Influence of Natural *Chlamydia* spp. Infection on the Health of the Ruminant Mammary Gland

by

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Abstract

Exposure to obligate intracellular *Chlamydia abortus* and *C. pecorum* bacteria is ubiquitous in ruminants worldwide, with high seroprevalence rates approaching 100% in some investigations. This study aimed to analyze the effect of these largely asymptomatic infections on the lactating mammary gland of ewes and dairy cows.

C. abortus is the most common abortion agent in sheep. After chlamydial abortion was identified in a flock of milk sheep early in the lambing season, a prospective observational study investigated the progression of the subsequent *C. abortus* abortion storm in flock. Abortion was associated highly significantly with the gestation phase at high-level exposure to *C. abortus* during the first abortion in the herd. Abortion sheep showed significantly increased complement-binding antibodies against *Chlamydia* spp. followed by a highly significant reduction in CFT titers. During the initial seven weeks of lactation, overall milk somatic cell counts in abortion sheep were lower than in live lambing sheep. *C. abortus* infection of the mammary gland moderately increased milk SCC, but abortion or the anti-chlamydial immune response associated with abortion reduced milk SCC and protected against streptococcal and staphylococcal mastitis.

In dairy cows, mastitis, the inflammation of the mammary gland, is the most prevalent and economically important production disease. To characterize mastitis, caused by natural *Chlamydia* spp. infection of dairy cows, 17 dairy cows in the second or

higher parity were sampled for 20 weeks after parturition. All cows (100%) were positive for anti-*Chlamydia* spp.IgM serum antibodies, indicating endemic infection. Twelve (70%) of the cows were PCR-positive for *Chlamydia* spp. in any of the samples, with 11 cows positive only in vaginal cytobrush specimens, 1 positive only in milk samples, and 2 cows in both types of specimens. Data analysis using principal components resulted in indices "Production loss index", "Anti-*Chlamydia* immune index", and "Inflammatory Index" which delineated the complete dataset in well defined, biologically significant natural clusters. In a multivariate logistic stepwise regression model, cows with chlamydial colonization of the mammary gland had significantly higher milk SCC and lower milk protein. Systemic chlamydial infection highly significantly associated with increased milk yield and milk fat, and decreased milk protein. These data confirm and characterize the influence of asymptomatic localized and systemic chlamydial infection on the health of the mammary gland of dairy cows and their milk production.

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List of Abbreviations

CD_4^+	Cluster of Differentiation 4
CFT	Compliment Fixation Titer
Chl immune	Anti-*Chlamydia* immune index
CMT	California Mastitis Test
DHIA	Dairy Herd Information Association
DTH	Delayed Type Hypersensitivity
EB	Elementary Body
ELISA	Enzyme Linked Immunosorbent Assay
IACUC	Institutional Animal Care and Use Committee
ICAR	International Committee for Animal Recording
IL	Interleukin
Inflam	Inflammation index
MIF	Microimmunofluorescene
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
Prod loss	Production loss index
RB	Reticulate Body
SAA	Serum Amyloid A
SCC	Somatic Cell Count

Chapter 1: Literature Review

Biology Of *Chlamydiaceae*

Bacterial lifecycle. Microorganisms of the family *Chlamydiaceae* are obligate intracellular bacteria that parasitize an extremely wide range of eukaryotic cells and replicate in a vacuole of these cells (Stephens, 1999). The developmental cycle of *Chlamydia*, first described by Bedson and Bland (Bedson and Bland, 1932), and later confirmed by electron microscopic studies (Eb et al., 1976, Matsumoto, 1988, Soloff et al., 1982), is biphasic consisting of small, extracellular infectious but metabolically inert elementary bodies (EB) and larger intracellular reticulate bodies (RB) that divide intracellularly. Conversion of EBs to RBs and reorganization of RBs back to EBs are unique stages in the chlamydial development cycle. EBs, approximately 0.3 μm in diameter, become metabolically inert by completely shutting down their metabolism and condensing their DNA into hyperpyknotic nucleoids (Stephens, 1999) and are refractory to extracellular physical and chemical duress. This resistance is achieved by a poorly permeable and osmotically stable cell envelope maintained by a complex of disulfide-crosslinked envelope proteins. The EBs attach to host cells by contact with

