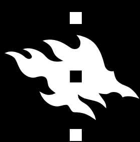
ACUTE PHASE PROTEINS IN DAIRY CALVES AND REINDEER

CHANGES AFTER BIRTH AND IN RESPIRATORY INFECTIONS

TOOMAS ORRO



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Department of Production Animal Medicine
Faculty of Veterinary Medicine
University of Helsinki
Finland

Acute phase proteins in dairy calves and reindeer
Changes after birth and in respiratory infections

Toomas Orro

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Supervisors: Professor Satu Pyörälä, DVM, PhD
Department of Production Animal Medicine
Faculty of Veterinary Medicine
University of Helsinki
Finland

Professor Timo Soveri, DVM, PhD
Department of Production Animal Medicine
Faculty of Veterinary Medicine
University of Helsinki
Finland

Docent Satu Sankari, DVM, PhD
Department of Equine and Small Animal Medicine
Faculty of Veterinary Medicine
University of Helsinki
Finland

Reviewers: Professor P David Eckersall BSc, MBA, PhD, FRCPath
Division of Animal Production & Public Health
Institute of Comparative Medicine
Faculty of Veterinary Medicine
University of Glasgow
UK

Professor Erkki Pesonen, MD, PhD
Department of Paediatrics
University Hospital, Lund
Sweden

Opponent: Docent Liisa Kaartinen, DVM, PhD
Finnish Food Safety Authority Evira
Finland

Layout and cover design by Vahur Puik

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To my father

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ABSTRACT

The early protection mechanism of the host against infection, trauma or other tissue damage comprises a set of reactions known as the acute phase response (APR). During APR, circulating concentrations of acute phase proteins (APPs) change. These proteins can serve as indicators of host response during various inflammatory conditions. In this thesis, APR in reindeer was investigated for the first time. Systemic concentrations of APPs during the neonatal period were studied in reindeer and cattle. APPs were also investigated during spontaneous bovine respiratory disease (BRD) in dairy calves.

An *Escherichia coli* endotoxin model was used in adult reindeer to obtain basic information on APR in this animal species. Endotoxin challenge triggered APR in reindeer, which was seen as a decrease in iron concentration and an increase in serum amyloid A (SAA) in all animals. Haptoglobin (Hp) showed a less pronounced increase. SAA and Hp were concluded to be acute phase reactants in reindeer, with SAA appearing to be a more sensitive inflammatory marker.

Age-related changes in serum concentrations of APPs were studied in reindeer and cattle. A total of 51 reindeer calves aged 0–32 days were sampled at regular intervals. SAA concentrations were low at birth, increasing during the first 2 weeks of life and decreasing by the age of 3-4 weeks. Serum Hp concentrations increased throughout the first month after birth. SAA concentrations in the second week had a negative association with weight gain at 4 months of age. In cattle, two groups ($n = 13$) of newborn dairy calves were sampled over a 3-week and a 2-month period, respectively. Both groups of dairy calves had a very similar pattern of APP concentrations in the blood, which stabilized around 3-4 weeks of age. SAA and lipopolysaccharide binding protein (LBP) concentrations were low at birth, but then increased, peaking at the second week of life and decreasing thereafter; the relative rise of SAA was more pronounced. The most marked changes of SAA and LBP were comparable with increased concentrations seen in calves suffering from spontaneous moderate respiratory disease. Concentrations of $alpha_1$-acid glycoprotein (AGP) were high at birth, gradually decreasing thereafter. Relative changes in Hp concentrations were very small, and values generally remained low. Identification of SAA isoforms in calves' serum and in colostrum of their dams showed that calves produced the same isoforms as adult cattle. Circulating SAA was thus not derived from colostrum.

Results of these two studies indicated that newborn reindeer and dairy calves have an inflammatory response during the first weeks of life. Possible reasons for this include presence of inflammatory mediators in the colostrum, stimulation by the birth process, age-related changes in hepatic synthesis of APP and exposure to pathogens after birth. Very similar SAA changes in the two ruminant species also suggest that this inflammatory response may have role in the adaptation process of newborns to extrauterine life. Moreover, the results stress the importance of taking the age of young animals into consideration when interpreting APP concentrations.

The effect of different bovine respiratory pathogens on concentrations of APPs (SAA, LBP, Hp, AGP and fibrinogen; Fb) was studied in 84 calves with spontaneous BRD, from 18 herds. Isolation of *Pasteurella multocida* was associated with increased concentrations of all APPs tested. For other pathogens, no significant relationships were observed. In another study, concentrations of APPs were investigated in 10 dairy calves during an outbreak of BRD over a 6-week period, starting one week before the outbreak of BRD. Calves presented mild to moderate signs of respiratory disease from the first to the fourth week. Serological and PCR findings confirmed the initial role of bovine respiratory syncytial virus (BRSV) in this BRD outbreak. Concentrations of SAA and LBP increased at week 1, peaked at week 3 and decreased at week 4. Some calves had high Hp concentrations at week 3, but AGP concentrations did not rise during the disease. Higher SAA, LBP and Hp concentrations at a later stage of BRD (week 3) were associated with lower BRSV-specific IgG_1 production, suggesting that these calves had enhanced inflammatory response to secondary bacterial infection. In conclusion, APPs proved to be useful in exploring host response in bovine respiratory infections. *P. multocida* can be considered a important infecting agent in BRD, as isolation of this pathogen was linked to strong APR.

LIST OF ORIGINAL ARTICLES

This thesis is based to the original articles (I-IV) and to the unpublished manuscript (V). These articles are referred in the text by their Roman numbericals.

I Orro T, Sankari S, Pudas T, Oksanen A, Soveri T. Acute phase response in reindeer after challenge with *Escherichia coli* endotoxin. Comp. Immunol. Microbiol. Infect. Dis. 2004; 27:413-422.

II Orro T, Nieminen M, Tamminen T, Sukura A, Sankari S, Soveri T. Temporal changes in concentrations of serum amyloid-A and haptoglobin and their associations with weight gain in neonatal reindeer calves. Comp. Immunol. Microbiol. Infect. Dis. 2006; 29:79-88.

III Orro T, Jacobsen S, LePage J-P, Niewold T, Alasuutari S, Soveri T. Temporal changes in serum concentrations of acute phase proteins in newborn dairy calves. Vet. J. 2007; doi:10.1016/j.tvjl.2007.02.010

IV Nikunen S, Härtel H, Orro T, Neuvonen E, Tanskanen R, Kivelä S-L, Sankari S, Aho P, Pyörälä S, Saloniemi H, Soveri T. Association of bovine respiratory disease with clinical status and acute phase proteins in calves. Comp. Immunol. Microbiol. Infect. Dis. 2007; 30:143-151.

V Orro T, Pohjanvirta T, Rikula U, Huovilainen A, Alasuutari S, Sihvonen L, Pelkonen S, Soveri T. Acute phase protein changes in calves during an outbreak of respiratory disease – differences between high and low IgG_1 responders to initial bovine respiratory syncytial virus infection. Submitted to Vet. Microbiol.

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ABBREVIATIONS

AGP	Alpha$_1$-acid glycoprotein
α_1-PI	Alpha$_1$-proteinase inhibitor
α_1-AT	Alpha$_1$-antitrypsin
APP(s)	Acute phase protein(s)
APR	Acute phase response
ASAT	Aspartate aminotransferase
BRD	Bovine respiratory disease
BUN	Blood urea nitrogen
Cp	Ceruloplasmin
CK	Creatine kinase
CRP	C-reactive protein
CV	Coefficient of variation
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GGT	Gamma-glutamyl transferase
Fb	Fibrinogen
Hb	Haemoglobin
Hp	Haptoglobin
HDL	High-density lipoprotein
IgG	Immuno-globulin G
IL	Interleukin
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
MAP	Major acute-phase protein
OD	Optical density
PCR	Polymerase chain reaction
pI	Isoelectric point
SAA	Serum amyloid A
SDH	Sorbitol dehydrogenase
TBL	Tracheobronchial lavage
TNF-α	Tumor necrosis factor alpha
WBC	White blood cell

1 INTRODUCTION

The acute phase response (APR) is a series of complex physiological events occurring in the host after a tissue injury or an infection. One of the main phenomena during the APR is the hepatic production of acute phase proteins (APPs; Baumann and Gauldie, 1994). APPs play a role in the defence response of the host (Vogels et al., 1993). Monitoring the blood concentrations of APPs can provide information on the progression of the inflammatory reaction (Kent, 1992). APPs are already used as markers of disease in veterinary clinical chemistry (Petersen et al., 2004). APPs could also serve as valuable research tools in studies of semi-domesticated reindeer. However, little is known about the inflammatory response and changes in concentrations of APPs not only in reindeer but also in other cervid species. Although APPs have been extensively investigated in various inflammatory and non-inflammatory conditions in cattle, knowledge of the behaviour of APPs in certain physiological and disease conditions is still limited. These conditions involve e.g. changes after birth or during infectious diseases in field conditions.

After birth, newborns go through a period of rapid growth and development, and adapt to various physiological functions. Exposure to the new environment and foreign antigens requires the establishment of appropriate defence responses. The neonate is immunocompetent, but the adaptive immune system is immature (Kovarik and Siegrist, 1998; Morein et al., 2002). Functional immaturity of neonatal lymphocytes during the first weeks of life has been reported in calves (Nagahata et al., 1991). Other non-specific defence mechanisms such as APP response may thus be important for the adaptation to the extrauterine life. Characterization of changes in concentrations of APPs after birth could elucidate the role of the inflammatory response in newborns' adaptation mechanisms. Possible age effect on the concentrations of APPs also complicates the use of APPs as host response markers in newborn animals.

Bovine respiratory disease (BRD) is one of the most important diseases in beef and dairy calves. Respiratory disease is a multifactorial disease complex, which is caused by a variety of aetiological agents. APPs can potentially be used to investigate the complex pathogenesis of BRD and to evaluate the role of different aetiological factors.

2 REVIEW OF THE LITERATURE

2.1 Acute phase response and APPs

Inflammatory response to tissue injury is a mechanism by which the host sets up defence against further injury and starts the healing process. The early and immediate set of reactions is known as APR (Baumann and Gauldie, 1994; Raynes, 1994; Koj, 1996). The APR process is initiated at the site of injury, leading to release of soluble mediators that mobilize the defence response of the whole organism. The cause of the injury can be infective, traumatic, immunological, neoplastic or other (Stadnyk and Gauldie, 1991). The APR is thus part of the non-specific innate immune response, and its components are relatively consistent despite the large variety of conditions that induce it. The APR is followed by the specific immune response. The function of the APR is to prevent ongoing tissue damage, to isolate and destroy the infective organisms and to activate the repair processes necessary to return the organism to the normal condition (Baumann and Gauldie, 1994). Initiation of APR most commonly starts by the release of inflammatory mediators from tissue macrophages or blood monocyte cells that gather at the site of damage (Baumann and Gauldie, 1994; Koj, 1996). These inflammatory mediators set off both the local and systemic inflammatory processes. Activated macrophages release a broad spectrum of mediators of which cytokines appear to be uniquely important in initiating the next series of reactions (Koj, 1996). At the reactive site, cytokines act on stromal cells, including fibroblast and endothelial cells, to cause a secondary wave of cytokines. This secondary wave augments the homeostatic signal and initiates the cellular and cytokine cascades involved in the complex process of the APR (Baumann and Gauldie, 1994). Interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor (TNF)-α have been identified as the predominant cytokines capable of stimulating the response (Baumann and Gauldie, 1994; Koj, 1996). As a result, the APR is expressed by such clinical, systemic inflammatory signs as fever, inappetite and depression, which are reflections of multiple endocrinological, haematological, immunological, metabolic and neurological changes in the diseased animal (Stadnyk and Gauldie, 1991).

One of the predominant features of APR is changes in the concentrations of a number of plasma proteins (APPs) associated with the host response. These changes are mainly the result of alterations in APPs synthesis in the liver (Eckersall and Conner, 1988; Baumann and Gauldie, 1994; Gruys et al., 1994; Raynes, 1994). An APP is a protein the concentration of which increases or decreases by more than 25% in causes of infection/inflammation (Kushner, 1982). The change in APP concentration in plasma can show either a major response by increasing from very low levels to over 10-fold or even up to 1000-fold (e.g. bovine haptoglobin; Hp), a moderate response between 2 and 10 times the normal concentration (e.g. bovine alpha$_1$-acid glycoprotein; AGP) or a minor response with a maximum increase of about twice the normal concentration (e.g. bovine fibrinogen; Fb). The concentration of APPs may decrease instead of increasing; negative APPs include albumin, transferrin and retinol binding protein (Jain, 1993; Gruys

et al., 1994). APPs represent a very heterogeneous group of plasma proteins, some of which are constitutively produced (e.g. Fb) or have very low systemic concentrations (e.g. bovine Hp) if no inflammation is present. The exact biological effects of different APPs are still under investigation, but APPs are thought to participate in innate defence mechanisms and in controlling inflammatory responses to infection by, for example, binding to foreign substances, having opsonizing activities and modulating phagocytic cell functions. Several studies on mice have shown protective properties of APPs against microbial challenge (Briles et al., 1989; Vogels et al., 1993; Hochepied et al., 2000; Szalai et al., 2000) or endotoxaemia and septic shock (Alcorn et al., 1992; Xia and Samols, 1997; Lamping et al., 1998). Several APPs are also produced extrahepatically in various tissues, which further supports the important role of APPs in non-specific defence of the host. For example, the bovine mammary gland produces locally Hp (Hiss et al., 2004), serum amyloid A (SAA; Jacobsen et al., 2005) and lipopolysaccharide binding protein (LBP; Bannerman et al., 2003) during mastitis. Mammary-associated AGP has also been identified in bovine colostrum and milk (Ceciliani et al., 2005).

Hepatic production of APPs is stimulated by pro-inflammatory cytokines (predominantly IL-1β, IL-6 and TNF-α) released into the circulation during APR. Other factors, such as growth factors (e.g. insulin and transforming growth factor beta), glucocorticosteroids and anti-inflammatory cytokines have important modulating effects on APP production (Richards et al., 1991; Baumann and Gauldie, 1994; Gabay and Kushner, 1999). APPs respond differently to the different combinations of cytokines, and individual APPs are regulated by complicated cytokine interactions and regulatory factors (especially glucocorticoids; Mackiewicz et al., 1991; Smith and McDonald, 1992; Wan et al., 1995). Concentrations and kinetics of systemic APPs during inflammatory response appear to be related to the severity of tissue damage and time course of the inflammation process (Kent, 1992). Measuring circulating levels of these proteins thus provides valuable quantifiable information about the ongoing APR and can be used as a non-specific disease marker (Thompson et al., 1992; van Leeuwen and van Rijswijk, 1994; Petersen et al., 2004).

A significant variation between different APP profiles has been noted, and not all of the proteins respond in the same way in different animal species (Kushner and Mackiewicz, 1987; Hayes, 1994; Petersen et al., 2004). Therefore, before we can use APPs effectively as research or clinical tools, different APPs should be investigated in different animal species in various disease and non-disease conditions.

2.2 Acute phase proteins in cattle

In cattle and other ruminants, Hp has been one of the APPs most commonly monitored as a marker of inflammation in cattle (see Table 1 for examples in respiratory infections studies). Hp is a major bovine APP that shows a high relative increase during APR. High serum Hp levels have been reported in cattle with bacterial infections (Eckersall and Conner, 1988; Conner et al., 1989; Skinner et al., 1991; Alsemgeest et al., 1994; Hirvonen et al., 1996). Hp has been found to be effective in detecting serious inflammatory conditions in cows such as traumatic reticuloperitonitis (Hirvonen et al., 1998). Increased concentration has been detected in bovine serum during the peripartum period (Humblet et al., 2006), in cows with fatty livers (Nakagawa et al., 1997), in bull calves after castration (Fisher et al., 1997a; 2001) and in heifers after tail docking

(Eicher et al., 2000). Long transport of beef calves has also triggered Hp response (Murata and Miyamoto, 1993). Some non-inflammatory conditions, such as hypocalcaemia and ketosis, have not been associated with increased Hp concentration (Skinner et al., 1991). Hp has been suggested to be a useful tool at the farm and slaughterhouse to improve food safety (Saini et. al., 1998; Tourlomoussis et al., 2004).

The main function of Hp is binding free haemoglobin and the haemoglobin binding property has a bacteriostatic effect, as it limits free iron available for bacteria (Eaton et al., 1982). Hp also has numerous other functions related to the host defence response in infection and inflammation, for example stimulation of angiogenesis and modulation of granulocyte activity (reviewed in Dobryszycka, 1997). The inhibitory effect of Hp on granulocyte activity has been suggested to be beneficial in acute inflammation by reducing the late inflammatory response, which can be harmful to the host (Rossbacher et al., 1999).

