations) = 109			

[a] I) not treated; II) treatment start was delayed or duration was < 7 days; III) treatment was done correctly (started in the first 48 h of life and lasted ≥ 7 days of treatment).

to decrease the number of *Cryptosporidium* oocysts shed in faeces, similar to what has been reported in one study (Weber et al., 2016), but contrary to another (Keidel and Daugschies, 2013). Nevertheless, the correctly performed prophylactic treatment had a delaying effect on the onset of shedding (Fig. 2 and Table 2), seemed to improve survival (Fig. 1S), but resulted in a poorer ADWG (Table 5). Previous investigations have also reported that HL can cause a delay in oocyst shedding (Jarvie et al., 2005; Trotz-Williams et al., 2011; Keidel and Daugschies, 2013), but not an impact on the survival of calves. Calves have higher risk of succumbing to dehydration and acidosis due to diarrhoea in their first week of life (Foster and Smith, 2009). Prophylactic HL treatment may delay the development of cryptosporidiosis and help calves cope with very strong infection pressure (Abeywardena et al., 2015). It has been suggested that HL may have a positive therapeutic effect in calves aged 8–14 days (Klein, 2008). Other authors (Silverlås et al., 2009; Almawly et al., 2013) have reported that the therapeutic treatment effect of HL on calves' health seems to be limited, similar to the findings in this study.

4.2. Survival

Most of the calves' deaths in the current study were concentrated in a relatively short period, and were found to be related to infections of the digestive system in the group that did not receive HL treatment. Shortly after the mass treatment with HL started, the death rate dropped (Fig. 1). This suggests that the mortalities were related. *Cryptosporidium* infections and the treatment may have reduced the severity of the illness and raised the chance of survival (Fig. 1S). Higher GGT activity had a positive effect on survival, which suggests colostrum quality and adsorption of antibodies had an important role in the animal's ability to survive the infection. It has been shown that high levels of immunoglobulin G and long fatty acids in colostrum have some protective effect against diarrhoea caused by *Cryptosporidium*, but not against the infection itself (Lopez et al., 1988; Schmidt and Kuhlenschmidt, 2008; Weber et al., 2016). This could mean that the animals who were at a weaker starting position due to poorer quality colostrum and lower birth weights more easily succumbed to *Cryptosporidium* infection. Only after starting the mass treatment with HL did the survival chances of these calves improve. Even incorrect treatment with HL seemed to have a positive effect on survival; thus, we could conclude that no treatment would be the worst option during a massive increase in cryptosporidiosis cases, especially when most of the deaths are diarrhoea-related.

The incubation period of *Cryptosporidium* infection is 5–7 days, which is so short that the adaptive immune response is unlikely to stop the development of clinical disease (Petry et al., 2010; Abeywardena et al., 2015). APR as an innate immune response is faster and more likely to play a role in controlling the infection and the development of disease in the early stages. Interestingly, higher SAA concentrations had a negative impact on the survival of calves. This suggests that APR was triggered more profoundly in severely affected animals (Fig. 3). Although we cannot rule out other common digestive system pathogens, the evidence suggests that *Cryptosporidium* played a major role in diarrhoeic calves, and that the correct HL treatment was able to delay the APR induction and decrease its magnitude (Fig. 4).

4.3. APPs

The HL prophylactic treatment delayed *Cryptosporidium* infection and seemed to affect the APR in the second week of life. HP, but not SAA, serum concentrations were significantly lower in the animal groups that were correctly treated compared to the untreated and incorrectly treated calves (Fig. 4). This increase in concentrations of APP coincided with an increased proportion of the calves shedding *Cryptosporidium* spp. oocysts (Fig. 2) and mortality (Fig. 1S) in the second week of life. The HP median concentration in heavily shedding animals was 4.8 times higher (950 mg/l) than the reference value (< 196 mg/l) of calves that age while the SAA median value (158 mg/l) did not exceed the reference value (< 178 mg/l) (Seppä-Lassila et al., 2013). We

Table 5
Results of weight measurement and average daily weight gain (ADWG) at 1 and 3 months (± SD) by different halofuginone lactate (HL) treatment groups.[a]