numerous, but poorly defined surface proteins, and are internalized into host cells via a process termed receptor-mediated endocytosis (Miyashita et al., 1993, Rockey and Matsumoto, 2000). Endocytosed EBs prevent phagosome-lysosome fusion and remain in vacuolar inclusions, which are modified membrane-bound compartments. Inside these vacuolar inclusion bodies, disulfide crosslinks within and among chlamydial envelope proteins become chemically reduced, allowing transformation and differentiation of EBs into RBs (Everett et al., 1999, Stephens, 1988). RBs may be up to 1.5 μm in diameter, take up nutrients from the host cell via type III secretion systems, and undergo multiple rounds of binary division. Finally, the RB reorganizes asynchronously into infectious EBs, which are released from the host cell to initiate a new round of infection. EBs and RBs both have inner and outer membranes and a distinct but variable periplasmic space. Due to the lack of a cell wall, chlamydiae appear gram negative in the gram stain. EBs, when stained with basic dyes such as furchsin in the Macchiavello or Gimenèz stains are visible as small red dots; however their appearance is purple-blue in Giemsa staining procedure (Fig. 1). Development and replication of *Chlamydiae* is dependent on the nutrient supply thus metabolic status of the host. Delayed development due to nutrient deprivation can lead to enlarged, aberrant and degenerative chlamydial forms (Fig. 2).

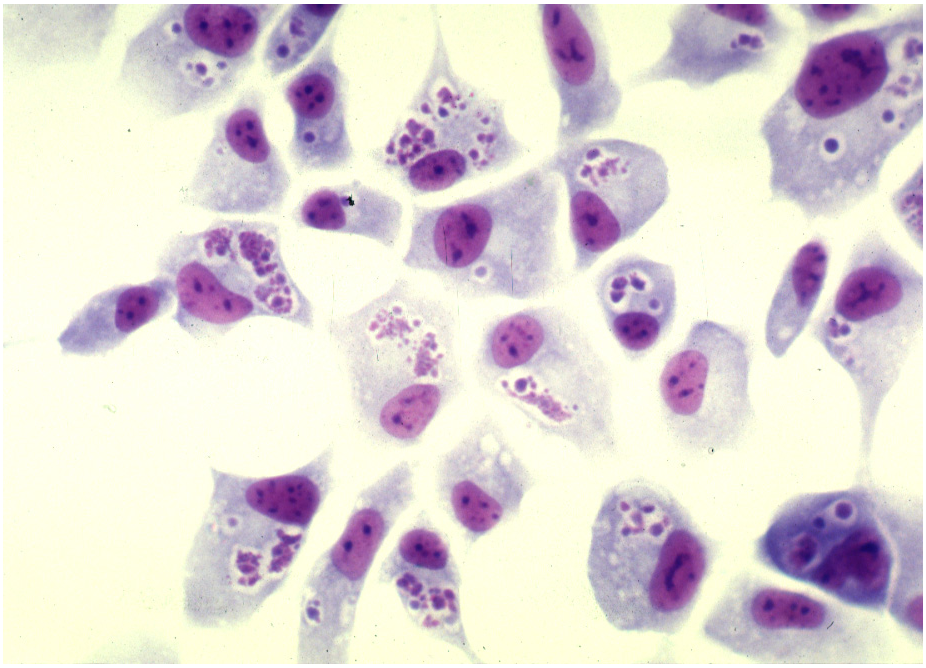

Figure 1. Mouse L 929 fibroblast monolayer cells infected with *Chlamydia psittaci* and stained by the Giemsa staining method. Cells were cultivated in Minimum Essential Medium with Eagle's salts (Eagle's MEM). The intracellular inclusions contain few chlamydial developmental forms such as RB and EB, but also enlarged aberrant chlamydial organisms. Image courtesy B. Kaltenboeck.