SAA is an APP in cattle as well as in humans (Steel and Whitehead, 1994) and in fact in the majority of animal species (Uhlar and Whitehead, 1999; Petersen et al., 2004). SAA has been shown to be increased during the course of infection or endotoxaemia (Gruys et al., 1994; Werling et al., 1996; Hirvonen et al., 1999; Jacobsen et al., 2004). Measuring SAA in milk has been shown to have diagnostic value in detecting mastitis (Eckersall et al., 2001; Weber et al., 2006). While increased concentrations have been found in acutely, sub-acutely and chronically diseased animals, SAA seems to be a better marker in more acutely diseased animals (Alsemgeest et al., 1994; Horadagoda et al., 1999). Alsemgeest et al. (1995a) reported higher SAA concentrations in calves housed on a slippery floor and suggested that SAA could be a marker of stress. Recently, this suggestion was supported by Saco et al. (2007), who found increased SAA concentrations in a group of cows living in stressful conditions. In both of these studies, Hp concentrations were not different, indicating that SAA is a more sensitive APP in bovines. This was shown also in other studies, where rise of SAA was seen during the first hours of *Mannheimia haemolytica* infection in calves (Horadagoda et al., 1994) and infusion of *E. coli* endotoxin in heifers (Werling et al., 1996), whereas Hp concentrations stayed constant. High sensitivity of SAA was further demonstrated by Karreman et al. (2000), as they reported elevated SAA concentrations in cows without clinical disease and suggested SAA for use in screening of subclinical diseases at the herd level.

SAA is a multifunctional apolipoprotein associated with high-density lipoprotein (HDL) during APR. The association of SAA with HDL interferes with cholesterol transport and metabolism (Steel and Whitehead, 1994) by facilitating the uptake and removal of cholesterol from destroyed cells at the inflammation site (Jensen and Whitehead, 1998). Other identified functions include several pro- and anti-inflammatory properties (reviewed in Uhlar and Whithead, 1999; Urieli-Shoval et al., 2000). For example, SAA has been shown to activate antimicrobial functions of polymorphonuclear cells and subsequent enhancement of antifungal activity in vitro (Badolato et al., 2000). SAA can be bound to outer cell wall of Gram-negative bacteria which enhances the capacity of phagocytic cells to engulf these bacteria (Hari-Dass et al., 2005, Shah et al., 2006). As SAA protein is highly conserved through evolution (Uhlar et al., 1994; Jensen et al., 1997) and a dramatic increase during infections occurs, some authors have suggested a critical protective role in pathogen defence for SAA (Uhlar and Whithead, 1999).

AGP (also called orosomucoid or seromucoid) is a highly glycosylated protein with a relatively slow and moderate response during APR in cattle (Conner et al., 1988; Dowling et al., 2002). Systemic concentrations of AGP have been shown to increase in cattle suffering from traumatic pericarditis, arthritis, pneumonia, mastitis and hepatic abscesses (Tamura et al., 1989; Motoi et al., 1992; Hirvonen et al., 1996; Eckersall et al., 2001). Increased AGP concentrations have also been reported in subcutaneous chamber fluid of calves after subcutaneous inoculation of *M. haemolytica* (Walker et al., 1994) or systemically after endotoxin administration (Conner et al., 1989). Similarly to Hp, AGP concentrations are elevated in cows during the peripartum period (Cairoli et al., 2006), and experimental inoculation with *Theileria annulata* (Glass et al., 2003) and *Strongyloides papillosus* (Nakamura et al., 2002) in calves increased AGP concentrations. AGP has been used as a research tool to investigate the effects of pre-shipping medication on beef cattle health (Duff et al., 2000), individual housing design and size on veal calves (Wilson et al., 1999) and tail docking on heifers (Eicher et al., 2000).

AGP is a immunocalin; a group of proteins that shows significant immunomodulatory effects (Logdberg and Wester, 2000). AGP regulates the inflammatory response of leucocytes (e.g. inhibits platelet aggregation, proliferation of lymphocytes and activities of neutrophils; Fournier et al., 2000; Hochepied et al., 2003). Immunomodulating effects of AGP are primarily downgrading the local inflammatory response to reduce potential tissue damage caused by inflammatory cells (Hochepied et al., 2003). Suppression of cattle leucocytes was also found to correlate with AGP concentrations during mastitis (Sato et al., 1995). One of the many functions of AGP is to protect cells from apoptosis (van Molle et al., 1997), and an anti-apoptotic effect on cattle monocytes has recently been reported (Ceciliani et al., 2007).

LBP is an APP in humans (Froon et al., 1995; Opal et al., 1999) and laboratory animals (Tobias et al., 1986; Gallay et al., 1993). It has been identified in bovine sera and found to have similar characteristics to human, murine and rabbit LBP (Khemlani et al., 1994; Horadagoda et al., 1995; Bochsler et al., 1996). LBP has also been recognized as an APP in cattle in experimental *M. haemolytica* infections (Horadagoda et al., 1995; Schroedl et al., 2001) and in several experimental mastitis models induced with LPS, *Mycoplasma bovis* and either Gram-negative or Gram-positive bacteria (Bannerman et al., 2003; 2004a; 2004b; 2004c; 2005; Vangroenweghe et al., 2004; 2005; Kauf et al., 2007). However, to my knowledge, LBP concentrations during spontaneous inflammatory conditions or infectious diseases in cattle have not been reported to date.

The classical function of LBP is to bind LPS and to form an LPS-LBP-CD14 complex that is essential for recognition of Gram-negative bacteria by inflammatory cells and early induction of the innate defence response to infection (Tobias et al., 1999; Guha and Macman, 2001). Whereas early recognition of Gram-negative bacteria is important for the host, regulation mechanisms are needed to prevent overreaction of the immune system, as observed in sepsis and septic shock. LBP has a dual and seemingly opposite role in inflammation. While low concentrations of LBP stimulate initiation of the inflammatory response to Gram-negative bacteria by binding to LPS, high concentrations (like those occurring during APR) have several mechanisms to inhibit innate immune cell activation (Hamann et al., 2005), and therefore, LBP as an APP has an host-protective role. High levels of LBP are beneficial in humans with severe sepsis and septic shock (Opal et al., 1999; Zweigner et al., 2001), and LBP protects mice from septic shock by Gram-negative bacteria (Lamping et al., 1998). The binding of bacterial

components by LBP is not limited to LPS, also including Gram-positive bacteria, and thus, LBP stimulates the innate immune response to several microbes (Zweigner et al., 2006).

Fb is a constitutive plasma protein that behaves as an APP in most species, including birds (Jain, 1993; Petersen et al., 2004) and cattle (Conner et al., 1988). It increases in various inflammatory diseases of cattle, such as peritonitis, endocarditis, pericarditis, pneumonia, mastitis, enteritis and nephritis (McSherry et al., 1970; Sutton and Hobman, 1975). The most consistent changes have been reported in peritonitis and pericarditis (McSherry et al., 1970), findings later supported by Hirvonen et al. (1998), who described Fb to be a good marker of traumatic reticuloperitonitis. During the last decades, Fb, together with Hp, is probably the APP most commonly used as a marker of host inflammatory response in research of cattle (see Table 1 for studies of experimental respiratory infections). Some other studies where Fb has been used include castration models in calves (Fisher et al., 1997a; 2001), evaluation of the effect of assembling and transport stress on beef calves (Phillips, 1984) and experimental lungworm *Dictyocaulus viviparus* infection in calves (Ganheim et al., 2004).

Fb, factor I of the coagulation system, is the circulating precursor of fibrin. This plasma protein plays an important role in haemostasis and thrombosis by its interaction with thrombin, factor XIII, plasminogen, glycoprotein IIb/IIIa and endothelial cells (Jain, 1993).

Ceruloplasmin (Cp; Conner et al., 1986; 1988; Arthington et al., 1996), protease inhibitors like alpha$_1$-proteinase inhibitor (α_1-PI; Honkanen-Buzalski et al., 1981; Conner et al., 1986; 1989), alpha$_2$-macroglobulin (Conner et al., 1989; Cheryk et al., 1998), alpha$_1$-antichymotrypsin (Conner et al., 1989) are some other plasma proteins reported to act as positive acute phase reactants in cattle. Recently, ITIH4 (inter-alpha-trypsin inhibitor heavy chain 4) has been described to be an APP in cattle (Pineiro et al., 2004). ITIH4 is also known as major acute-phase protein (MAP) and has previously been established as an APP in pigs (Heegaard et al., 1998; González-Ramón et al., 2000) and rats (Daveau et al., 1998).

2.3 Acute phase proteins in cervids

Studies on APR in cervids are relatively scant. Some studies have been published about methods to determine Fb and physiological concentrations of Fp in reindeer (Halikas and Bowers, 1972; Catley et al., 1990). Increased Fb concentrations have been observed in sick reindeer (Catley et al., 1990). In other cervid species, such as red deer, Fb has also been noted to behave as an acute phase reactant. Concentrations of Fb have been shown to increase after experimental infection of red deer with malignant catarrhal fever (Sutherland et al., 1987). Increased concentrations of Fb and Hp have been reported in red deer after tuberculine testing (Cross et al., 1991) and after inoculation with *Yersinia pseudotuberculosis* (Cross et al., 1994). In red deer, systemic Hp showed high predictive value in identifying animals with proliferative tuberculosis (Griffin et al., 1992). However, experimental infection with bluetongue virus (Howerth et al., 1988) or chemical immobilization (Kocan et al., 1981) did not affect Fb concentrations in captive white-tailed deer. Sedation with xylazine resulted in lower Fb concentrations in red deer (Cross et al., 1988). In other cervid species, occasional studies on Fb or Hp

are available, and physiological values have been reported for Hp in roe deer (Hartwig et al., 1983), and for Fb in fallow deer (Sutherland et al., 1985), axis deer, Pere David's deer and barashingha (Hawkey and Hart, 1985).

2.4 Endotoxin challenge as an inducer of APPs

The cell wall of Gram-negative bacteria consists of three layers, with the outer cell layer containing phospholipids, membrane proteins and lipopolysaccharide (LPS). Lipid-A is the lipophilic, inner part of LPS, and it is responsible for the toxic effects of LPS (Hogan and Smith, 2003). The terms endotoxin and LPS are frequently used as synonyms. A systemic endotoxin model has been used in animal experiments to simulate infections due to Gram-negative bacteria like *Escherichia coli*. Endotoxin mainly induces inflammatory response during *E. coli* infection, although other minor contributors may also exist (Gonen et al., 2007). Effects of LPS are based predominantly on activation of pro-inflammatory cytokines, like TNF-α, IL-1β and IL-6 (Lohuis et al., 1988), released by monocytes and macrophages in response to LPS (Henderson and Wilson, 1996). This makes systemic LPS challenge a useful model for APP research, and it has been widely used in cattle (Boosman et al., 1989; Conner et al., 1989; Werling et al., 1996; Jacobsen et al., 2004) as well as in other animal species, e.g. pigs (Dritz et al., 1996; Wright et al., 2000). *E. coli* endotoxin challenge is also a standard method for APP research in laboratory animals (Dowton et al., 1991; Rygg et al., 1996).

2.5 Acute phase proteins after birth

Adaptation of neonatal animals to extrauterine life is a complicated physiological process involving many different mechanisms. APR is one of the essential mechanisms to regain homeostasis. Consequently, it may be reasonable to hypothesize that initiation of inflammatory response, reflected as changes in APP concentrations, would be seen in newborns. Possible factors affecting the concentrations of APPs after birth include foetal synthesis of APPs, APP stimulation by birth trauma, intake of colostrum containing APP or their stimulants and immaturity of synthesis capacity of the newborn liver. Introduction to the extrauterine environment, which contains various microbes, could also trigger an inflammatory response.

Colostrum contains high quantities of inflammatory mediators (Munoz et al., 1990; Bocci et al., 1993). Transfer of colostral cytokines to the blood of calves has been reported (Goto et al., 1997; Yamanaka et al., 2003a). Colostral inflammatory mediators may thus induce APR in the newborn. Direct transfer of APPs from colostrum to newborns may potentially occur. Schroedl et al. (2003) found that bovine colostrum contained high levels of C-reactive protein (CRP), and calves had elevated systemic CRP concentrations after colostrum consumption. Although CRP is a major APP in many species (e.g. pigs, dogs and humans), it is a constitutive protein in cattle, and blood concentrations do not change markedly during inflammation (Maudsley et al., 1987). Schroedl et al. (2003) concluded that transfer of CRP from colostrum was the reason for elevated serum concentrations of CRP in newborn calves, and higher CRP levels contribute to protection against infections. McDonald et al. (2001) reported high concentrations of mammary-associated SAA in the colostrum of healthy cows, and they suggested a possible role for mammary SAA in supporting the welfare of calves.

Human mammary-associated SAA was shown to have a primarily protective effect on the gastrointestinal tract of neonates by stimulating mucin production and reducing adherence of pathogens (Larson et al., 2003). Potential transfer of SAA from colostrum to the circulation of newborns has not been investigated.

Differences in neonatal and adult AGP isoforms (Itoh et al., 1993a) and a rise in systemic AGP concentration already during the foetal stage in calves and piglets have been reported (Stone and Maurer, 1987; Itoh et al., 1993a). These findings indicate that neonatal AGP is probably differently regulated than in adults. Existence of neonatal and adult SAA isoforms and different regulation of systemic SAA production in adult and newborn cattle are also possible, as multiple SAA isoforms have been found (Alsemgeest et al., 1995c).

Physical stress or trauma during parturition may induce a rise in systemic APP concentrations of the neonate. This has been proposed as a reason for high SAA concentrations in infants immediately after birth (Marchini et al., 2000). LBP concentrations, by contrast, have not been reported to be affected by labour in humans (Behrendt et al., 2004). Concentrations of systemic CRP in infants at birth were negatively associated with the Apgar score used to assess the fitness of the baby and positively associated with a birth complication, namely rupture of membranes for 18 h or longer (Chiesa et al., 2001). However, these associations were no longer significant when CRP was measured at 24 and 48 h after birth (Chiesa et al., 2001). Babies delivered by Caesarean section also had lower peak CRP values at 48 h than vaginally born babies, but by the end of the first week concentrations decreased to baseline levels (Ishibashi et al., 2002). Results from an animal study (Richter, 1974) also support theories of the effect of birth trauma and/or colostrum on systemic APP concentrations of the newborn; piglets born by Caesarean section and deprived of colostrum had only temporal and low elevation of serum Hp compared with conventionally reared piglets.

Immaturity of the neonatal liver to mount an APP response to an inflammatory stimulus could affect APP concentrations in neonatal animals. In humans, Hp, SAA and AGP have been reported to be lower at birth and to increase progressively to normal adult concentrations by 6 months of age (Kanakoudi et al., 1995; Brunn et al., 1998). Low Hp concentrations are common in newborn infants. This has been related to immaturity of the liver to produce Hp in a situation where Hp consumption is increased because of haemolysis of foetal erythrocytes (Dobryszycka, 1997). Studies in laboratory animals indicate that APP gene expression in hepatocytes is age-dependent. Newborn rats had lower APP mRNA expression than adults after stimulation by turpentine injection (Schwarzenberg et al., 1991), reaching adult levels by day 7–19. Neonatal rabbits had only a 1.2-fold increase in CRP mRNA expression, but adult rabbits a 20-fold increase after turpentine stimulation (Baker and Long, 1990).

Transient changes in APP hepatic gene expression seen in neonatal laboratory animals (Glibetíc et al., 1992; Rygg, 1996) or temporal changes in APP concentrations in newborn piglets (Martin et al., 2005) may reflect the adaptation mechanisms necessary for extrauterine life, as suggested by the authors. However, the question of a possible effect of exposure to the extrauterine environment and predisposing pathogens on APP response in newborn animals has not yet been thoroughly addressed.

Very few studies on the concentrations of APP in bovine calves after birth are available. Alsemgeest et al. (1993) did not report changes in concentrations of SAA in four cannulated foetuses before and after birth. Unfortunately, they only measured SAA concentrations for 24 h after birth. The same authors found low SAA and Hp concentrations in calves sampled within 10 minutes of parturition (Alsemgeest et al., 1995b). Knowles et al. (2000) reported considerable fluctuation and some very high Hp concentrations during the first 2 weeks of life in a group of 14 calves. In the same calves, temporal elevation of mean Fb concentration during the first 2 weeks after birth was evident, although the rise was relatively small and concentrations did not exceed the reference limit (Knowles et al., 2000). Very similar transient and relatively small increases in Fb concentrations during the first 2 weeks of life in calves have been reported earlier (Gentry et al., 1994). In the study by Schroedl et al. (2003), Hp concentrations in newborn calves did not differ between samples obtained at birth, at 1 day of age and at 10 days of age. Itoh et al. (1993a) reported a rise in AGP concentrations in foetuses before birth, the highest concentrations being reached at birth, followed by a decrease during the first 3 weeks of life to adult levels.