HL treatment group	n (observations)	Age (days)	Weight (kg)	ADWG (g/day)
1 month of age				
Not treated	22	29.2 ± 4.4	54.1 ± 5.7	433.6 ± 160.2
Incorrectly treated	44	27.8 ± 3.8	53.4 ± 6.3	449.5 ± 168.2
Correctly treated	56	31.2 ± 4.6	53.9 ± 5.7	388.4 ± 122.8
Total	122	29.6 ± 4.5	53.8 ± 5.9	418.6 ± 148.9
3 months of age				
Not treated	19	108.6 ± 12.2	134.5 ± 17.3	861.6 ± 121.0
Incorrectly treated	43	99.8 ± 12.9	120.4 ± 19.3	794.4 ± 129.4
Correctly treated	58	100.4 ± 6.6	117.7 ± 14.9	751.3 ± 123.7
Total	120	101.5 ± 10.6	121.3 ± 17.9	784.2 ± 130.3

[a] I) not treated; II) treatment start was delayed or duration was < 7 days; III) treatment was done correctly (started in the first 48 h of life and lasted ≥ 7 days of treatment).

speculate that this drastic difference was caused by the nature of the *Cryptosporidium* infection and likely because localised damage to the small intestine was more prone to trigger an immunological response that increased HP rather than SAA concentrations. Previously, relatively small studies (1 to 6 animals) reported an increase in HP and SAA in dairy calves as a response to *Cryptosporidium* infections, especially before the onset of diarrhoea (Enemark et al., 2003a,b; Pourjafar et al., 2011). Although this study shed more light on the subject, there is a lack of research on the role of APR in *Cryptosporidium* and *Giardia* infections in cattle.

4.4. Weight gain

Although about 67% of the calves died in the group that did not receive HL treatment, it was surprising to find that the treatment had significant negative effects on the daily weight gain when the calves reached 3 months of age. In general, the calves' weight gain met the expectations of Holstein breed calves at 3 months of age (Retamal and Risco, 2011), averaging around 121.3 kg. We expected that *Cryptosporidium* infection would have a lasting negative effect on the growth of the surviving calves. The effect of the infection should have been most obvious in calves that were infected but not treated. However, the largest effect was observed in animals that had the correct treatment. Other authors have not found a significant positive or negative effect of HL treatment on the growth rate (Jarvie et al., 2005; Trotz-Williams et al., 2011). It is important to remember that HL treatment does not stop calves from being infected and shedding large numbers of *Cryptosporidium* oocysts, damaging the host's cells and consuming resources for replicating (Silverlås et al., 2009). There were no significant changes in the feeding regimens in first 3 months of the calves' lives, ruling out differences in nutrition as an explanation. A possible explanation for the observed effect is the delay and possible expansion of the parasites' life cycle in the host due to the effect of HL. The categorisation of different HL treatment groups was very strongly influenced by birth order. As a result, we were not able to exclude time as a confounding factor in average daily weight regression models.

Elevated concentrations of SAA in the second week of life have been negatively associated with the growth rate in reindeer calves (Orro et al., 2006), lambs (Peetsalu et al., 2013) and beef calves (Seppä-Lassila et al., 2017). In the current study, *Cryptosporidium* shedding was associated with higher serum concentrations of SAA and HP in the second week of the calves' life, indicating that this period of adaptation was critical. However, only the HP overall response had a negative association with short-term weight gain at 3 months of age. The elevated HP concentrations may have triggered a stronger APR as a response to the infection and consequently affected the growth of the calf.

5. Conclusions

In the outbreak, there was a strong association between *Cryptosporidium* infection and diarrhoea, but not with *Giardia* or *Eimeria* infections. Correctly performed prophylactic HL treatment against cryptosporidiosis delayed the onset of oocyst shedding and improved the chances of survival. However, the growth rate was negatively affected by correct treatment and a strong APR. Correct treatment was associated with lower HP concentrations in the second week of life. The study demonstrates a possible connection between *Cryptosporidium* infection and APR in dairy calves.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2017.12.015.

Conflict of interest

None.

Acknowledgements

We would like to thank veterinary students for helping with sample collection, farm veterinarians Kärt Kalvet and Kadri Kaugerand, and laboratory help by Pille Paats, Külli Must, Azzurra Santoro and Niina Sidarova. The project was funded by the Estonian Research Council project IUT8-1.