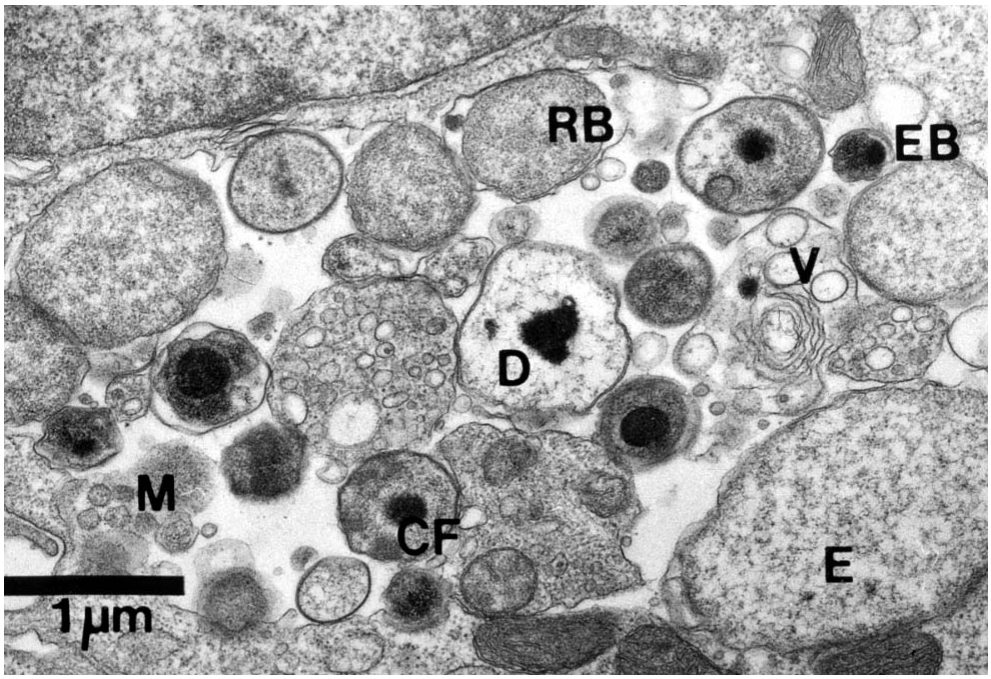

Figure 2. Developmental stages of *Chlamydia psittaci* under electron microscopy. An inclusion in a L929 mouse fibroblast cell as shown in Fig. 1 is shown containing various stages of the normal chlamydial developmental cycle such as elementary bodies (EB), reticulate bodies (RB), and condensing forms (CF) in the transition from RB to EB. Also present are several developmental forms arrested due to nutrient deprivation in abnormal morphologies such as enlarged (E), degenerate (D), vacuolar (V) and microvacuolar (M) reticulate bodies. Image courtesy B. Kaltenboeck.

Microbiological culture. The obligate intracellular nature of C*hlamydiaceae* bacteria prevents the use of cell-free culture for propagation of these organisms. Traditionally, *Chlamydiaceae* were cultured in yolk sacs of chicken embryos (Storz and QLOC VETM, 1971), but cultivation in cell culture has become increasingly popular due to the ease of use. Using a saturated equilibrium monolayer cell culture system for growing *C. pneumoniae*, *C. trachomatis*, *C. pecorum*, or *C. abortus* , Li et al. (2005) have achieved production of approximately 4×10^8 *Chlamydiaceae* genome copies per ml of culture after 10 days of cultivation. *Chlamydiaceae* EBs were used to infect Buffalo Green Monkey Kidney (BGMK) cells cultivated in Iscove's Modified Dulbecco's Medium (IMDM) with a multiplicity of infection (MOI) of 10 or above.

Taxonomy of *Chlamydiaceae*. The family *Chlamydiaceae* is the only family in the order *Chlamydiales*, and all chlamydial species belong to this family. In the early years of the chlamydial taxonomy, the family *Chlamydiaceae* consisted of the single genus *Chlamydia* that had two species, *C. trachomatis* and *C. psittaci*. This classification was based on the sensitivity to sulfadiazine of *C. trachomatis*, but not of *C. psittaci*. In subsequent years *C. pneumoniae* (Grayston et al., 1989) and *C. pecorum* (Fukushi and Hirai, 1992) were speciated on the basis of genomic DNA cross-hybridization data. Nevertheless, strains within *C. psittaci* and *C. trachomatis* were clearly sufficiently divergent to be considered different species but were not recognized by this classification (Palys et al., 1997, Tanner et al., 1999).