2.6 Acute phase proteins as markers of BRD

2.6.1 Bovine respiratory disease

Bovine respiratory disease (BRD) is one of the most important health problems in beef and dairy calves (Lekeux, 1995). It causes major economic losses in both production systems (Kapil and Basaraba, 1997; van der Fels-Klerx et al., 2001). BRD is a multifactorial disease complex, and it is used to refer to different infectious conditions of the respiratory tract in cattle of different ages and in various management systems. BRD is predominantly associated with young (<2 years) calves, heifers and steers (Ames, 1997) starting from the first weeks of life (Virtala et al., 1996; Crowe, 2001). The most common form of BRD is enzootic calf pneumonia, a clinical respiratory disease seen periodically or year round, mostly in young animals. Epizootic calf pneumonia or BRD outbreaks occur when a group of calves suddenly shows signs of BRD. Outbreaks are mostly caused by a primary viral infection. In beef calves, the term ”shipping fever” is used because in most cases pneumonia develops after transport of the animals to rearing units or feedlots (Ames et al., 2002; van der Fels-Klerx et al., 2002).

BRD is caused by one or more respiratory pathogens (viral, bacterial or mycoplasmal), and a synergistic effect between pathogens is common. Different viruses, such as bovine respiratory syncytial virus (BRSV), bovine herpes virus 1 (BHV-1), bovine parainfluenza virus 3 (PIV-3), bovine coronavirus (BCV), bovine adenovirus (BAV) and bovine viral diarrhoea virus (BVDV), have been identified in BRD (Bryson et al., 1978; Stott et al., 1980; Kapil and Basaraba, 1997; Hägglund et al., 2006). BRSV is one of the most important aetiological factors of BRD, especially in young calves (Baker et al., 1986; Uttenthal et al., 1996). The most common bacteria isolated in BRD are M. haemolytica, Pasteurella multocida, Histophilus somni and Arcanobacterium pyogenes (Babiuk et al., 1988; Autio et al., 2006; Duff and Galyean, 2007). Bacteria and viruses may also interact synergistically with Mycoplasma spp. (e.g. M. bovis and M. dispar), causing a more severe disease (Virtala et al., 1996; Thomas et al., 2002). In general, viral or Mycoplasma spp. infections damage the respiratory tract defence mechanisms, enabling secondary bacterial colonization, which leads to a more severe clinical disease (Babiuk et al., 1988). Most infectious agents involved in BRD are ubiquitous in cattle

populations, and bacteria associated with the disease may be isolated in the respiratory tract of healthy animals (Autio et al., 2006), highlighting the complexity of respiratory disease. In addition, BRD is heavily influenced by various predisposing environmental and host risk factors, e.g. lack or poor quality of colostrum, transport, commingling of animals, poor ventilation of barns and crowded housing. In addition, other diseases like diarrhoea can compromise the immunity of the calf, making it more susceptible to BRD (Ames et al., 2002).

2.6.2 Acute phase proteins in experimental BRD infections

Several studies have been published during the past two decades on bovine APPs in experimental respiratory infections. *M. haemolytica* alone or in combination with other pathogens has been the infectious agent most frequently used (Table 1). Changes in the concentrations of APPs have been used to evaluate the host response to the respiratory infection. Fb and Hp are the most common markers used for this purpose. For example, responses of gnotobiotic or conventional calves (Vestweber et al., 1990) and the effect of copper deficiency or some other treatment (Babiuk et al., 1985; Arthington et al., 1996; Corrigan et al., 2007) have been studied in these experimental models. Furthermore, APPs have been used to validate respiratory infection models (Ciszewski et al., 1991; Dowling et al., 2002), to assess the effect of vaccines (Blanchard-Channell et al., 1987; Antonis et al., 2007) or to explore immunological responses (Grell et al., 2005). Another group of experimental studies has been carried out especially to investigate bovine APP responses during the inflammatory response, starting with the experimental *M. haemolytica* infection by Conner et al. (1989). Since then, different bovine APPs have been explored in respiratory bacterial, viral or combined infections. A list of published APP studies using experimental infection models of cattle is shown in Table 1.

Bacterial respiratory infection (mainly *M. haemolytica*) seems to be a potent APP inducer, as APP responses of varying degree can be seen in all experiments using bacterial or combined infection models. APP response to viral infections appears to be more variable. BVDV increased systemic concentrations of Hp, SAA and Fb (Ganheim et al., 2003; Schaefer et al., 2004). BRSV caused an increase in SAA and Hp (Heegaard et al., 2000; Grell et al., 2005; Antonis et al., 2007), but not in Fb (Ciszewski et al., 1991), and some calves did not show an Hp response to BRSV infection in the study of Heegaard et al. (2000). However, in another study, BAV-3 and BHV-1 caused an increase in Fb concentration (Cole et al., 1986). BHV-1 infection resulted in an increase of Cp, but not in Fb (Arthington et al., 1996), and when the same authors repeated the study with the same infection model using younger calves, neither APP responded to BHV-1 infection (Arthington et al., 1997). Blanchard-Channell et al. (1987) and Godson et al. (1996) described a substantial Fb and Hp response to *M. haemolytica* inoculation after BHV-1 infection. No reports on the effects of *Mycoplasma* spp. and some viruses (PIV-3 and BCV) are thus far available. The APP response in viral respiratory infections in calves appears to be variable and generally weaker than that in bacterial infections. These experimental studies confirm that measurement of APPs could be useful in exploring host responses to spontaneous respiratory infections.

Table 1. Published studies of APPs in calves in experimental models of respiratory infections.

Infection agent used (in experimental infection)	APPs measured	Main objective	Reference
Bacteria			
M. haemolytica	Hp, AGP, Cp, α_1-PI, α_2-macroglobulin, α_1-antichymotrypsin	APP research	Conner et al., 1989
	Fb	Host response	Vestweber et al., 1990
	SAA	APP research	Horadagoda et al., 1993
	SAA, Hp	APP research	Horadagoda et al., 1994
	LBP	APP research	Horadagoda et al., 1995
	Hp, Fb, albumin, α_2-macroglobulin	Host response	Cheryk et al., 1998
	SAA	APP research	Yamamoto et al., 1998
	Hp	APP research	Katoh and Nakagawa, 1999
	SAA, Hp	APP research	Nakagawa and Katoh, 1999
	LBP, Hp	APP research	Schroedl et al., 2001
	SAA, Hp, Fb	APP research	Ganheim et al., 2003
	Hp, Fb	Host response	Corrigan et al., 2007
P. multocida	SAA, Hp, AGP	Host response	Dowling et al., 2002
	SAA, Hp, AGP	Host response	Dowling et al., 2004
	SAA	Vaccine effect	Hodgson et al., 2005
H. somni	Hp	APP research	McNair et al., 1997
Viruses			
BHV-1	Fb	Host response	Cole et al., 1986
	Fb, Cp	Host response	Arthington et al., 1996; 1997
	SAA, Hp	APP research	Nakagawa and Katoh, 1999
BRSV	Fb	Host response	Ciszewski et al., 1991
	Fb	Host response	Bingham et al., 1999
	SAA, Hp	APP research	Heegaard et al., 2000
	Hp	Host response	Grell et al., 2005
	Hp	Vaccine effect	Antonis et al., 2007
BVDV	SAA, Hp, Fb	APP research	Ganheim et al., 2003
	Hp	Host response	Schaefer et al., 2004
BAV-3	Fb	Host response	Cole et al., 1986
Combinations			
BHV-1 and *M. haemolytica*	Fb	Host response	Babiuk et al., 1985
	Fb	Vaccine effect	Blanchard-Channell et al., 1987
	Hp	APP research	Godson et al., 1996
BHV-1 and *P. multocida*	Fb	Vaccine effect	Chengappa et al., 1989
BVDV and *M. haemolytica*	SAA, Hp, Fb	APP research	Ganheim et al., 2003

2.6.3 Acute phase proteins in BRD studies in the field conditions

Despite extensive information from experimental infection models, limited data are available on APPs related to spontaneous BRD. Published studies have mainly aimed at evaluating the potential of APPs as diagnostic tools in veterinary practice for individual animal or herd health monitoring. For example, Ganheim et al. (2007) reported higher APR scores (combined APP results and total leukocyte counts) in a group of calves with a high prevalence of respiratory disease and diarrhoea, supporting the use of APPs in herd health monitoring. Host response to respiratory infections caused by specific pathogens has rarely been evaluated, and practically the only APPs investigated are Hp and Fb. One exception is the early study by Thomson et al. (1975), where calves were divided in to healthy and sick groups based on high concentrations of Fb and rectal temperature. In the sick group, the mean nasal colonization by *M. haemolytica* was higher, but in the nasal colonization of *P. multocida* or PIV-3 serum antibody titres, no differences were seen. Concentrations of Fb remained high in the group of sick calves throughout the study (Thomson et al., 1975).

Studies on systemic Hp concentrations associated with clinical BRD have been published, with somewhat contradictory results. Wright et al. (1995) reported that concentrations of Hp in serum of calves decreased after treatment for BRD, but the initial values were not different from healthy calves. Other studies have indicated that serum Hp concentration may be an indicator of treatment efficiency but was unrelated to disease severity or need for treatment (Wittum et al., 1996). Hp alone was inadequate for predicting clinical BRD, at least when cross-sectional sampling was applied (Young et al., 1996). A positive association was found between systemic Hp concentration and subsequent clinical BRD and pulmonary lesions at slaughter (Young et al., 1996). Svensson et al. (2006) concluded that serum Hp was not very useful in diagnosing BRD in calves; however, in combination with rectal temperature, it could be used as a BRD marker in heifer calves, especially for herd-level diagnostics. Others have shown that Hp would be useful in identifying beef calves with BRD needing treatment and in monitoring treatment efficacy (Carter et al., 2002; Berry et al., 2004; Humblet et al., 2004). Soethout et al. (2003) successfully used systemic Hp to quantify the severity of spontaneous BRD in a study on the role of α4-integrin expression in calf lung neutrophils during pneumonia. Fb concentration has also been shown to be a useful clinical tool in BRD (Berry et al., 2004; Humblet et al., 2004). However, calves needing treatment were identified more efficiently using a combination of Hp and Fb (Humblet et al., 2004). SAA and AGP were not particularly useful markers of BRD in feedlot calves (Carter et al., 2002; Berry et al., 2004). Berry et al. (2004) claimed that SAA would be a poor diagnostic tool because of its sensitivity to other stress factors. Overall, Hp and Fb seem to be best candidates as clinical tools in BRD, but more studies with different APPs in different management conditions are warranted. To evaluate APPs as research tools in spontaneous respiratory infections, differently designed experiments are needed.

3 AIMS OF THE STUDY

Specific aims of the study were as follows:

1. To characterize SAA and Hp as potential acute phase proteins in reindeer using an *E. coli* endotoxin challenge model.

2. To investigate systemic concentration in acute phase proteins during the first weeks of life in reindeer and dairy calves.

3. To investigate concentrations of acute phase proteins in relation to respiratory infections during naturally acquired respiratory disease in dairy calves.

4 MATERIALS AND METHODS

4.1 Animals, study design, sampling and clinical evaluation

4.1.1 *Escherichia coli* endotoxin challenge in reindeer (I)

Eight adult female reindeer were randomly divided into two equal groups. The mean weight of the animals was 76.7 (range 67.5–90.5) kg and the mean age was 5 (3–6) years. The groups were kept in two separate corrals (about 650 m^2 each) in which they adapted before the experiment was initiated. The first group was challenged with 0.1 mg/kg *E. coli* 0111:B4 lipopolysaccharide B (LPS; 1 mg/ml Bactoq, Difco Laboratories, Inc., Detroit, MI, USA) administered into the jugular vein. The second group received the same volume of physiological saline solution (0.1 ml/kg). After 7 weeks, the procedure was repeated in reverse. The group of reindeer was herded into the small pen near the corrals and then caught and manually restrained. Blood samples were drawn from the jugular vein into plain tubes before the challenge (0 h) and then at 1, 4, 8, 12, 24, 48, 96 and 168 h. The serum was separated and stored at -20°C for further analysis. Rectal temperature was recorded when blood was taken during the first day of the experiments. Animals were observed without manual handling every hour during the first 12 h. After the experiments, the animals were killed and autopsies performed.

After laboratory analysis, two reindeer were excluded from the statistical analysis, one from each group. One reindeer had shown considerably increased Hp (over 3-fold) and SAA (over 10-fold) concentrations compared to other reindeer in the 0-h sample, and the concentrations remained high throughout the first experiment. Clear arthritic changes were found in the left tarsal joint of this animal at autopsy; in all other reindeer, no pathological changes were found. The second excluded reindeer reacted very strongly to the restraint procedures, and its serum creatine kinase (CK) and aspartate aminotransferase (ASAT) activities increased markedly during the first 24 h of experiments, indicating muscle injury.

4.1.2 Newborn reindeer calves (II)

Reindeer calves born from 9 to 23 May 2002 in the Kaamanen experimental herd, Finnish Lapland, were blood-sampled four times during the calving season. The time periods between samplings were 7, 7 and 11 days. Two to four blood samples from each of the 51 reindeer calves (23 males, 28 females) were obtained during the first month after birth, for a total of 174 blood samples. The ages of the calves at sampling varied from 0 to 32 days. Calves and their hinds were kept in outdoor pens during the calving season, and the calves' main source of food was milk from the hinds. The herd was released after calving to summer pastures and allowed to graze under natural conditions.

Blood was drawn into 10-ml evacuated glass tubes. The serum was separated, frozen in portions and stored at -20°C for further analysis. At the time of sampling, clinical status of the calves was evaluated. The calves were weighed within 24 h of birth, at the end

of the calving season before being put out to pasture (10 June, at age 18–32 days) and in autumn (11 September, at age 111–125 days). Daily weight gain was calculated for the neonatal period and for the entire study period. Two samples from one calf (from days 9 and 16) were excluded from the study as outliers because of exceptionally high SAA and Hp concentrations. SAA concentrations in these samples were 1271.8 and 1226.7 mg/l, respectively (range of other samples 0.0–181.9 mg/l), and Hp concentrations 1.85 and 4.57 g/l (range of other samples 0.38–0.88 g/l). This calf and two other calves were slaughtered before weighing in autumn, and a fourth calf disappeared in the summer. Because time periods between samplings were different (see above), data from 51 calves were pooled into 5 age groups (0, 1-7, 8-14, 15-21 and 22-32 days; white bars in Fig 3). Data from calves sampled at least three times were used for statistical analysis for age dependent changes (age groups 1–7, 8–14 and 22–32 days; black bars in Fig 3).

4.1.3 Newborn dairy calves (III)

A group of 13 Holstein Friesian calves (7 males, 6 females; Group A), born on the Helsinki University Suitia Research Farm, was used to study changes in APPs during the first 3 weeks of life. Calves were raised according to the regular routine of the farm. Within 3 hours of birth, calves received colostrum from their dams. Calves were kept in individual pens and fed milk from their dams 3 times a day for 5 days, after which they were adapted to the automated feeding system with milk powder. At the age of 1-1.5 weeks, they were moved to group fences with an automatic feeding system and free access to water, silage and hay. Blood samples were collected at the age of 0 or 1 day (median time from birth 18 h, range 4-32 h) and at 3, 7, 10, 14 and 21 days. The second group of 13 Holstein Friesian calves (5 males, 8 females; Group B), born on the same farm and raised similarly, was sampled during the first 2 months of life, first at 3 days of age and then weekly. The mean ages of the calves at the time of weekly sampling were 10, 17, 24, 31, 38, 45, 52 and 59 days. Serum was separated and stored in aliquots at -20°C. Colostrum samples from the dams of calves in Group A were also stored at -20°C. At each sampling point, every calf was examined clinically and rectal temperature was measured.

The need for obstetric assistance was recorded for every calf and graded as spontaneous parturition (no assistance), extraction by one person or forceful extraction by two persons. If a calf had clinical signs of disease at the time of sampling (signs of respiratory disease, diarrhoea) or a rectal temperature >39.5°C, the sample was excluded from the analysis at that time point.