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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. DOI: 10.1016/j.rvsc.2018.10.013

Research in Veterinary Science 121 (2018) 117–123

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

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

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ARTICLE INFO

Keywords:
Sheep
Neonatal immune response
Parasitic infection
Domestic ruminant
Organic farming

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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https://doi.org/10.1016/j.rvsc.2018.10.013
Received 25 May 2018; Received in revised form 24 October 2018; Accepted 28 October 2018

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

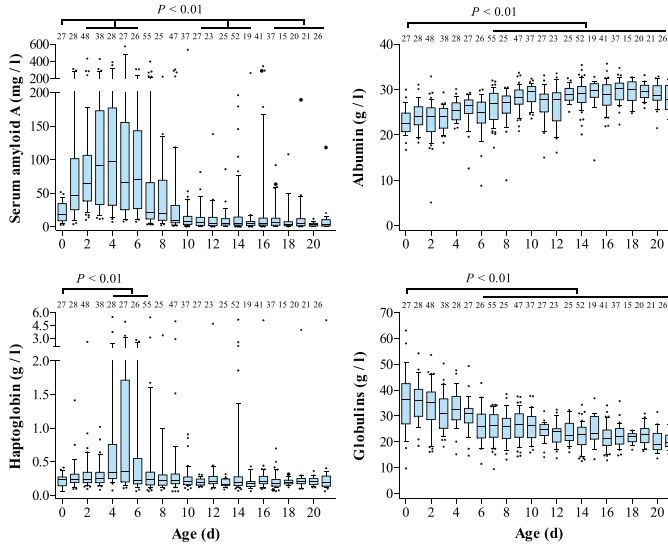

Fig. 1. Concentrations of serum amyloid A (SAA), haptoglobin (Hp), and serum proteins (albumin, ALB, and globulins, GLOB) in lambs during the first 3 weeks of age. The whiskers of the box plot represent 10–90% of results. The numbers on top of the box plot bars represent the number of lambs at different 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

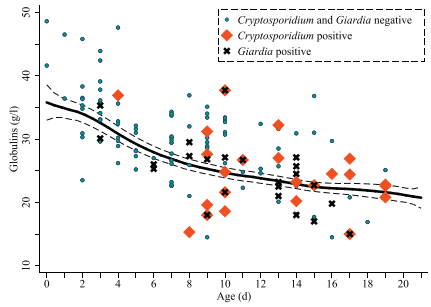

Fig. 2. Scatterplot of globulin (GLOB) serum concentrations in lambs with *Cryptosporidium* or *Giardia* positive (both $n = 23$) faecal samples ($n = 141$). The thick black line represents the relation/function of GLOB on age by local polynomial smooth. The degree of the polynomial used in the smoothing 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

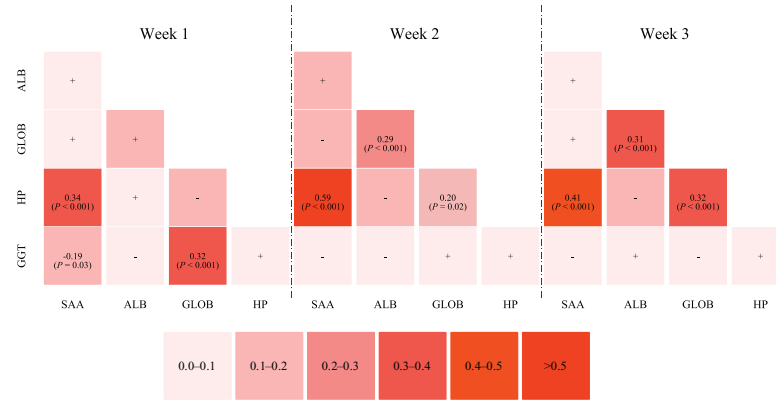

Fig. 3. Heat map matrix of partial correlations between serum amyloid A (SAA), haptoglobin (Hp), globulin (GLOB), and γ-glutamyltransferase (GGT) by week of age. The strength of the correlation is indicated by the colour tone. Only significant correlation coefficients are presented on corresponding squares 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.

Acknowledgements

We would like to thank veterinary students, Pille Paats, Külli Must and Niina Sidorova for helping with sample collection and laboratory assistance. This project was funded by the Estonian Research Council project IUT8-1.