In 1999, Everett et al (1999) proposed a new classification based on genome size, 16S and 23S ribosomal RNA gene sequence similarity, DNA-DNA reassociation, coding

sequence similarity, and number of ribosomal operons and advocated dividing the family *Chlamydiaceae* into two genera, *Chlamydia* consisting of species *C. trachomatis, C. suis* and *C. muridarum,* and *Chlamydophila* consisting of the species *C. pneumoniae*, *C. psittaci*, *C. abortus*, *C. pecorum*, *C. caviae* and *C. felis* (Fig. 3). However, protein evolution patterns based on the complete chlamydial genome sequences are not consistent with this separation (Griffiths et al., 2006, Gupta and Griffiths, 2006). These findings challenge and undermine the validity of the proposal for separate genera. There is general agreement that these chlamydial strains may be defined as different species but researchers argue that the available biological evidence contradicts the genus separation. Thus, separation into additional species has been widely accepted but the division of the family *Chlamydiaceae* into two genera has largely been rejected (Schachter et al., 2001, Stephens et al., 2009). Thus, the forthcoming volume 4 of the second edition of the authoritative Bergey's Manual of Systematic Bacteriology will contain the single genus *Chlamydia* in the family *Chlamydiaceae*, but will retain 9 species and their epithets within this genus (B. Kaltenboeck, oral communication).

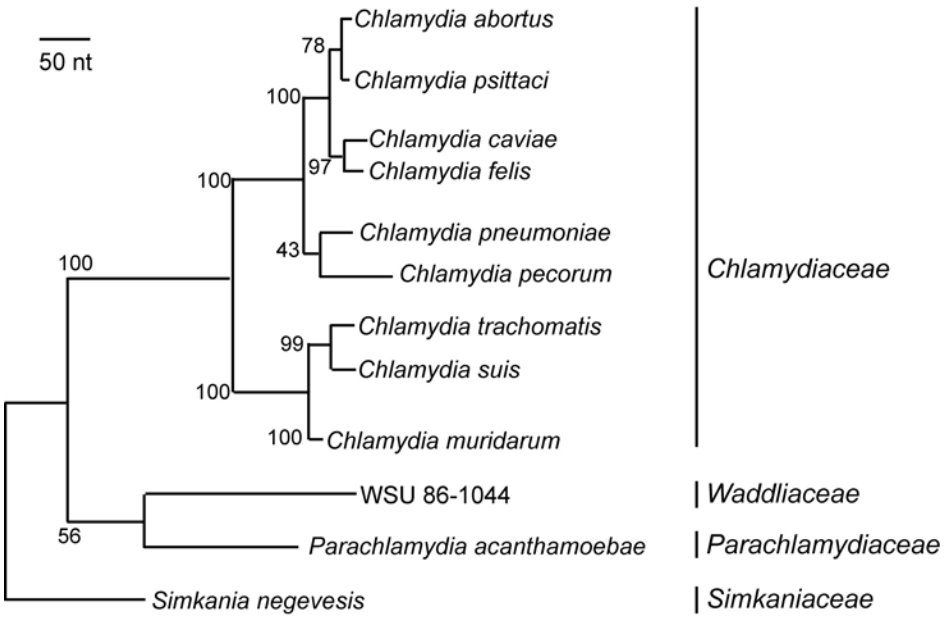

Figure 3. Phylogeny of the order *Chlamydiales* based on full-length 16S rRNA genes (Everett et al., 1999, Horn et al., 2000). Branch lengths are measured in nucleotide substitutions, and numbers show branching percentages in bootstrap replicates. Only bacteria of the family *Chlamydiaceae* have fully established pathogenic potential for vertebrates. Strains of the remaining families *Waddliacae*, *Parachlamydiaceae*, and *Simkaniaceae* have been variably and inconsistently associated with diseases.

CHLAMYDIAL DISEASES

Epidemiology. A myriad of acute and chronic diseases has been attributed to the obligate intracellular bacteria of the family *Chlamydiaceae* in a very wide range of hosts including invertebrates, amphibians, reptiles, birds, and mammals. In humans and livestock most *Chlamydia* spp. infections are asymptomatic, but still pose a serious challenge to public health in humans, and lead to substantial economic losses in the livestock industries.