4.1.4 Spontaneous respiratory disease in calves – cross-sectional study (IV)

Eighteen herds were included into the study (10 fattening units and 8 dairy herds). The fattening units had 48–217 young cattle, and dairy herds had 30–130 cows. When BRD problems were discovered by farmers, the farms were visited by veterinarians who examined all calves with clinical signs. Calves' heart rate, respiratory rate and rectal temperature were measured, and respiratory sounds and the appearance and amount of nasal discharge were recorded. Any coughing or diarrhoea was noted. A total of 90 calves (5 calves from each herd) were chosen for tracheobronchial lavage (TBL) sampling. The pre-set criterion for including a calf in the study was abnormal sounds on auscultation of the respiratory tract. In addition, all of the chosen calves had at least one of the following signs: increased respiratory rate (>40/min), rectal temperature

>39.5°C, cough or nasal discharge. The calves were mostly of the Ayrshire or Holstein–Friesian breed. The mean age and weight of calves were 98 (range 59–137) days and 88 (range 61–115) kg, respectively.

TBL and blood samples were taken from the calves without sedation. A double catheter sampling method for TBL was used (Bengtsson et al., 1998). The calf was restrained by assistants and a sterile plastic double catheter was inserted through the ventral nose duct into the trachea. Then the inner catheter was pushed through the silicone plug of the outer catheter as far as possible. Sterile phosphate-buffered saline (30-40 ml; PBS, Dulbecco's phosphate-buffered saline, Gibco TM, Invitrogen Corporation, Paisley, Scotland, UK) was injected into the catheter and aspirated immediately. The TBL sample was divided into test tubes with a glucose calf serum broth (GS) for mycoplasma and for bacteria isolation to the transport media (Portagerm multi-transport medium, BioMerieux, Lyon, France). The mycoplasma samples were kept frozen at -70°C before cultured.

Blood samples were collected into plain tubs for serum samples and into EDTA tubes for WBC count and Fb determination. Serum was separated and stored at -20°C for serological tests and at -70°C for determination of APPs (Hp, SAA, AGP and LBP). The second serum sample was collected from the same calves 3–4 weeks later. Finally, only those 84 calves for which all TBL samples could be obtained and analysed were included in the study.

4.1.5 Spontaneous respiratory disease in calves – longitudinal study (V)

The original purpose of this study was to investigate the effects of age on the physiological concentrations of APPs. Ten Holstein-Friesian calves (7 males, 3 females) from the Helsinki University Suitia Research Farm were followed up weekly over a 6-week period. The ages of the calves at week 0 (first sampling) were 9-32 days. Calves were housed in two group fences (calf nos. 1-5 in one and nos. 6-10 in another fence) with an automatic milk feeding system (one nipple in fence) with milk powder and free access to water, silage and hay. After weaning (approximately at 8 weeks of age), calves were moved into a group fence with older calves.

Calves were exposed to an initial BRS virus infection probably around the week 0 sampling time. Four days before the week 0 sampling, one older calf was brought back from a veterinary clinic after having surgery due to umbilical hernia. This calf showed clinical signs of BRD on arrival and was treated with antibiotics. Blood sampling started one week (week 0) before the manifestation of the first clinical signs of BRD. Serum was separated and stored at -20°C for further analysis. Calves were clinically examined in conjunction with each blood sampling. The overall clinical score for respiratory disease was calculated according to Hägglund et al. (2004).

TBL samples were taken at week 2 and at the end of the experimental period (week 6) by the double catheter method described earlier (see paragraph 7.1.4.), with some differences. Isotonic saline solution was used instead of PBS and 0.5 ml of TBL samples were transferred into mycoplasma D medium (Friis and Krogh, 1983). TBL samples from three calves at week 6 could not be investigated because of difficulty in obtaining TBL samples. Calves were retrospectively divided into low and high (n = 5) BRSV IgG_1 antibody response groups (Fig. 9) for exploring possible effect of antibody production to inflammatory response.

4.2 Ethical considerations

All studies were approved by the local Ethics Committees for Animal Experiments.

4.3 Analytical methods

4.3.1 Acute phase proteins

4.3.1.1 Serum amyloid A (I-V)

SAA concentrations in serum of reindeer, reindeer calves, dairy calves and in colostrum of cows were measured with a commercially available solid phase sandwich ELISA kit (Phase SAA Assay, Tridelta Development Ltd., Maynooth, Co. Kildare, Ireland) according to the manufacturer's instructions for cattle. A standard curve, consisting of six points, was used (0.0, 9.4, 18.7, 37.5, 75 and 150 ng/ml), and optical density of ELISA plates was measured at 450 nm with the reference at 620 nm using a spectrophotometer (Multiskan MS, Labsystems Oy, Helsinki). According to the manufacturer, the detection limit of the assay for bovine samples is 0.3 mg/l. The initial dilution for serum samples was 1:500 and for colostrum samples 1:50. Intra-assay coefficients of variation (CV) were <7% (mean concentrations of control samples 18.9 mg/l; n = 20 and 89.5 mg/l; n = 19). Inter-assay CVs, were <16% (I) (mean concentrations of control samples 9.9 mg/l; n = 6 and 76.7 mg/l; n = 6), <12% (II) (mean concentrations of control samples 12.7 mg/l; n = 8 and 72.0 mg/l; n = 8), <9% (III) (mean concentrations of control samples 8.9 mg/l; n = 6 and 65.0 mg/l; n = 6), <13% (IV) (mean concentrations of control samples 12.4 mg/l; n = 4 and 73.3 mg/l; n = 4) and <15% (V) (mean concentrations of control samples 16.6 mg/l; n = 6 and 133.0 mg/l; n = 6).

4.3.1.2 Denaturing isoelectric focusing and Western blotting of SAA (III)

Denaturing isoelectric focusing (IEF) and Western blotting were used to identify SAA isoforms in calves' serum and colostrum of their dams, as described by Jacobsen et al. (2005).

4.3.1.3 Haptoglobin (I-V)

Serum Hp was determined using the haemoglobin binding assay described by Makimura and Suzuki (1982), with the modification of tetramethylbenzidine (0.06 mg/ml) being used as a substrate (Alsemgeest et al., 1994). Twenty microliters of standard solutions, controls and test serum samples were mixed with 100 μl of a methaemoglobin (metHb) solution (0.3 mg/ml) in 10-ml plastic tubes.

A metHb stock solution was prepared from bovine erythrocytes by washing EDTA stabilized bovine blood five times with isotonic saline solution. After washing, erytrocytes were mixed with distilled water in equal proportions. The mixture was centrifuged at 3500 rpm for 15 min, clear supernatant was removed, and 4 drops of 10% potassium ferricynide was added per 6 ml of haemolysate. Mixture was allowed react for 10 min. Haemolysate was filtered (filter size 0.45 μm) and metHb concentration was quantified with standard metHb. Solution was diluted to 3 mg/ml with isotonic saline, and stored in 1 ml aliquots at -70°C. Before the use, stock metHb solution was diluted with isotonic saline (working concentration 0.3 mg/ml).

After a 10-min incubation of samples, standards and controls with metHb at room temperature, 2.5 ml of 0.9% saline solution was added. After mixing, 20 µl of the standard, control and sample solutions were applied in triplicate to the 96-well microplate. A chromogen solution was prepared by dissolving tetramethylbenzidine (Tetramethylbenzidine dihydrochloride, Sigma-Aldrich Co., St Louis, MO, USA) in chromogen buffer (final concentration of 0.06 mg/ml). A chromogen buffer was prepared by dissolving 0.5 g of di-Na EDTA and 15.6 g of NaH_2PO_4-$2H_2O$ in 500 ml of distilled water, and the solution volume was extended to 1000 ml. The chromogen buffer pH was adjusted to 3.8. To each well, 200 µl of chromogen solution was added. After incubation for 1 h at 37°C, 50 µl of substrate (12 µl of 30% H_2O_2 solution in 10 ml of sterile water) was added. The plate was allowed to stand at room temperature for 15 min for colour formation. The reaction is stopped by adding 50 µl of sulphuric acid (20% solution), and the absorption of the wells was read at 450 nm using a spectrophotometer (Multiskan MS, Labsystems Oy, Helsinki). Pooled and lyophilized aliquots of bovine acute phase serum were used to create standard curves by serial dilutions with isotonic saline. To calibrate the assay, a bovine serum sample with a known Hp concentration provided by the European Commission Concerted Action Project (number QLK5-CT-1999-0153) was used. The range of the standard curve was 0.04-1.16 g/l, and a zero standard was included in the assay. If a sample's Hp concentration was higher, the sample was diluted with isotonic saline and reassayed. The intra-assay CVs, were <12% (mean concentrations of control samples 0.10 g/l; n = 20 and 0.98 g/l; n = 20). Inter-assay CVs were <14% (I) (mean concentrations of control samples 0.11 g/l; n = 6 and 0.98 g/l; n = 6), <18% (II) (mean concentrations of control samples 0.14 g/l; n = 11 and 1.02 g/l; n = 11), <11% (III) (mean concentrations of control samples 0.13 g/l; n = 8 and 0.96 g/l; n = 8), <7% (IV) (mean concentrations of control samples 0.15 g/l; n = 5 and 2.3 g/l; n = 5) and <9% (V) (mean concentrations of control samples 0.24 g/l; n = 4 and 1.08 g/l; n = 4). Because haemolysis can interference the results, 12 (III) and 18 (II) haemolysed samples (detected by visual examination) were removed from the Hp analysis.

4.3.1.4 *Alpha$_1$-acid glycoprotein (III-V)*

Serum AGP was analysed using a commercial radial immunodiffusion kit for cattle (Bovine AGP, Tridelta Development Ltd., Maynooth, Co. Kildare, Ireland). Five microliters of high (1000 mg/l) and low (250 mg/l) bovine purified AGP standard solutions and test samples were applied to the wells of test plates (10-well plates). Plates were incubated for 24 h at 37°C in humidified chambers, and the diameter of precipitin rings (mm) was measured. High and low standard sample diameters were plotted on semilogarithmic graph paper to yield a linear standard curve, with the y-axis representing the ring diameter and x-axis the concentration of AGP. Using this standard line, AGP concentrations of test samples were obtained. Samples with a high result (over 1000 mg/l) were diluted with isotonic saline and reassayed. The intra-assay CV was <4% (mean concentrations of control samples 332 mg/l; n = 10 and 893 mg/l; n = 10).

4.3.1.5 *Lipopolysaccharide binding protein (III-V)*

Serum LBP concentrations were determined using a commercially available ELISA kit with cross-reactivity to bovine LBP (Bannerman et al., 2003) (LBP ELISA for various species, HyCult Biotechnology, Uden, The Netherlands). Serum samples were initially diluted 1:1000. Optical density of plates was read at 450 nm using a spectrophotometer (Multiskan MS, Labsystems Oy, Helsinki). The concentration of LBP was calculated from a standard curve of known amounts of human LBP included with every plate.

The range of the human LBP standard curve was 1.6-100 µg/l. Intra-assay CVs were <9% (mean concentrations of control samples 21.3 mg/l; $n = 10$ and 81.2 mg/l; $n = 10$) and inter-assay CVs <13% (III, V) (mean concentrations of control samples 17.9 mg/l; $n = 10$ and 91.5 mg/l; $n = 10$) and <26% (IV) (mean concentrations of control samples 10.1 mg/l; $n = 4$ and 76.4 mg/l; $n = 4$).

4.3.1.6 Fibrinogen (IV)

Fb concentration in plasma was measured by the heat precipitation method (Millar et al., 1971). Two microhaematocrit tubes were filled with EDTA whole blood and sealed from one end for each individual measurement. After centrifugation for 5 min at 15 000 r/min, the tubes were placed in a water-bath at 56°C for 3 min. The precipitated Fb was packed on top of the blood cell column after a second centrifugation for 3 min. The lengths of the precipitated Fb column and the total plasma column were measured using a microscope with an ocular micrometer. The plasma Fb concentration (g/l) was determined by calculating Fb column percentage relative to the plasma column length. The average of the two microhaematocrit tubes was used as the test result.

4.3.2 White blood cell count (IV)

White blood cell (WBC) count was determined by an automatic cell counter adjusted for animal cell counting (Coulter-Counter Model T850, Coulter Electronics Ltd., Luton, UK).

4.3.3 Blood chemistry (total protein, albumin, iron, urea, enzymes and cortisol) (I)

The activities of ASAT and CK were determined following the recommendations of the Scandinavian Society for Clinical Chemistry and Clinical Physiology (1974, 1979). Spectrophotometric methods were used for the determination of serum total protein (Weichselbaum, 1946), urea (Gutmann and Bergmeyer, 1974), sorbitol dehydrogenase (SDH) (Gerlach and Hiby, 1974), albumin (Doumas et al., 1971) and gamma-glutamyl transferase (GGT) according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Expert Panel on Enzymes (1983). Serum iron was determined with a colorimetric method (Persijn et al., 1971). The analyses were performed with an automatic chemistry analyser (KONE Pro, Konelab, Thermo Clinical Labsystems Oy, Vantaa, Finland). Cortisol was measured in 25-µl duplicates using radioimmunoassay with Coat-A-Count RIA kits obtained from Diagnostic Products Corporation (Los Angeles, CA, USA).

4.3.4 Gammaglobulins and viral antibody detections (II, IV, V)

Reindeer calves serum concentrations of gammaglobulins were quantified by serum protein electrophoresis of the agarose gel using a Paragon® electrophoresis system (Becman Coulter, Inc., Fullerton, CA, USA). Relative amount of gammaglobulins to the total proteins were used to calculate concentrations. Serum total protein concentration was determined by a colorimetric method (Weichselbaum, 1946) (II).

Serum samples from the dairy calves were tested for antibodies to bovine parainfluenza virus-3 (PIV-3) (IV, V), bovine respiratory syncytial virus (BRSV) (IV, V), bovine coronavirus (BCV) (IV, V), bovine adenovirus-3 (BAV-3) (IV) and bovine adenovirus-7

(BAV-7) (IV). An ELISA test was used for antibodies to PIV-3, BRSV and BCV. A virus neutralization test was used for BAV-3 and BAV-7. ELISA kits (SVANOVA Biotech, Uppsala, Sweden) were used according to the manufacturer's instructions (IV).

4.3.5 Bacteria, mycoplasma and virus detection from TBL (IV, V)

The bacterial tests were performed according to standard procedures. Details of bacterial and mycoplasma identification methods for cross-sectional study (IV) are described by Härtel et al. (2004) and for longitudinal study (V) by Autio et al. (2006). The nested RT-PCR method described by Vilcek et al. (1994) was used for detection of BRSV, and the RT-PCR method (Tsunemitsu et al., 1999) for detection of BCV (V).

4.3.6 Pulsed-field gel electrophoresis of Pasteurella multocida isolates (V)

P. multocida isolates were grown overnight at 37°C in a brain-heart infusion broth (Difco, Sparks, MD, USA). Cells (0.9 ml) were harvested by centrifugation and washed with 2 ml of PIV buffer (10 mM Tris-HCl, pH 7.5, 1 M NaCl). Cells were resuspended in 0.25 ml of PIV and mixed with an equal volume of 1.8% InCert agarose (Cambrex, Rockland, ME, USA) and then dispensed into plug moulds (Bio-Rad, Richmond, CA, USA). Bacteria in plugs were lysed as described previously (Cameron et al., 1994). After lysis, plugs were washed in TEN buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, pH 8.0, 50 mM NaCl), and slices of plugs were digested with 10 U *Sal*I restriction enzyme (Roche, Mannheim, Germany). Fragments were separated in 1% SeaKem Gold agarose (Cambrex, Rockland, ME, USA) in 0.5 x TBE by using a CHEF-DR III system (Bio-Rad, Richmond, CA, USA). Ramp time was 1-10 s for 17 h at 6 V/cm, and the temperature was 12°C. *Salmonella* Braenderup H9812 (*Xba*I-digested) was used as a molecular marker. Restriction fragment patterns of the isolates were compared visually.

4.4 Statistics

Carry-over and period effects were tested using the t-test procedures of Jones and Kenward (1989). These tests were run for selected time points. In cases of insignificant carry-over effects in the examined parameters, the data from both challenges were combined (I). Repeated measures analyses of variance was used to analyse *E. coli* LPS treatment effects in APPs and blood chemistry parameters (I) and to evaluate time-related changes in APPs and serum gammaglobulins between age groups of neonatal reindeer calves. Data from calves sampled at least three times were used (age groups 1–7, 8–14 and 22–32 days). A repeated contrast method was used to compare adjacent age groups (II). Greenhouse–Geiser adjusted p-values were used for evaluation of results (I, II).

General linear model of analyses of variance was used to examine associations of SAA, Hp and gammaglobulins concentrations with daily weight gains, with gender as a fixed factor and protein levels from the age groups as covariates (II).