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01.09.2017–... Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences, Chair of Veterinary Bio- and Population Medicine, Veterinary immunology and physiology, Teaching assistant (1.00)

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Education

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2004–2010 Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences, Veterinary Medicine

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1992–2001 Kabala Basic School

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2018−... A member of the Society for Veterinary Epidemiology and Preventive Medicine

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Course: „Bayesian Disease Mapping workshops“, Prof. Andrew Lawson, Medical University of South Carolina, Charleston, South Carolina, 12.-16.3.2018.

Course: „Quantitative Risk Analysis With R“, Dr. Francisco Zagmutt, Epix Analytics, Fort Collins, Colorado, 1.-4.12. 2015.

Course: „Multilevel Modelling“, Prof. Ian Dohoo, Estonian University of Life Sciences, Tartu, 8.-12.6.2015.

Course: „EpiNOVA Advanced Course – Data Quality“, Prof. Ian Dohoo, NOVA University Network, Tallinn, 17.-23.9.2014.

Academic degrees

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Haridustee

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Veterinaarimmunoloogia, veterinaarepidemioloogia

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COST Action CA15116 ”Understanding and combating African Swine Fever in Europe (3.05.2016−2.05.2020)”, Arvo Viltrop, Eesti Maaülikool, veterinaarmeditsiini ja loomakasvatuse instituut.

IUT8-1 ”Piimalehmade sigimine ja tervis (1.01.2013−31.12.2018)”, Andres Valdmann, Eesti Maaülikool, Veterinaarmeditsiini ja loomakasvatuse instituut.

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Kursus: „Bayesian Disease Mapping workshops“, Prof. Andrew Lawson, Medical University of South Carolina, Charleston, South Carolina, 12.-16.3.2018.

Kursus: „Quantitative Risk Analysis With R“, Dr. Francisco Zagmutt, Epix Analytics, Fort Collins, Colorado, 1.-4.12.2015.

Kursus: „Multilevel Modelling“, Prof. Ian Dohoo, Eesti Maaülikool, Tartu, 8.-12.6.2015.

Kursus: „EpiNOVA Advanced Course – Data Quality“, Prof. Ian Dohoo, NOVA University Network, Tallinn, 17.-23.9.2014.

Teaduskraadid

Tarmo Niine, magistrikraad (veterinaarmeditsiin), 2010, (juh) Tõnu Järveots, Patohistoloogiline leid sigade tsirkoviirus tüüp 2 nakkuse korral, Eesti Maaülikool.

LIST OF PUBLICATIONS

1.1 Articles in journals. Scholarly articles indexed by Web of Science Science Citation Index Expanded, Social Sciences Citation Index, Arts & Humanities Citation Index and/or indexed by Scopus (excluding chapters in books)

Nurmoja, I.; Mõtus, K.; Kristjan, M.; Niine, T.; Schulz, K.; Depner, K.; Viltrop, A. (2019). Epidemiological analysis of the 2015–2017 African swine fever outbreaks in Estonia. Prev. Vet. Med., S0167-5877(18)30361-1.

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.

Niine, T.; Dorbek-Kolin, E.; Lassen, B.; Orro, T. (2018). *Cryptosporidium* outbreak in large dairy farm calves: Effect of treatment, association with inflammatory response and short-term weight gain. Res. Vet. Sci., 117, 200−208.

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.

3.5. Articles/presentations published in local conference proceedings

Niine, T.; Nurmoja, I.; Viltrop, A. (2018). Estimating Africa Swine Fever outbreaks in domestic pigs by using Bayesian hierarchical models. SVEPM Conference & Annual General Meeting of the Society, Tallinn, 21-23 March. Tallinn: Society for Veterinary Epidemiology and Preventive Medicine

Niine, T.; Nurmoja, I.; Viltrop, A. (2018). Sigade aafrika katku puhangute kodusigadel tekkimise seos haigusjuhtude arvuga metssigadel, jahipidamise ja metsaraie intensiivsusega. Terve loom ja tervislik toit:

Terve loom ja tervislik toit 2018. Editor: Marko Kass. Tartu: Eesti Maaülikool, 52−56.