Chlamydia trachomatis with approximately 4 million cervical infections annually is the most common sexually transmitted bacterial disease in the United States (Washington et al., 1987). The US Centers for Disease Control and Prevention recommends routine testing based on age, risk behavior, and clinical findings in all clinics and group practices that provide reproductive health care to adolescent and young women (Dowell et al., 2001). Although 70% of infected women have few or no symptoms (Cates Jr and Wasserheit, 1991), these infections can persist for up to 15 months (Lycke et al., 1980) and may progress to involve the upper reproductive tract which results in serious complications. The cost of treating these chlamydial infections and conditions associated with these infections is estimated to be close to $2 billion annually in the US alone. There are reports of human abortion, or severe respiratory disease, contracted from chlamydiae from aborting sheep or infected birds (Mare, 1994).

Chlamydial infections in animals are ubiquitous worldwide with high seroprevalence rates approaching 100% in some investigations (Cavirani et al., 2001, Kaltenboeck et al., 1997, Wang et al., 2001). In a study by DeGraves et al. (2003a) in

Alabama, USA, 53% of the vaginal cytobrush swabs taken once a week for a month from herd of 51 Holstein heifers were found positive by 23S and *omp1* FRET PCRs. Positive percentages of *C. abortus* and *C. pecorum* were 24% and 39% respectively (DeGraves et al., 2003a, DeGraves et al., 2004).

Abortion in sheep, goats, and cattle, and occasionally in horses, swine, rabbits, and guinea pigs has been attributed primarily to *C. abortus* (Appleyard et al., 1983, McCauley and Tieken, 1968). *C. abortus* is transmitted by infected animals via contact, secundinae, uterine discharge, or feces of asymptomatic carrier animals. *C. abortus* and *C. pecorum* are the two most important *Chlamydia* species routinely detected in cattle (Everett et al., 1999, Schachter et al., 1975).

Diagnosis. The microimmunofluorescence (MIF) test is recognized as the gold standard for serodiagnosis in humans (Dowell et al., 2001, Wang, 2000) whereas complement fixation test (CFT) is still used as a standard procedure in livestock (Kaltenboeck et al., 1997). Enzyme-linked immunosorbent assays (ELISA) also receive wide acceptance due to limited sensitivity of the CFT test (Kaltenboeck et al., 1997). The high seroprevalence of chlamydial infections is also a problem in research studies because of the difficulty in defining a reliable serological cut-off in ELISA assays of anti-chlamydial antibodies.

Direct detection of chlamydiae has been classically performed by culture in the yolk sac of chicken embryos (Storz and QLOC VETM, 1971). This method has largely been replaced by cultivation in monolayer cell cultures (Li et al., 2005). Nevertheless, these methods all are cumbersome and insensitive. In recent times, high-sensitivity and high-specificity diagnostic methods based on nucleic acid detection by PCR is becoming

increasingly popular (Kaltenboeck and Wang, 2005). A real-time PCR platform capable of detecting single genome copies of chlamydiae (DeGraves et al., 2003a) has been developed and used to detect *C. abortus* and *C. pecorum* in bovine vaginal cytobrush specimens (DeGraves et al., 2003b)

Pathogenesis and immunity. *Chlamydia* spp. are not known to produce toxins or toxic by-products in the host cell. Chlamydiae modulate their growth based on availability of nutrients in the host cytoplasm. Chlamydial replication, host response in conjunction with many factors like host immune status, concurrent diseases, age, stress of production etc. determine the outcome of chlamydial infection(Ramsey, 2006, Stephens, 1999, Wang et al., 2009). Common disease conditions are manifested as chronic granulomatous lesions of mononuclear cell aggregates and fibrosis. However repeated infection or superinfections along with host genetic susceptibility are the key factors that exacerbate chlamydial infections. $CD4^+$ lymphocytes are crucial for host protection by restricting chlamydial replication via a Th1-type immune response (Wang et al., 2009) (Morrison et al., 1995, Patrick and Priscilla, 2009). Th1 cells are the inducers of a protective delayed type hypersensitivity DTH response since they secrete interferon gamma (IFN-γ), a potent stimulator of macrophages (Perry et al., 1997, Rottenberg et al., 2000). The DTH response features tissue infiltration of $CD4^+$ T cells and macrophages, and release of proinflammatory Th1 cytokines such as IL-1, IL-2, IL-12, IFN-γ, or TNF-α. These cytokines increase the production of free radical molecules such as reactive oxygen species (ROS) and reactive nitrogen oxide species (RNOS) (Swindle and Metcalfe, 2007) and play a pivotal role in cellular signaling and regulation of the immune response to

chlamydial infection (Huang et al., 2002, Moore et al., 2001). This Th1 cytokine storm can also cause the early death of the host. Therefore, protective immunity against chlamydiae has an absolute requirement for a Th1 cell response that mediates elimination of chlamydiae. However, an excessive Th1 response also causes disease and therefore needs to be balanced by some Th2 cells in order to limit host inflammation and pathology (Huang et al., 2002, Moore et al., 2001).