Linear regression analysis was used to study the relationships of results from seroconversion tests (BAV-7, BAV-3), mycoplasmal findings (*M. dispar, Mycoplasma sp.*) and other bacterial findings (*P. multocida, Fusobacterium nechrophorum*) to concentrations of APPs, WBC count and clinical signs. If the response variable was dichotomous

(cough, diarrhoea, changed respiratory sounds, nasal discharge), logistic regression analysis was applied. Logarithmic transformations were used for all APPs and WBC count (IV).

Non-parametric Wilcoxon signed-rank test was applied to evaluate changes in concentrations of APPs after birth. In Group A, every age point was compared with the 21-day sample and with the next sample in the series. In Group B, every age point was first compared with the 59-day sample to identify the age at which the concentrations stabilized. Each sampling time before this time point was then compared with the next sample (III). Two-sample Wilcoxon signed-rank test was used to explore age differences between BRSV antibody response groups at the beginning of the observational period (V).

The above-described statistical analyses were carried out using the Statistical Package for Social Sciences, versions 10.0 (I, II), 11.0 (III) and 12.0 (IV) (SPSS for Windows, SPSS Inc., Chicago, IL, USA) and R version 2.3.1 (V) (R Development Core Team, 2006). A linear random-intercept model was used for exploring time changes in serum concentrations of APPs during the respiratory disease outbreak using nlme package (Pinheiro et al., 2006) with R version 2.3.1 (R Development Core Team, 2006). APP changes in all calves during the observation period and differences between BRSV antibody response groups were analysed using contrasts where every time point was compared with the previous time point. Logarithmic transformation of SAA, LBP and AGP and reciprocal transformation of Hp were used. Changes between time points in clinical scores of all calves and differences between BRSV antibody response groups were analyzed using a generalized linear mixed model (GLMM) fitted by the GLIMMIX procedure with SAS/STAT 9.1 software (SAS Institute Inc., Cary, NC, USA). Poisson distribution was used for a response variable (clinical score). Calf was included as a random effect and a first-order autoregressive (AR1) correlation structure was used for modelling serial correlations of repeated measurements in all mixed models (V).

5 RESULTS

5.1 Acute phase response to the *Escherichia coli* endotoxin challenge in reindeer (I)

5.1.1 Clinical response

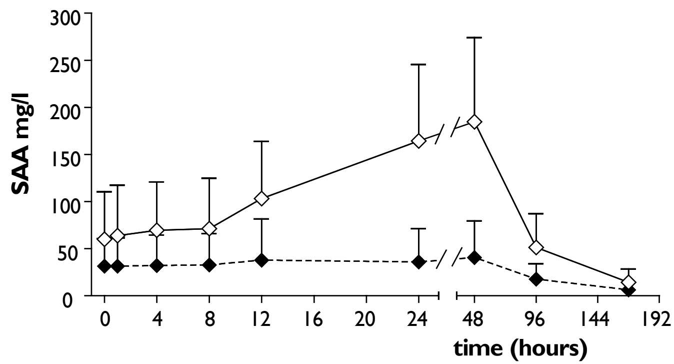
In the first experiment, all animals receiving LPS showed clear signs of general depression (they kept their heads down and moved slowly) and two had tremor of the legs. The clinical signs were observed during the first 4 h after LPS administration. The two reindeer with tremors had loose droppings the following day. The signs of depression were less obvious during the second experiment. No significant differences in rectal temperature were found between treatments.

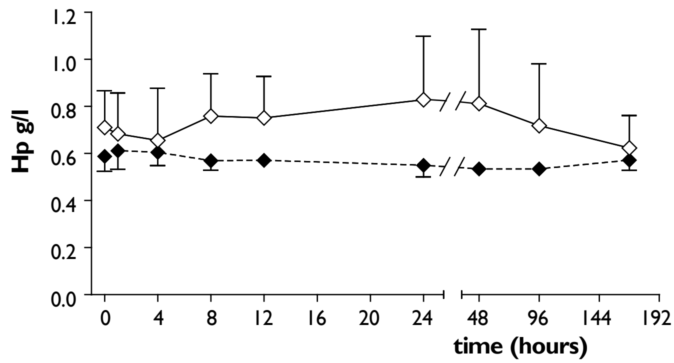
5.1.2 Acute phase proteins response

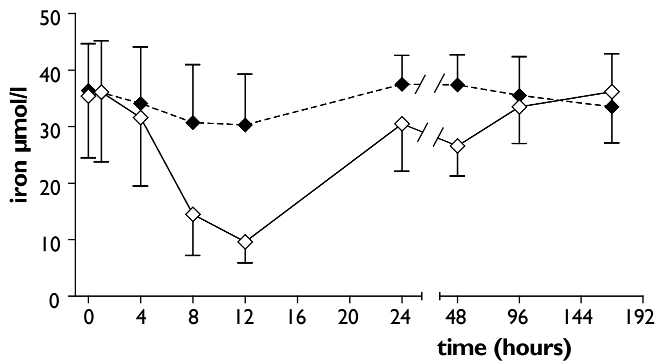
Endotoxin caused a significant increase in the systemic concentration of SAA (Fig. 1; $p < 0.001$) The rise in SAA in the LPS group was seen in the 12-h sample and peaked at 48 h (184.7 ± 89.4 mg/l), when the LPS-treated animals had a 2- to 29-fold increase of serum SAA. The mean serum Hp concentrations had a small tendency to decrease during the first 4-h period in the LPS group, increasing slightly in the 8-h sample and attaining a maximum value at 24 h (0.87 ± 0.25 g/l). The Hp concentrations remained relatively stable in the control group throughout the experiment (Fig. 1); however, these differences between treatments were not statistically significant. Individual Hp response to endotoxin administration varied, and only one reindeer showed a clear increase (almost 2-fold) in the 48-h sample, and one showed no increase at any time.

5.1.3 Other biochemical changes

Serum iron concentrations decreased in all endotoxin-treated animals and were significantly lower ($p < 0.01$) at 8 and 12 h (Fig. 1). A carry-over effect was found in cortisol, and data were analysed separately in both experiments. The serum cortisol concentrations showed wide variability among animals, but no consistent increases in cortisol in the LPS group were detected during the follow-up period (0–96 h). Some of the reindeer had high cortisol values already in the pre-treatment sample in both experiments (mean 102.9 nmol/l, range 9.8–240 nmol/l; Fig. 2). The endotoxin challenge did not result in significant changes in concentrations of serum total protein, albumin, ASAT, CK, GT, SDH and urea recorded during the follow-up period.







a
b
c

Figure 1. Mean (± SD) serum concentrations of SAA (a), Hp (b) and iron (c) in reindeer (n = 6) after IV administration of *E. coli* LPS (◊; 0.1 µg/kg) or saline (♦) at 0 h. The data are combined from two successive challenges.

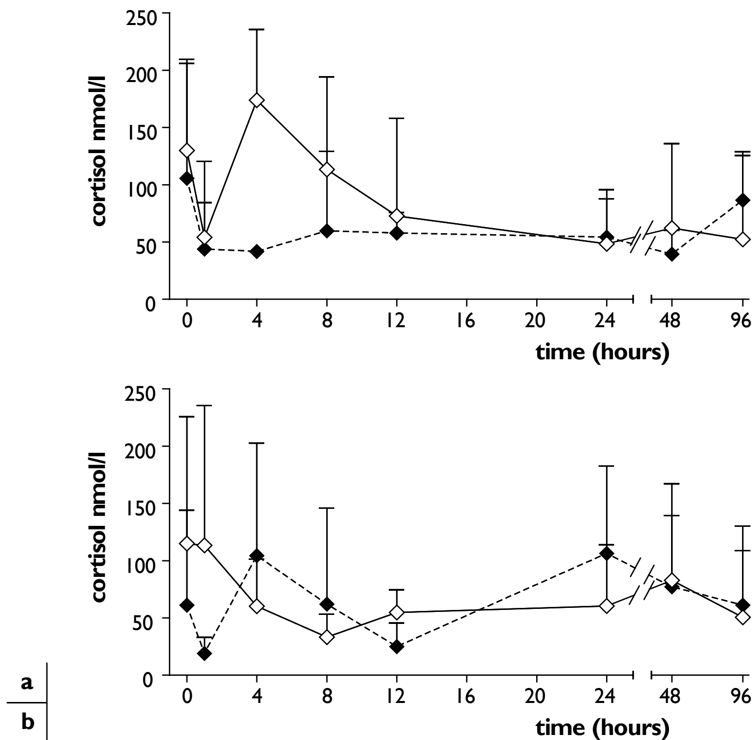


Figure 2. Mean (± SD) serum concentrations of cortisol from first set (a; n = 3) and from second set of the experiment (b; n = 3) in reindeer after IV administration of *E. coli* LPS (◊; 0.1 mg/kg) or saline (♦) at 0 h.

5.2 Acute phase proteins concentrations in newborn reindeer calves (II)

Calves showed no clinical signs of disease during the entire experimental period. Serum Hp concentrations were at the lowest on day 0 and during the first week (0.49 ± 0.07 and 0.54 ± 0.07 g/l, respectively), with concentrations increasing thereafter. Although this increase was relatively small, it was significant (from 1–7 to 8–14 days of age, $p < 0.001$; and from 8–14 to 22–32 days, $p < 0.001$, $n = 30$; black bars in Fig. 3).

Serum SAA concentrations largely varied among calves, but a significant increase was seen between the first and second weeks (i.e. between ages 1–7 and 8–14 days; $p < 0.016$, $n = 37$). However, by age 22–32 days, SAA concentrations decreased ($p < 0.001$; black bars in Fig. 3). Three calves had undetectable levels of SAA; all of these samples were obtained on days 0 and 1. The highest concentrations of gammaglobulins in the serum were recorded in calves sampled on days 0 and 1 (13.8 ± 4.0 and 15.5 ± 4.9 g/l, respectively). This was followed by a sharp decrease in concentrations (during 2 weeks), after which they stabilized, but showed a significant decreasing tendency throughout the observation period ($n = 37$, $p < 0.001$; black bars in Fig. 3).

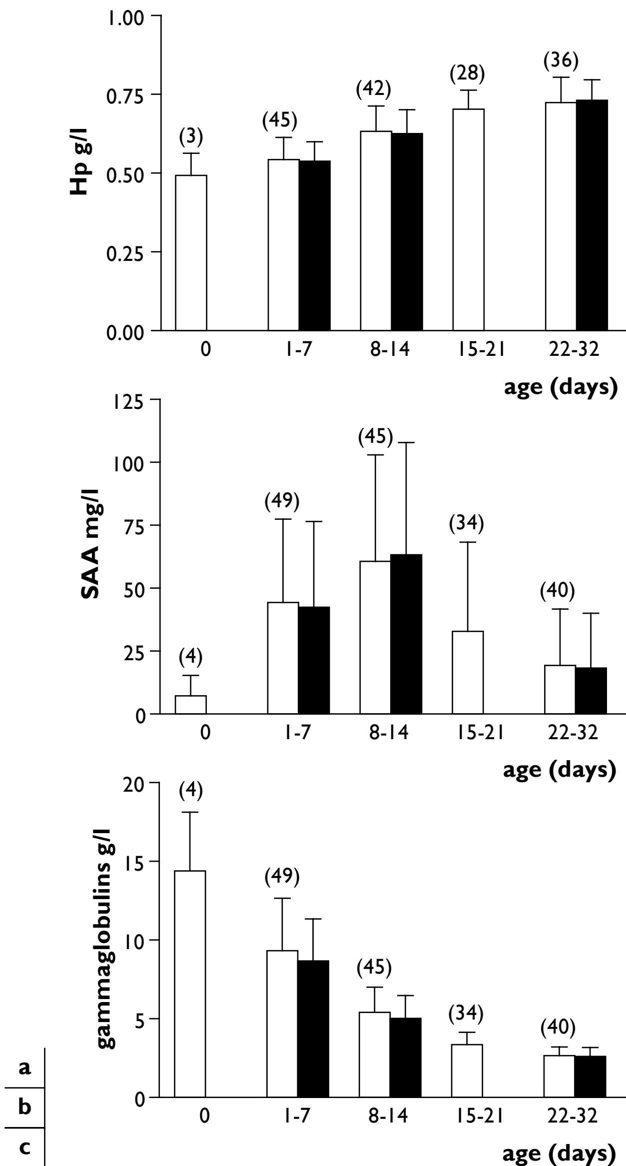


Figure 3. Mean (± SD) serum concentrations of Hp (a), SAA (b) and gammaglobulins (c) in 51 reindeer calves during the first month of life by age groups (open bars). The numbers of samples are shown above the bars. Black bars represent data for 37 calves (30 calves for Hp) used to test age-related changes statistically.

The mean daily weight gain during the neonatal period was 0.332 ± 0.045 kg/day ($n = 51$) and during the entire experimental period 0.375 ± 0.037 kg/day ($n = 47$). Male reindeer gained more weight than females during the study ($p < 0.001$). High SAA values at age 8–14 days were associated with smaller daily weight gain throughout the study ($p < 0.01$, $n = 43$).

5.3.1 Clinical signs and obstetric aid

In Group A, one episode of diarrhoea in one calf was recorded (at the age of 7 days). In Group B, six calves had signs of disease at the time of sampling (3 calves once, 3 calves twice). One high rectal temperature, one episode of cough and seven episodes of diarrhoea were recorded. Three calves (2 from Group A, 1 from Group B) needed forceful extraction at delivery, and 10 were born without any assistance (5 from both groups). The remaining calves were extracted by one person.

5.3.2 Acute phase proteins during 3 weeks after birth

Mean concentrations of SAA and AGP from all time points were higher than 21-day concentrations. After birth, mean serum concentrations of SAA increased, decreasing after 10 days of age. The two youngest calves at the first sampling had the lowest SAA values (7.4 and 6.4 mg/l at 4 h and 6 h from birth, respectively). The same calves had the lowest and the third lowest LBP concentrations (4.5 and 13.8 mg/l, respectively). Two calves with forceful extraction had the highest SAA values (134.7 and 129.2 mg/l) in the first sample (sampling times from birth of 11 and 32 h, respectively). Concentrations of AGP decreased gradually over the 3-week period. Serum concentrations of LBP and Hp were more stable (Fig. 4). Three calves had high Hp concentrations (0.49 and 0.38 g/l at day 10 in two calves, and 0.72 g/l at day 14 in the third calf) compared with the other values.

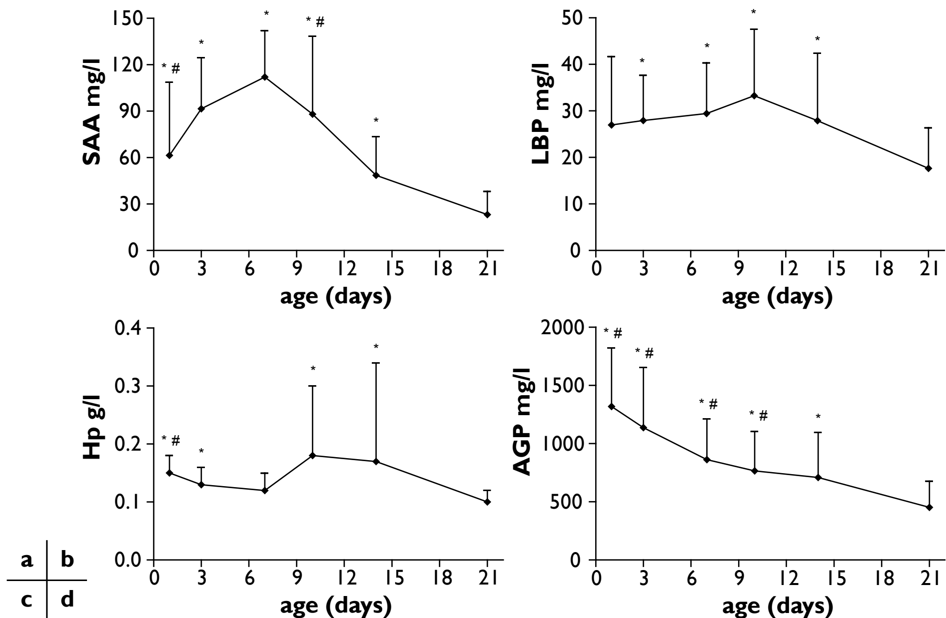


Figure 4. Mean (± SD) serum concentrations of SAA (a), LBP (b), Hp (c) and AGP (d) in calves sampled during a 3-week period after birth (Group A; $n = 13$). (*) Significant difference ($p < 0.05$) from the last sampling (21 days of age). (#) Significant difference ($p < 0.05$) from the next sampling.

SAA and LBP concentrations varied greatly between calves, but changes were very similar in most of the calves (Fig. 5). LBP concentrations seemed to peak later and concentrations decreased more slowly (see calves 1167, 1168, 1169, 1172, 716, 717 and 718; Fig. 5).