Niine, T.; Lassen, B.; Orro, T. (2017). Piimaveise vasikate *Cryptosporidium* spp. nakkuse ravi neonataalperioodil halofuginoon laktaadiga. Terve Loom ja Tervislik Toit: Terve Loom ja Tervislik Toit 2017. Editor: Marko Kass. Tartu: Eesti Maaülikool, 159−159.

5.2 Published meeting abstracts. Conference abstracts which are not indexed by Thomson Reuters Web of Science

Niine, T.; Lassen, B.; Orro, T. (2016). Treatment of *Cryptosporidium* spp. infections in neonatal dairy calves with Halofuginone lactate. Veterinaarmeditsiin 2016, Tartu, 28.-29. oktoober. Eesti Loomaarstide Ühing.

Niine, T.; Oksanen, A.; Soveri, T.; Orro, T. (2015). *Giardia intestinalis* impact on reindeer calves systemic innate immune response. First International congress on Pathobiome: Pathogens in microbiotas in hosts, National Veterinary School (ENVA), Maisons-Alfort, France. Maisons-Alfort, France: INRA Editions, 9−9.

Niine, T.; Peetsalu, K.; Soveri, T.; Orro, T. (2014). Serum amyloid A (SAA) concentrations in newborn reindeer calves serum during first months of life and its association with daily weight gain at age of 2 months. Proceedings of the sixteenth biennial congress of the international society for animal clinical pathology: The 16th Biennial Congress of the International Society for Animal Clinical Pathology. Editors: Kjelgaard-Hansen, Mads; Jacobsen, Stine. Copenhagen, Denmark: Copenhagen University Press, 55−55.

Järveots, T.; Saar, T.; Põdersoo, D.; Blank, I.; Niine, T. (2010). About histopahological changes in swine affected with Porcine Circovirus-2. Abstract book of 28th Meeting of the European Society of Veterinary Pathology and European College of Veterinary Pathologists 2010. From: Serbia, Belgrade, September 8-11 2010. Belgrade:, 2010, 138.: 28th Meeting of the European Society of Veterinary Pathology and European College of Veterinary Pathologists 2010, Serbia, Belgrade, September 8-11 2010. Editors: Jovanovic, M. Serbia, Belgrade: Sava Centar, Belgrade, 138.

VIIS VIIMAST KAITSMIST

PEETER PADRIK
FACTORS INFLUENCING THE QUALITY OF SEMEN FROM ESTONIAN HOLSTEIN AI BULLS, AND RELATIONSHIPS BETWEEN SEMEN QUALITY PARAMETERS AND *in vivo* FERTILITY
EESTI HOLSTEINI TÕUGU SUGUPULLIDE SPERMA KVALITEET, SEDA MÕJUTAVAD TEGURID NING SEOS *in vivo* VILJAKUSEGA
Professor **Ülle Jaakma**, Professor Emeritus **Olev Saveli**
1. november, 2019

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SÜNTEETILISTE JA BIOLOOGILISTE PESTITSIIDIDE MÕJUD MEEMESILASTELE JA KIMALASTELE
Professor **Marika Mänd**, Dr. **Reet Karise**
26. september, 2019

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PÕLEVKIVIREGIOONI TERVIKLIK ARENDAMINE TÖÖSTUSPÄRANDI-, PUHKE-, SPORDI- JA TURISMIPIIRKONNANA
Professor **Kalev Sepp**
27. juuni, 2019

KERSTI VENNIKU
THE EFFECT OF MILITARY VEHICLES ON RUT FORMATION ON ESTONIAN SOILS AND NATURAL RECOVERY OF THE RUTS.
EESTI MULDADEL MILITAARSÕIDUKITE ÜLESÕITUDE TULEMUSENA KUJUNENUD ROOPAD JA NENDE LOODUSLIK TAASTUMINE
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KARIN NURME
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KÕRGETE VÄLISTEMPERATUURIDE SENSOORNE KODEERIMINE PUTUKATE ANTENNAALSETE TERMO- JA HÜGRONEURONITE TRIAADI POOLT
Dr. **Enno Merivee**, Dr. **Anne Must**, Dr. **Ivar Sibul**
19.juuni 2019

ISSN 2382-7076
ISBN 978-9949-629-97-8 (trükis)
ISBN 978-9949-629-98-5 (pdf)