Classical acute disease manifestations in livestock. *Chlamydia suis* in swine is associated with enteritis, pneumonia, conjunctivitis, polyserositis, pericarditis (Schiller et al., 1997, Woollen et al., 1990).

Abortion in sheep, goats, and cattle, and occasionally in horses, swine, rabbits, and guinea pigs has been attributed primarily to *C. abortus* (Appleyard et al., 1983, McCauley and Tieken, 1968). *C. abortus* has also been sporadically associated with naturally occurring clinical bovine mastitis (Kaltenbock et al., 1997, Kaltenboeck et al., 1992, Wehnert et al., 1980). Experimental inoculation of *C. abortus* via the teat canal produces a severe acute mastitis of the inoculated mammary glands accompanied by fever and anorexia followed by reduction in milk production and mammary gland atrophy (Corner et al., 1968) (Andreani et al., 1987, Mecklinger et al., 1980, Stephens, 1999).

Bovine encephalitis caused by *C. pecorum* was one of the first chlamydial diseases in cattle identified by McNutt (1940). *C. pecorum* infections are endemic in the intestinal tract of sheep and cattle populations. Acute infections with *C. pecorum* can cause purulent endometritis and fertility disorders including occasional abortions, and sporadic encephalomyelitis, kerato-conjunctivitis, pneumonia, enteritis, and polyarthritis

(Bowen et al., 1978, Griffiths et al., 1995, McKercher et al., 1966, Otter et al., 2003, Storz et al., 1968, Twomey et al., 2003, White, 1965, Wilson and Thomson, 1968, Wittenbrink et al., 1993).

Subclinical infections, "the hidden-epidemic" of chlamydial diseases in livestock. With the advent of improved and sensitive diagnostic tools, such as the Polymerase Chain Reaction (PCR), the prevalence and incidence of *Chlamydia* spp. has been redefined and a high prevalence of chlamydial infections in livestock is seen without any clinically apparent infection. Over the past few years many researchers have focused on elucidating the health impact of these ubiquitous subclinical or latent chlamydial infections.

Chlamydia suis is the primary chlamydial species reported in swine. MMA-syndrome (mastitis, metritis, agalactia), limited infant mortality, reproductive failure and infertility in sows has been found in conjunction with low level chlamydial infections *C. suis* (Schiller et al., 1997, Woollen et al., 1990).

C. pecorum infection of cattle resides mostly in the mucosal epithelium and is somewhat analogous to *C. trachomatis* infection in humans and can be asymptomatic but simultaneously leading to chronic sequelae such as salpingitis, infertility, airway obstruction, pulmonary inflammation, and reduced weight gains (Everett et al., 1999, Fukushi and Hirai, 1992, Hitchcock, 1999).

Reinhold et al. (Reinhold et al., 2008) studied the impact of subclinical chlamydial infections in dairy calves and observed intermittent shedding of *Chlamydia*

spp. in carrier calves without any correlation to serum CFT of antibodies against chlamydiae. This also emphasizes the importance of multiple sampling and sensitive tests to identify the true *Chlamydia* carrier status of animals. The core body temperature of *Chlamydia* spp. carrier calves was significantly higher compared to *Chlamydia*-free calves. In addition, a significant effect on hematological status of the calves was also seen. *Chlamydia* carrier calves were consistently and significantly lower in haematocrit values, and hemoglobin and serum iron concentrations. Differences in these physiologically important parameters indicate a strong effect of persistent subclinical infection and continued engagement of host defense mechanisms. In the same study, the authors also studied the consequences of these infections on the respiratory system and evaluated the lung function of calves by repeated non-invasive impulse oscillometry tests. A profound effect of chlamydial infections on *Chlamydia*-carrier calves was observed as evidenced by significantly higher peripheral airway obstruction, increase in respiratory rates and minute volume of respiration. These changes are consistent with chronic inflammation of lung and bronchiolar tissue. The most important observation with the significant detrimental economic effect of chlamydial infection was consistent evidence of lower body weight of *Chlamydia*-carrier calves.