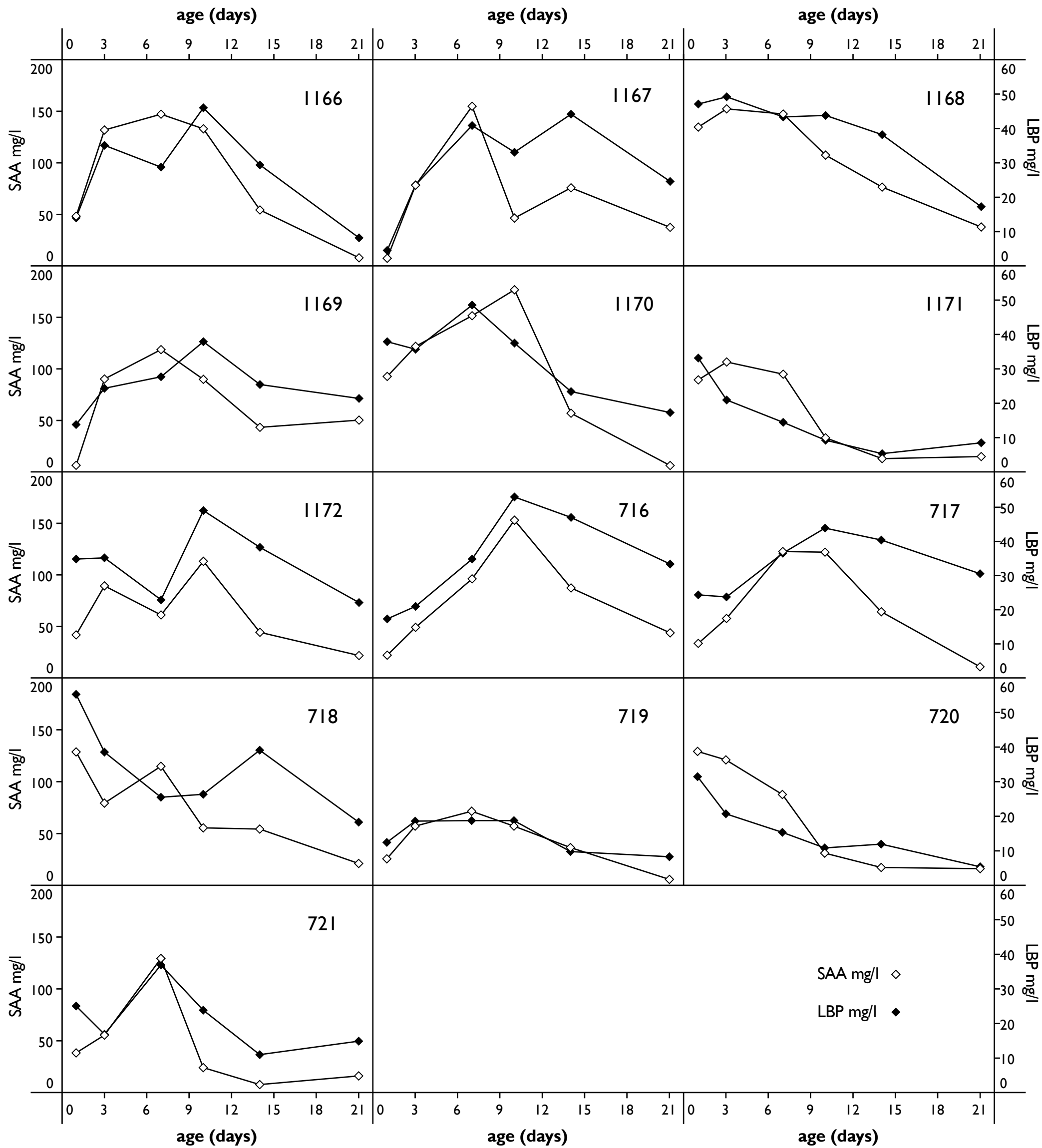


Figure 5. Individual serum concentrations of SAA (◊) and LBP (♦) of 13 dairy calves during a 21-day period (x-axis) after birth.

The mean SAA concentration in the colostrum of the eight cows investigated was 25.8 ± 26.8 mg/l. All colostrum samples contained one or more alkaline SAA bands with isoelectric point (pl) values >9.3 (Fig. 6). Four colostrum samples also contained an isoform with an apparent pI of 6.8 (Fig. 6, panels a-d). None of the serum samples of the calves contained alkaline isoforms. Two isoforms with a pI of 6.8 and 6.2 were found in the serum of all eight calves. Additional isoforms (pI 5.8 and 7.4) were found in two calves (Fig. 6, panels d and g).

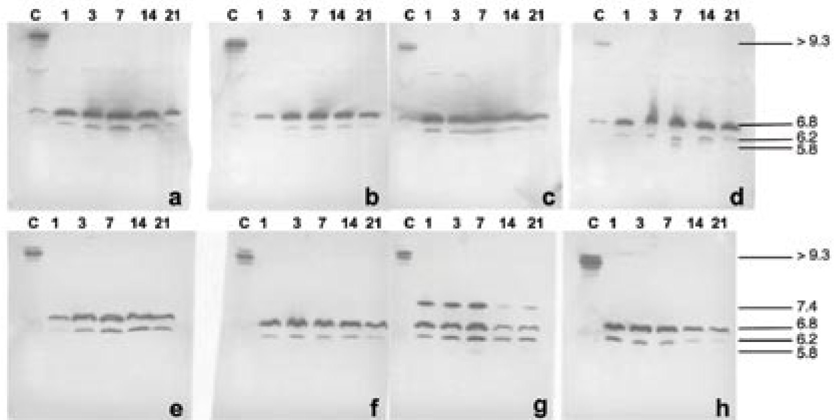


Figure 6. Serum amyloid A isoforms by denaturing isoelectric focusing and Western blotting of serum samples from 8 calves (panels a–h; sampling age 1, 3, 7, 14 and 21 days) and a colostrum sample from their dams (c).

5.3.3 Acute phase proteins during 2 months after birth

The highest mean serum concentrations of all APPs occurred in the first sample taken 3 days after birth. From a mean age of 24 days onwards, APP levels remained stable. Mean concentrations of SAA and AGP decreased relatively more during this period than other APPs (75% and 73%, respectively). Although mean concentrations of LBP were higher in the first 3 samples than in the last sample, the decrease in concentrations from 3 to 24 days of age was relatively smaller (40% decrease). Concentrations of Hp at the second sampling were even lower than those at the last one, and concentrations remained low (<0.2 g/l) throughout the observation period (Fig. 7).

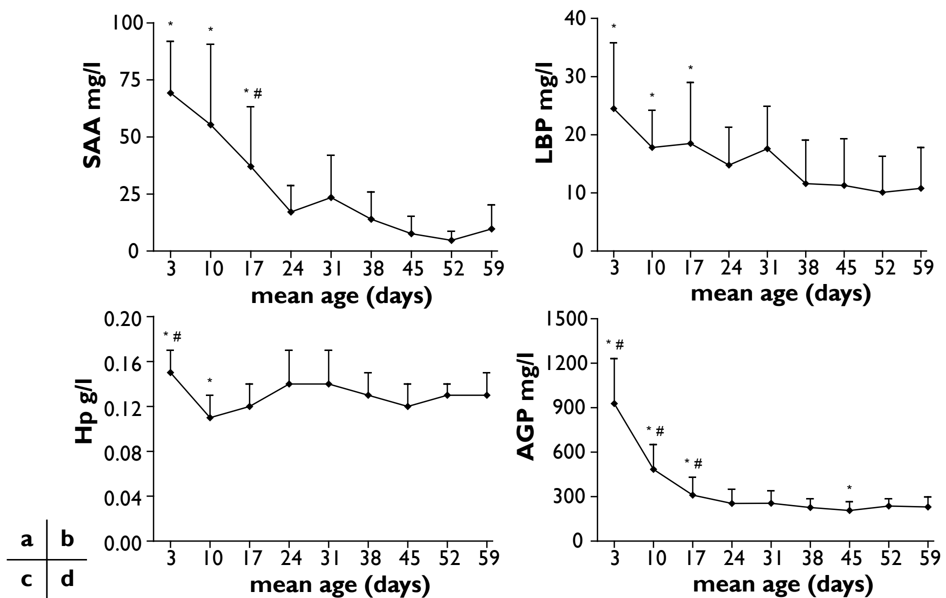


Figure 7. Mean (± SD) serum concentrations of SAA (a), LBP (b), Hp (c) and AGP (d) in calves sampled during a 2-month period after birth (Group B; $n = 13$). (*) Significant difference ($p < 0.05$) from the last sampling (mean age 59 days). (#) Significant difference ($p < 0.05$) from the next sampling.

5.4 Association of respiratory infections with APP concentrations (IV)

Diarrhoea, increased rectal temperature, increased respiratory rate, crackling respiratory sounds, cough and nasal discharge were present in 14.3%, 15.5%, 44.1%, 41.7%, 78.6% and 45.2% of calves, respectively. Associations between *P. multocida* and elevated respiratory rate, increased rectal temperature, crackling respiratory sounds and nasal discharge were found. A negative association was present between seroconversion to BAV-7 and crackling respiratory sounds.

Seroconversion to viruses BAV-7, BAV-3, BCV, PIV-3 and BRSV was observed in 22.6%, 13.1%, 4.8%, 3.6% and 0% of calves, respectively. *M. dispar*, other mycoplasmas, *P. multocida*, *Pasteurella* sp., *F. necrophorum* and *A. pyogenes* could be isolated in 90.5%, 60.7%, 14.3%, 1.2%, 6.0% and 2.4% of calves, respectively. Isolation of *P. multocida* was associated with increased concentrations of all tested APPs (Table 2). No significant relationships were found between APPs and other pathogens and virus seroconversions.

Table 2. Serum/blood concentrations (mean ± SE; *p*-value), of fibrinogen (Fb; g/l), haptoglobin (Hp; g/l), serum amyloid A (SAA; mg/l), alpha$_1$-acid glycoprotein (AGP; mg/l), lipopolysaccharide binding protein (LBP; mg/l) and white blood cell count (WBC; x10^9/l) in calves with respiratory disease, with and without *Pasteurella multocida* in tracheobronchial lavage fluid.

	Fb	Hp	SAA	AGP	LBP	WBC
Absence of *P. multocida*						
Number of calves	65	72	72	72	72	69
Mean	6.02	0.228	18.4	377.1	6.3	11.68
± SE	0.16	0.0	2.13	24.64	0.72	0.382
Presence of *P. multocida*						
Number of calves	11	12	12	12	12	12
Mean	7.73	0.465	31.5	580.0	13.5	9.58
± SE	0.58	0.125	5.97	91.17	3.29	0.758
p-value	0.002	0.031	0.016	0.018	0.006	0.073

5.5 Changes of acute phase proteins during an outbreak of respiratory disease (V)

5.5.1 Clinical signs

Calves presented no signs of respiratory disease or any other disease at week 0 (three calves had a clinical score of 1 at week 0 because of a rectal temperature of 39.0-39.5°C). All calves showed signs of BRD starting at week 1, and clinical disease became more pronounced at weeks 2, 3 and 4 (Fig. 8). Clinical scores of all calves increased from week 0 to 1 ($p < 0.001$) and from week 1 to 2 ($p < 0.01$). Clinical scores decrease from week 4 to 5 ($p < 0.001$). No differences were seen in clinical signs between BRSV antibody response groups. Overall, only mild to moderate (maximal score 8) clinical res-

piratory disease was recorded. Two calves were treated with antibiotics based on the clinical evaluation at week 3. These calves also had relatively high Hp values at that time (0.43 and 0.67 g/l, respectively; Fig. 10).

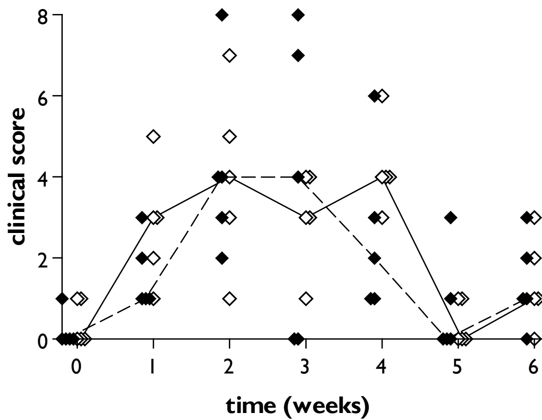


Figure 8. Clinical score values of 10 calves from low IgG_1 response (--♦--; *n* = 5) and high IgG_1 response (–◊–; *n* = 5) groups over a 6-week period. Lines connect median values of IgG_1 response groups by weeks.

5.5.2 Respiratory infections and antibody responses

Microbial findings are presented in Table 2. BRSV was detected in 5 calves in the 2-week TBL samples, but in none in the 6-week TBL samples. No BCV was found in any of the TBL samples investigated. The other microbes most commonly isolated from the TBL samples were *M. dispar* and *P. multocida*. All available *P. multocida* isolates had an identical *Sal*I restriction pattern. More bacterial species were isolated in the middle of the BRD outbreak (week 2) than at the later stage (week 6). The only calf (no. 8) with no *P. multocida* infection at week 2 had an increase of SAA and LBP concentrations from week 0 to week 1 (from 13.4 to 23.8 mg/l and from 2.6 to 9.8 mg/l, respectively). No change was seen thereafter (range of 2.7-5.2 mg/l and 2.0-4.3 mg/l in subsequent samples, respectively) and no rise in Hp. This calf also had the lowest peak in the clinical score (Table 3). All calves were seronegative to BRSV at week 0. By week 6, all calves had become seropositive (Fig. 9). Calves were seronegative to BCV throughout the observation period. Antibodies to the PIV-3 virus were present in all calves at week 0, with these gradually decreasing.

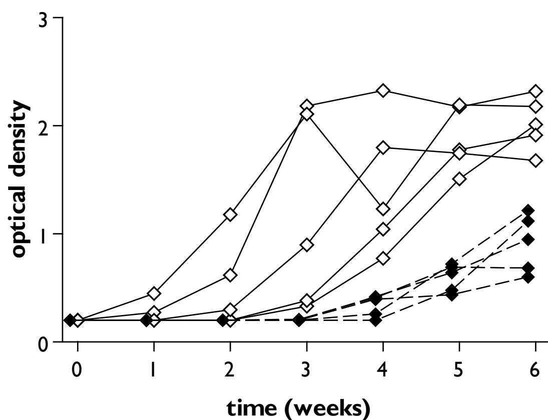


Figure 9. Bovine respiratory syncytial virus (BRSV) serum IgG_1 corrected optical density values of 10 calves from low IgG_1 response (--♦--; $n = 5$) and high IgG_1 response (–◊–; $n = 5$) groups over a 6-week period.

Table 3. Age of calves, bovine respiratory syncytial virus (BRSV) antibody response group, peak clinical score, presence of BRSV and bacterial growth in tracheobroncheal lavage (TBL) samples.

Calf no.	Age of calves at week 0 (days)	BRSV antibody response group	BRSV in TBL at week 2	Peak clinical score (week)	Bacterial growth in TBL sample at week 2	Bacterial growth in TBL sample at week 6
1	32	Low	neg	7 (3)	Pm[a], Md[b], Mb[c]	Pm, Md, Mb
2	30	High	neg	4 (2; 4)	Pm, Md, Mb	Pm, Md
3	27	High	pos	7 (2)	Pm, Md	not investigated
4	24	High	neg	6 (4)	Pm, Psp[d], Ss[e], Md, Mb	Md
5	23	Low	pos	8 (2; 3)	Pm, Fsp[f], Md, Mb	Pm, Md, Mb
6	21	Low	pos	4 (3)	Pm, Ss, Ap[g], Fsp, Md, Mb	not investigated
7	18	High	pos	4 (3; 4)	Pm, Md, Mb	Pm, Md, Mb
8	14	Low	neg	3 (2)	Ss, Md, Mb	Pm, Md, Mb
9	13	Low	neg	4 (2)	Pm, Mb	not investigated
10	9	High	pos	5 (2)	Pm, Fsp, Md	Pm, Md, Mb

[a] *Pasteurella multocida*
[b] *Mycoplasma dispar*
[c] *Mycoplasma bovirhinis*
[d] *Pasteurella sp.*
[e] *Streptococcus suis*
[f] *Fusobacterium sp.*
[g] *Arcanobacterium pyogenes*

5.5.3 Acute phase protein concentration changes

Mean SAA and LBP concentrations of all calves increased between weeks 0 and 1 ($p < 0.001$) and then decreased between weeks 3 and 4 ($p < 0.01$). Mean Hp concentrations of calves decreased between weeks 3 and 4 ($p < 0.05$), and mean AGP concentrations decreased between weeks 0 and 1 ($p < 0.05$).

No differences in the initial APP concentrations or age between BRSV antibody response groups were seen before the outbreak of BRD. In general, APP patterns changed differently in the antibody response groups. Mean SAA concentration increased in the low antibody group between weeks 2 and 3 ($p < 0.001$) compared with the high antibody group, where the mean concentration decreased. Mean LBP concentrations decreased between weeks 1 and 2 ($p < 0.05$) and increased between weeks 2 and 3 ($p < 0.001$) in the low antibody group compared with the high response group, where the mean concentrations increased and decreased, respectively. In the low antibody group, Hp concentrations increased between weeks 2 and 3 ($p < 0.05$) and decreased between weeks 3 and 4 ($p < 0.01$) compared with the high response group, where no other clear changes occurred, except two high values at week 2 (Fig. 10).

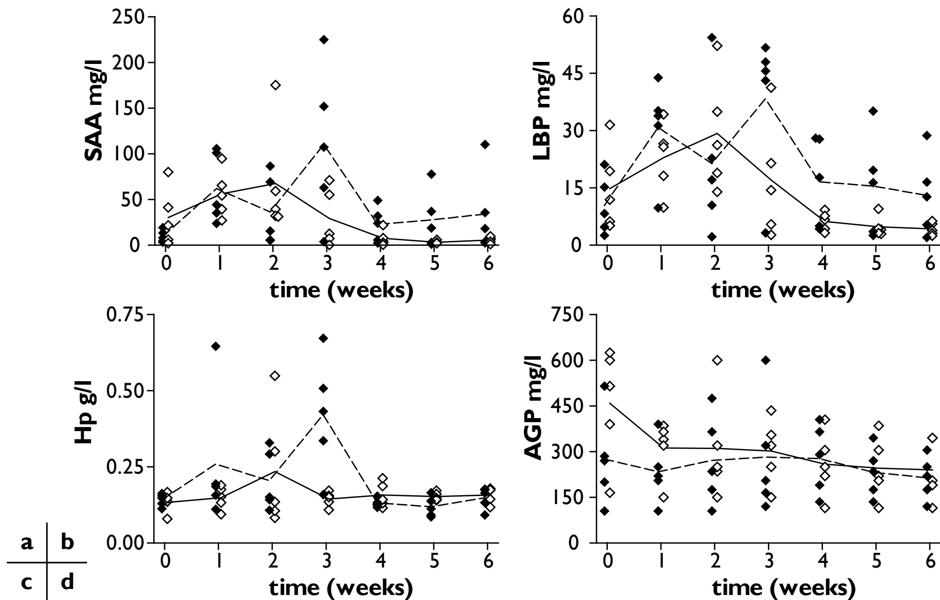


Figure 10. Serum concentrations of SAA (a), LBP (b), Hp (c) and AGP (d) of low bovine respiratory syncytial virus (BRSV) IgG_1 response (--♦--; $n = 5$) and high BRSV IgG_1 response (–◊–; $n = 5$) groups of calves over a 6-week period. Lines connect mean concentrations of each APP in different IgG_1 response groups by weeks.

6 DISCUSSION

6.1 Acute phase proteins in reindeer (I)

To our knowledge, no previous reports exist on SAA and Hp in reindeer. Consequently, some limitations regarding laboratory methods used for APP detection arose for this "new" species. The assay for SAA is an ELISA kit that has been developed for and used in five animal species: cattle (Jacobsen et al., 2004; Lehtolainen et al., 2004), pigs (Heinonen et al., 2006; Tecles et al., 2007), dogs (Martinez-Subiela et al., 2005; Dabrowski et al., 2007), cats (Giordano et al., 2004) and horses (Duggan et al., 2007; Paltrinieri et al., 2007). However, the standard curve should be calibrated separately for each species. The same assay calibrated for cattle has been successfully used for sheep to measure SAA concentrations in serum and milk (Winter et al., 2003). For reindeer, we also used cattle-specific calibration of the SAA assay. Hp concentration in the reindeer was measured with a biochemical method based on the Hp hemoglobin binding property. The biochemical method is anticipated to reflect Hp concentration more accurately than the immunological SAA method, which is dependent on the cross reactivity of SAA antibodies between species. However, for Hp assay, we also used the cattle-specific calibration, and the results should therefore be interpreted with caution. Taking into account these considerations, the discussion and conclusions about reindeer APPs are based on the relative changes in SAA and Hp concentrations observed.

E. coli LPS induced APR in reindeer, as shown by a decrease in serum iron concentration in all animals (Fig. 1) and clinical response in some animals. Decreases in iron concentration were similar to those previously reported in domestic ruminants (Boosman et al., 1989; van Miert et al., 1990). As iron is important for survival of bacteria, hypoferraemia during APR can be considered as one of the defense mechanisms of the host. A recently discovered peptide hepcidin produced by the liver (Park et al., 2001) regulates iron homeostasis by inhibiting iron absorption from the small intestine (Nicolas et al., 2002a) and preventing iron releasing from the macrophages (Nicolas et al., 2001). During inflammation, hepatic production of hepcidin is up-regulated very quickly by pro-inflammatory cytokines (Nemeth et al., 2004; Kemna et al., 2005; Lee et al., 2005). This peptide probably has a key role in decreasing iron during APR, as turpentine-induced hypoferremia was not observed in hepcidin-deficient mice (Nicolas et al., 2002b). Activation of pro-inflammatory cytokines in reindeer was further confirmed by the increased concentrations of SAA in all LPS-treated animals (Fig. 1); these changes are in agreement with earlier studies in cattle (Boosman et al., 1989; Werling et al., 1996; Jacobsen et al., 2004).

Hp concentrations increased in the challenged reindeer, but the relative increases were small and the patterns differed widely between individuals. Nevertheless, the serum Hp concentration could rise several-fold over baseline and appears to act as an APP in reindeer. This was seen in one animal, which had to be excluded from the study due to exceptionally high SAA and Hp concentrations already before endotoxin administra-

tion. In the study of reindeer calves (II), very high SAA and Hp concentrations were simultaneously seen in two samples of one calf, which supports the findings in adult reindeer.

6.1.1 Possible effect of LPS dose and stress response of reindeer

LPS dose (0.1 mg/kg) used may explain the minor Hp response. No reports of the use of the endotoxin challenge model in reindeer are available, and the LPS dose was chosen based on studies in goats (van Miert et al., 1983, 1992; Massart-Leen et al., 1992), as goats have similar body weight to reindeer. In those experiments, the same endotoxin dose resulted in a clear activation of APR (significant decrease in serum iron concentrations and increase in rectal temperature and serum cortisol concentrations). In our study on reindeer, the similarities in mean cortisol concentration patterns within the animal groups during both experiments, the wide individual variability and the carry-over effect (Fig. 2) suggest that cortisol was more affected by the stress responses of reindeer to handling than by LPS administration. No clear temperature response was seen. A rise in body temperature and Hp was reported in cows challenged with the same dose of *E. coli* LPS (Jacobsen et al., 2004). This supports further the conclusion that the low-dose LPS used here caused a milder APR in reindeer, as compared with domestic ruminants.

Differences have been found in the effects of cytokines and other mediators on the synthesis of APPs in humans (Mackiewicz et al., 1991). The stimulating mechanisms of APPs in reindeer are unknown. In bovines, cytokine stimulation of SAA and Hp is different *in vitro* (Alsemgeest et al., 1996). Studies in cattle have also demonstrated that SAA is more sensitive than Hp during APR (Horadagoda et al., 1994; Werling et al., 1996; Müller-Doblies et al., 2004). The cytokine response sequence is unaffected by LPS dose or administration route, but the amplitude of the response is dose-dependent (Gerros et al., 1993) and the single-bolus low dose of *E. coli* LPS may have been insufficient to initiate Hp hepatic production. Similarly, an evident, but non-significant elevation of Hp was reported after a single-bolus LPS dose administration in pigs (Wright et al., 2000). However, repeated injections induced a clear Hp response in swine (Dritz et al., 1996). This is supported by studies in cattle with experimental Gram-negative bacterial infections (Godson et al., 1996; Horadagoda et al., 2001). In these infections, a stronger, prolonged endotoxin release resulted in Hp increasing substantially. The same was shown in endotoxin challenge study, where a higher LPS dose was used (Conner et al., 1989).

Elevated cortisol levels observed already before LPS challenge (likely caused by handling) probably altered the response to endotoxin and the subsequent cytokine response. Endogenous or exogenous glucocorticosteroids can modulate the subsequent APR and production of APPs. Cortisol can directly stimulate APP production in the liver, but the response is not believed to be very strong (Baumann and Gauldie, 1994), and adrenocorticotrophic hormone (ACTH) did not stimulate APP response in cows (van der Kolk et al., 1992). Cortisol also failed to cause systemic Hp and Fb response in bull calves (Fisher et al., 1997a), but a partial medical suppression of cortisol response after castration of bulls resulted in lower Fb and Hp response 7 days later (Fisher et al., 1997b). High cortisol concentrations may also inhibit APP production by decreasing the response of some pro-inflammatory cytokines to endotoxin (Zuckerman et al., 1989; Smith and McDonald, 1992), which is a more likely explanation in the present study.

6.2 Acute phase proteins changes during the neonatal period (II, III)

6.2.1 Changes of APPs in dairy calves (III)

The concentrations of APPs in dairy calves changed within the first 3 weeks of life (Figs. 4 and 7) stabilizing thereafter. The relative changes were biggest in the concentrations of SAA and AGP. A different AGP isoform in neonatal calves compared with the isoform present in adults has been described (Itoh et al., 1993a). A rise of AGP already in the foetal stages has been reported in calves and piglets, followed by a gradual decrease in concentrations after birth (Stone and Maurer, 1987; Itoh et al., 1993a; Itoh et al., 1993b), in agreement with the findings of our study (Figs. 4 and 6). These studies indicate that neonatal AGP is probably foetally, regulated and a high serum concentration of AGP after birth is not necessarily a sign of the activation of APR by some external stimulus. On the contrary, the lowest SAA concentrations were seen in calves within a few hours of birth, but the concentrations then increased rapidly (Fig. 5), suggesting the presence of some external stimulatory factor for SAA at birth or soon after. Low SAA concentrations immediately after birth have been previously reported in cattle (Alsemgeest et al., 1993, 1995a). Unfortunately, the follow-up period was only 24 h after birth, and the subsequent rise of concentrations was not seen (Alsemgeest et al., 1993). We did not find SAA isoforms in serum of newborn calves to be different from the isoforms in adults. Isoforms observed in eight calves corresponded to those reported earlier in adult cattle during APR, including the exceptional isoforms with pI 5.8 and pI 7.4 in two calves (Alsemgeest et al., 1995c; Jacobsen et al., 2005; Fig. 6). This indicates that the systemic SAA response seen in calves was due to the activation of APR and not maternal transfer.

Concentrations of LBP changed after birth in a very similar pattern to SAA (Figs. 5 and 7). In some calves, the LBP concentrations peaked later and the decrease was slower (Fig. 5). The relative changes for SAA were considerably bigger (Figs. 4 and 7). Unfortunately, no reports of the cytokine stimulation patterns of hepatic production of LBP in cattle are available. An *in vitro* study revealed that LBP is stimulated by cytokines similarly but not identically to SAA in humans (Vreugdenhil et al., 1999). Interspecies differences probably exist, as a poor correlation between serum LBP and SAA values was found in horses with colic (Vandenplas et al., 2005). Recently, Bannerman et al. (2005) and Kauf et al. (2007) reported that SAA and LBP plasma concentrations showed a parallel increase in cows during experimental *Pseudomonas aeruginosa* and *M. bovis* mastitis. We found very similar systemic SAA and LBP patterns in calves during an outbreak of BRD (V). This suggests that hepatic regulation factors of SAA and LBP are similar in cattle and further supports the presence of some external stimulatory factor for synthesis of APPs after birth in calves.

Higher mean Hp concentrations were seen within 3 days of birth. After a small decrease (Figs. 4 and 7), the concentrations stayed relatively stabile. The majority of the values were comparable with levels seen in healthy cows tested with the same method in the same laboratory (Hyvönen et al., 2006). This difference of Hp from SAA and LBP may be explained by different regulatory mechanisms for hepatic synthesis (Alsemgeest et al., 1996); stimulatory factors may have been insufficient to cause Hp response. High endogenous cortisol values of newborn calves (Hammon et al., 2002) may also influence APP production by stimulating some APPs and inhibiting others (Smith and McDonald, 1992; Wan et al., 1995).

The decrease of Hp values during the first week of life is hard to explain. As concentrations remained low and the decrease was very small, it can be considered coincidence. On the other hand, as similar phenomena were observed in two different groups of calves, it may have some physiological background. The decrease may be related to the increased consumption of Hp due to haemolysis of foetal red cells and the functional immaturity of the neonatal liver to compensate for this, as proposed by Dobryszycka (1997). A decrease of Hp during the early stages of inflammatory response, exactly opposite to the other APPs (Fb, Cp and SAA), has been described in cattle (Arthington et al., 2003). Serum concentrations of Hp were found to be lower in transported than non-transported weaned calves in two separate experiments (Arthington et al., 2003). This suggests that the initial decreasing pattern of Hp during inflammatory stimulation may occur.

Physical stress or trauma during parturition may induce a rise in concentrations of APP immediately after birth (Marchini et al., 2000; Chiesa et al., 2001; Ishibashi et al., 2002). We found high SAA and LBP concentrations in two calves needing forceful extraction. As overall only three calves needed forceful extraction and the highest systemic SAA and LBP concentrations were noted during 7-14 days of life in most calves (Fig. 5), trauma from the birth process probably had only a minor effect on age-dependent changes in SAA and LBP.

6.2.1.1 Possible effect of colostrum

Theoretically, APPs can be directly transferred from the colostrum to calves' circulation, similarly to immunoglobulins. McDonald et al. (2001) have reported high concentrations of mammary-associated SAA in the colostrum of healthy cows. At least a partial effect of colostral pig-MAP (APP in pigs) on the elevation of piglet pig-MAP serum concentrations after birth has been proposed (Martin et al., 2005). Similarly as previously demonstrated in colostrum and mastitic milk (McDonald et al., 2001; Jacobsen et al., 2005), the SAA isoforms in colostrum samples in our study had highly alkaline apparent pI values (pH > 9.3; Fig. 6), and this isoform was not found in any of the serum samples from the eight calves tested (Fig. 6). Despite the lack of evidence of a direct transfer of APPs, free pro-inflammatory cytokines present in colostrum (Sordillo et al., 1991; Goto et al., 1997; Hagiwara et al., 2000) may have crossed the neonatal intestine and stimulated hepatic production of APPs. After colostrum intake, systemic concentrations of pro-inflammatory cytokines in calves increase, with the highest concentrations occurring on the first day of life (Yamanaka et al., 2003a). Concentrations then gradually decrease and are undetectable around 3–4 weeks of life. Possibly, free pro-inflammatory cytokines from the colostrum directly stimulate the production of APPs or trigger cytokine production of the newborn (Bessler et al., 1996; Hagiwara et al., 2001; Yamanaka et al., 2003b). Colostrum also contains cytokine antagonists and soluble cytokine receptors (Buescher and Malinowska, 1996; Hagiwara et al., 2000). However, concentrations of these factors are not probably sufficient to markedly interfere with the biological effects of colostral cytokines (Yamanaka et al., 2001; Yamanaka et al., 2003b).

6.2.2 Changes of APPs in reindeer calves (II)

In reindeer calves, the highest concentrations of serum SAA were seen at the second week of life, while systemic Hp increased until 3–4 weeks of age. The mean concentrations of both proteins increased to higher levels than those observed in adult reindeer (Figs. 1 and 3), indicating activation of the inflammatory response in newborn rein-

deer. Similar temporal changes in concentrations of SAA in serum of reindeer and dairy calves suggest similarity in the underlying mechanisms provoking this response, while Hp behaved differently in these ruminants. Functional immaturity of the neonatal liver discussed earlier (Dobryszycka, 1997) may be the reason for low Hp levels shortly after birth. At least from the second week of life, reindeer can have a Hp response to stimulation, as noticed in one of the reindeer calves with very high SAA and Hp concentrations in samples from the second week.

Piglets have been reported to have low serum concentrations of Hp after birth, with concentrations reaching adult levels when piglets were 2-3 weeks old (Richter, 1974). After this, serum Hp concentrations increased even more, remaining higher than in slaughter pigs until the end of the experimental period at 50 days of age. Special pathogen-free piglets born by Caesarean section and deprived of colostrum had lower, delayed and highly variable initial increasing patterns of Hp concentrations during the first month of life, followed by a decrease of Hp at 2 months of age (Richter, 1974). A similar initial increase of Hp in piglets after birth, followed with some decrease and stabilization already by 2 weeks of life was reported later (Martin et al., 2005; Sorrells et al., 2006). In these studies, the piglets were from a high health-status farm and an experimental unit, and consequently, lower immunological stimulation from the environment can be expected. Although the study by Richter (1974) confirms the effect of colostrum and/or the birth process on the concentrations of APPs in swine, it also suggests the involvement of other factors, such as subclinical infections.

6.2.2.1 Weight gain associations with APPs

In newly weaned beef calves, higher Hp and Cp concentrations were reported to correlate with a lower weight gain (Arthington et al., 2005). Several studies in growing pigs have found a negative relation between Hp concentrations and body weight gain (Eurell et al., 1992; Hiss and Sauerwein, 2003; Sauerwein et al., 2005). The findings could be explained by subclinical infections or other stressors. These factors may trigger a decrease in food intake through the effect of pro-inflammatory cytokines on the central nervous system (Johnson, 1998; Langhans and Hrupka, 1999) or by stimulating catabolism of body tissues (Johnson, 1997; Webel et al., 1997). Mounting an immunological response against pathogens also consumes more energy (Demas et al., 1997). We did not find a significant association between APP levels and weight gain during the first month of age in reindeer calves. One calf with very high APPs had the second lowest weight gain during the neonatal period. As APP concentrations in other calves were considerably lower, inflammatory stimulation was probably insufficient to alter food intake or metabolic level in most of the calves. However, the negative association observed between high SAA values during the second week of life and weight gain over 3 months may indicate the presence of some subclinical infection. Studies in laboratory animals and children have also shown the inhibiting effect of systemic APR on linear bone growth, and a contributing role of pro-inflammatory cytokines to that effect has been proposed (Skerry, 1994; Koniaris et al., 1997). This may be a reason for the reduced long-term weight gain of young animals with higher APP concentrations. The immune system of the newborn has been hypothesized to go through an educational process to obtain a proper helper T cell subset (Th1/Th2) balance (Rook and Stanford, 1998), which is dependent on the cytokine milieu present (London et al., 1998). Neonates have been reported to have a biased innate immune response towards the Th2 response (Adkins and Du, 1998; Adkins et al., 2001), and in humans this has been related to a higher risk for some infections (Kovarik and Siegrist, 1998). However, the

potential effect of inflammatory response shortly after birth on immunological maturation and susceptibility to subsequent infections thus far remains mostly unsolved and warrants further investigations.

6.2.3 Interspecies comparison and possible role of APPs in newborns

The analogous changing pattern of the concentrations of SAA in serum of semi-domesticated reindeer and in cattle after birth suggests a role of SAA in the life of newborns, at least in ruminants. Unfortunately, no reports exist of systemic SAA concentrations after birth for other ruminants. A short-lived (up to one week) elevation of systemic SAA concentration immediately after birth has been described in other species, e.g. in pigs (Llamas Moya et al., 2007), horses (Nunokawa et al., 1993; Stoneham et al., 2001; Duggan et al., 2007) and humans (Marchini et al., 2000).

Hp baseline serum concentrations seem to be relatively higher in reindeer than in dairy calves (Figs. 3 and 4) and the changes seen after birth are more comparable with pigs than with cattle. Pigs have a high constitutive Hp concentrations and a relatively small rise during inflammation (Eckersall et al., 1996), whereas cattle have a low basal levels of Hp (Eckersall et al., 1988) and a relatively higher increase during APR. This indicates a different regulation of Hp in reindeer than in cattle.

Another interesting similarity between reindeer calves and piglets which, is different from cattle, should be noted. In our study (II) and in a previous study (Nieminen et al., 1980) gammaglobulins concentrations in newborn reindeer decreased by approximately 50% by the end of the second week of life (Fig. 3). In piglets, a similar relative drop in overall IgG concentrations occurs during the same period (Martin et al., 2005; Sorrells et al., 2006). In neonatal dairy and beef calves, a considerably smaller (approximately 20-30%) relative decrease in total immunoglobulins during the first weeks of life has been described (Rajala and Castrén, 1995; Suh et al., 2003). Although speculative, this may support the different hepatic regulation of Hp production in reindeer calves and piglets as compared with cattle. The background of a rapidly decreasing passive immunity and the simultaneous protective function of increasing levels of Hp (Eaton et al., 1982; Dobryszycka, 1997) may play a role in the intrinsic defence mechanisms of newborn reindeer and piglets.

Unfortunately, we had too few dairy calves to explore the association between APP concentrations and weight gain in order to evaluate involvement of possible subclinical infections (III). Some of our results suggest that concentrations of APPs may have been influenced by the presence of subclinical disease processes during the second week of life also in dairy calves. Although we removed all samples from the animals in which any clinical signs of disease were recorded, 3 calves in group A had high Hp concentrations during the second week of life, and some of the calves had an additional elevation of concentrations of SAA and LBP at the same time (Fig. 5). The presence of subclinical infection would also explain the higher SAA and LBP concentrations during the second week in group A calves than in group B calves (Figs. 4 and 7).

In conclusion, patterns of systemic SAA response in dairy calves and reindeer calves and patterns of systemic Hp concentrations in reindeer calves and piglets (Richter, 1974; Martin et al., 2005) are comparable, although these species live in very different environmental conditions. This suggests that the changes in APPs described here are not merely caused by some coincidental disease-related mechanisms, but may re-

sult from the multiple overlapping factors discussed above. In general, they reflect the physiological adaptation of newborns to extrauterine life. Comparison of SAA and LBP results from the outbreak of respiratory disease in calves (V) also shows that the inflammatory reaction in calves during the first weeks of life is more or less similar to the response during minor to moderate clinical respiratory disease (Figs. 5 and 10), further supporting the role of APP response in the adaptation processes of neonatal animals.

6.3 Acute phase proteins in spontaneous BRD in dairy calves (IV, V)

6.3.1 Cross-sectional study (IV)

In the cross-sectional study, all APPs investigated had a positive association with *P. multocida* infection (Table 2). Clinical signs were related to the isolation *P. multocida*, which supports the strong APP response against this pathogen. These results were supported by a more recent study from Finland, where *P. multocida* was found to be more common in clinically ill animals, but this association was not present if *P. multocida* was the only pathogen isolated (Autio et al., 2006). *P. multoc*ida is considered to be more a secondary than a primary causative agent in BRD (Maheswaran et al., 2002). We detected *P. multocida* infection in calves after clinical BRD outbreak (V), suggesting the opportunistic nature of *P. multocida*. No other primary agents of BRD, such as *M. haemolytica*, *H. somni* or *M. bovis*, were isolated, and no seroconversion to BRSV was seen. Calves were from farms free of BVDV and BHV-1 is very rare in Finland. However, as animals were around 3 months old and high antibody levels against BRSV were found in calves from 12 farms used in the present study (Härtel et al., 2004), a possible suppressive effect of maternal antibodies on the own antibody production of the calves cannot be ruled out. BRSV may still be involved in the development of clinical BRD, and a predisposing effect of other viruses potentially involved (BCV, PIV-3, BAV-3 and BAV-7) shall also be taken into account. In the same time, almost all calves were infected by mycoplasmal species (not *M. bovis*), and a synergistic effect of mycoplasmas and *P. multocida* is possible (Virtala et al., 1996). Low numbers of other bacteria (*Pasteurella* sp., *F. necrophorum* and *A. pyogenes*) were also isolated from some calves. In the longitudinal study, we found other bacterial species besides *P. multocida* in 5 of the 10 calves investigated (Table 3). In the cross-sectional study, the mean age of the calves was higher, that may have affected the respiratory infection status of the calves. Despite these considerations, the strong association between APP response and infection with *P. multocida* indicates an important role of this pathogen in BRD.

6.3.2 Longitudinal study (V)

In this study, results of the isolation of viruses and the antibody response of calves in a dairy herd indicated that the BRD outbreak most probably started with a BRSV infection. In addition, a synergistic effect between BRSV and bacteria was observed, as more bacterial species were isolated in the TBL samples taken around the middle of the outbreak than in the samples taken after the clinical manifestation of BRD (Table 3). This suggests the multi-infection nature of this outbreak.

The first inflammatory reaction, reflected as an increase in systemic concentrations of SAA and LBP at week one and commencement of clinical BRD in all calves, was probably a response to BRS virus. A pronounced elevation of the concentrations of SAA, LBP and especially Hp in serum at week 3 is more likely a sign of a secondary bacterial infection. In a BRSV challenge study published earlier, a two-stage elevation of SAA

and Hp in one calf was reported and *P. avium* was found at necropsy (Heegaard et al., 2000). In the same study, only the high SAA responders to the virus also showed an Hp response, while medium and low SAA responders did not. This suggests that a systemic Hp response requires more severe inflammatory stimulation, which is more likely in bacterial infections. Godson et al. (1996) reported an Hp rise in only 10% of calves after BHV-1 challenge, although the virus did initiate APR, reflected as increased temperatures at 2 days post-infection. However, 43% of calves showed an Hp response at 24 h and 84% at 72 h after a subsequent *M. hemolytica* inoculation (Godson et al., 1996). Grell et al. (2005) showed in an experimental BRSV infection model that Hp concentrations decreased to initial levels by day 13 post-infection, which supports the role of secondary bacterial infections in higher Hp values at week 3, as seen in our study.

P. multocida is the most probable candidate for the enhanced responses of APPs during the later stages of outbreak, based on the literature (Autio et al., 2006) and on our cross-sectional study (IV). The only calf that had no *P. multocida* infection at week 2 had the mildest clinical response (at week 2) and showed no systemic APP elevation after week 1. *M. dispar* was also found in the majority of calves, but *M. dispar* is considered not to be a highly pathogenic agent in BRD (Virtala et al., 1996; Tegtmeier et al., 2000), although Tanskanen (1984) did report mild clinical respiratory disease signs in calves after inoculation with *M. dispar*. In addition, all *P. multocida* isolates investigated here had an identical *Sal*I banding pattern. Predominant strains of *P. multocida* have been suggested to be more pathogenic (Davies et al., 2004). We cannot, however, exclude an additional synergistic effect of other bacterial and mycoplasmal infections detected at week 2 on the inflammatory response of calves (Table 3). Allen et al. (1992) reported re-infection of *P. multocida* in the lower respiratory tract of calves after treatment with antibiotics and disappearance of clinical BRD. This is in agreement with our results, as *P. multocida* infection detected after the clinical disease at week 6 seemed to no longer have a significant effect on inflammatory response.

A stronger APP response at week 3 was connected with calves' lower specific IgG_1 response to BRSV infection. These differences in APP response patterns could easily be explained by a different onset of infection. However, all calves had similar SAA, LBP and clinical responses at week 1 and BRS virus findings at week 2 (2 from low-response and 3 from high-response groups; Table 3). There was also a comparable distribution of different antibody response groups between the two fences, and one nipple of an automatic feeder in both fences may have assisted the quick spread of the virus. As this was not a controlled experimental infection, we cannot confirm that all calves became infected with the same virus dose and at the same time. However, the initial onset of infection very likely occurred over a relatively short time. The groups of calves were also of a similar age and no maternal antibodies were present, so these likely did not serve as confounding factors. One explanation may be that early and sufficient production of antibodies to the virus protected calves from exacerbated bacterial infection.

6.3.3 Acute phase proteins as infection markers in spontaneous BRD

Our results indicate that SAA and LBP are sensitive markers of respiratory infections in calves and support the results from the experimental challenge studies (Horadagoda et al., 1994; Heegaard et al., 2000; Schroedl et al., 2001; Ganheim et al., 2003). Our findings on the systemic Hp response are in agreement with other reports (Wittum et al., 1996; Young et al., 1996; Svensson et al., 2006). Hp seems to have a limited capacity to serve as a sensitive indicator of respiratory disease; an initial virus infection failed to

trigger Hp response except in one calf (V). A subsequent substantial Hp rise was seen in only some of the calves, probably due to a secondary bacterial infection. Svensson et al. (2006) suggested that the reason for the poor association of systemic Hp with clinical signs of BRD was that a large proportion of the cases of acute BRD were probably caused by viral infection. Nevertheless, Hp and Fb can be a useful and reliable marker of inflammation in, for example, selecting animals needing antimicrobial treatment or following up the effects of treatment (Carter et al., 2002; Berry et al., 2004; Humblet et al., 2004). This is especially true for young calves because concentrations of Hp are less affected by age than the other APPs investigated here (III). In our study on spontaneous BRD outbreak, the two calves requiring antimicrobial treatment at week 3 simultaneously had high Hp values.

In our longitudinal study, clinical BRD was only mild to moderate and the time between samplings was relatively long. We probably missed some time points with more severe clinical signs and greater APP responses. This can explain the difference between the results of the cross-sectional and longitudinal studies, as no marked alterations of AGP concentrations were seen in the later study. AGP increases more gradually and at later stages of the infection than SAA and Hp (Dowling et al., 2002). Another reason might be the age effect since AGP is already elevated after birth (III) and the isoform is different in newborn calves (Itoh et al., 1993a), possibly suppressing their AGP response.

In conclusion, APPs can be used to investigate different aspects of host-pathogen interaction in respiratory diseases of calves. They are more sensitive markers for inflammation than monitoring clinical response alone (V) and probably better than clinical response for exploring the host responses. Our results suggest that SAA and LBP are the best candidates for this purpose. At the same time, parallel measuring of systemic Hp or possibly AGP can be valuable in improving the evaluation of the severity of inflammation. In addition, Fb can be a valuable tool, as the strongest association was seen with the isolation of *P. multocida* and Fb (Table 2) in the cross-sectional study.

6.4 Possible use of APPs in reindeer and dairy calves

SAA appears to be a more sensitive indicator than Hp of APR in the ruminants investigated here. Measuring of both proteins in parallel can provide more information (e.g. evaluation of disease severity) on the host's ongoing APR. However, in the reindeer, stress reactions from handling (blood sampling) develop easily and their potential effects on the results must be addressed. Moreover, proper validation of analytical methods for APPs in reindeer is needed before further investigation of the expression of these proteins in naturally occurring diseases and experimental studies.

We conclude that newborn reindeer and dairy calves show a mild APR after birth, which may be related to the adaptation of these young animals to extrauterine life. We also found a negative correlation of SAA after birth with long-term weight gain in reindeer calves. This indicates that investigating changes in concentrations of APPs after birth and their relation to environmental factors in more detail could shed light on the adaptation mechanisms of newborns to the environment and on the impact of these events on the performance of reindeer as well as dairy calves.

Our studies in spontaneous BRD in calves support the view that APPs are valuable research tools in clinical (e.g. for evaluating the effect of anti-inflammatory and anti-bacterial treatments) and other field studies (e.g. in epidemiological studies as markers of host response) of calves. Unfortunately, in our cross-sectional study, results were so consistent between APPs that evaluation of different APP values in epidemiological studies was not feasible. In the future, APPs should be examined using larger study populations and including apparently healthy animals to yield more variable infection status of calves. A larger study with variable infection status would also permit evaluation and quantification of the impact of different aetiological factors on host response using APPs. As APPs are non-specific, potential confounding factors, such as age in younger animals and presence of other diseases and infections, should be controlled.

Sensitive APPs (SAA and LBP) seem to be the best candidates as research tools in clinical and field studies. SAA may have some benefits as a research tool for quantifying inflammatory response. The relative increase of SAA is higher, which makes the measurement scale of SAA wider, possibly allowing more detailed information to be gained. However, in Study IV, APPs with a relatively small rise (LBP and Fb) effectively separated calves with *P. multocida* infection, and concentrations of LBP remained elevated for a longer period (Fig. 5), rendering sampling time in relation to APR not as critical. Fb in particular, seems to have potential as a research and clinical tool in cattle because inexpensive and easy methods for Fb are available. A possible suppressive effect of the high foetal and neonatal concentrations on AGP response makes AGP more useful in older animals.

Nevertheless, in veterinary practice, potential confounders (e.g. age, stress factors and subclinical infections) hinder the usefulness of SAA and LBP as inflammatory markers and exact cut-off values may be difficult to establish. In this respect, Fb and Hp may have more use (Orro et al., 2006).

7 CONCLUSIONS

1. SAA and Hp both serve as APPs in reindeer. SAA seems to be a more sensitive inflammatory marker than Hp.

2. Newborn reindeer and dairy calves have an inflammatory response during the first weeks of life.

3. Age of neonatal ruminants should be considered when interpreting results of APPs.

4. *P. multocida* can be considered an important infection agent in calves suffering from respiratory disease.

5. Measurements of APPs are useful when exploring host-pathogen interaction of calves in field studies.

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