The impact of chlamydial infection is also seen in the fertility of clinically healthy cows (DeGraves et al., 2004, Wehrend et al., 2005). DeGraves et al. investigated the effects of controlled re-infection on the fertility of cattle naturally pre-exposed to *C. abortus*. No animal showed signs of clinical disease but pregnancy outcome (infertility) correlated highly significantly with a rise in post-challenge anti-*C. abortus* IgM. In the same study a subunit *C. abortus* vaccine (Stemke-Hale et al., 2005) was used as a

prophylactic approach in heifers already exposed to, and immune against *C. abortus*, to modulate this existing immune response, and the authors reported a 32% increase in fertility in vaccinated heifers. These results underscore the impact of subclinical *C. abortus* infection. These studies give strong evidence of economic losses due to long term health impact of clinically inapparent chlamydial infections.

Most cases of mastitis in dairy cattle are clinically inapparent, and typical mastitis pathogens such as *Streptococcus agalactiae* are detected only in a fraction of the cases. Subclinical mastitis is nevertheless of major interest to production medicine because of the large impact on profit margins of dairy farms. With the advent of intensive commercial dairy farming and concerns of economic losses, numerous parameters have been established for routine monitoring of udder health (Green et al., 2004, Radostitis et al., 1994). Widely used and accepted among these parameters is the number of somatic cells in milk (SCC) which is less than 100,000 somatic cells per milliliter in a healthy bovine mammary gland. Higher numbers of SCC in bovine milk indicate inflammation of the mammary gland. Clinically manifest mastitis is typically associated with SCC above 10^6 (Green et al., 2004). Milk with 200,000 or more cells per ml is of reduced value due to reduction in manufacturing properties and yield of cheese (Dohoo and Leslie, 1991, Seegers et al., 2003). In a prospective cohort study in a herd of 140 Holstein dairy cows, Biesenkamp et al. (2007) examined the influence of chlamydial infection detected by PCR on subclinical inflammation of the bovine mammary gland as characterized by elevated somatic cell counts (SCC) in milk. Not surprisingly, 100% seroprevalence was observed in the study, however low anti-chlamydial serum antibody levels were significantly associated with bovine subclinical mastitis. In the same study,

an Alum-QuilA-based vaccine of inactivated *C. abortus/C. pecorum* elementary bodies was used which reduced milk SCC significantly and also improved the body condition score.

Thus, the majority of frequently detected low-level infections with *Chlamydia* spp. infections in cattle are not associated with obvious clinical disease and detection is possible only with repeated sampling and sensitive PCR methods (Jee et al., 2004). Thus a well balanced host-parasite relationship appears to exist in natural *Chlamydia* spp. infections (Shewen, 1980). Chlamydial infections in livestock are of huge economic and health significance of but impact of the ubiquitous subclinical chlamydial infections remains underestimated, underdiagnosed, and thus unknown.

Systematic investigations of the involvement of *Chlamydia* spp. in bovine mastitis have not been reported. In an epidemiological study of *Chlamydia* spp. infection in calves, Jee et al. (2004) detected low copy numbers of *C. abortus* DNA in the milk of 15% of the dams without any signs of disease, which suggests low-level natural *Chlamydia* spp infection of the bovine mammary gland as well. In the above study one-hundred micro liter milk samples from a single udder quarter were tested per week for a 12 weeks period post partum. Thus, sampling intensity was low, and higher prevalence of *Chlamydia* spp. in milk may possibly be detected with higher sampling intensity.

Continuous simultaneous detection of chlamydial infection, inflammatory status, and production of the mammary gland by PCR, SCC, and milk yield and protein and fat percent, respectively, would allow for long-term assessment of the impact of chlamydial infection on the health of the isolated mammary gland. This is important not only for cattle, but also for the understanding of human chronic inflammatory diseases such as

pelvic inflammatory disease and reactive arthritis, or coronary heart disease, for which a strong association with *C. trachomatis* and *C. pneumoniae* infection, respectively, has been found (Danesh et al., 1997, Saikku et al., 1988, Stephens, 1999).

This dissertation will correlate low-level *Chlamydia* spp infections of dairy cows, both as systemic infections and localized to the mammary gland, and their kinetics and cyclical characteristics with milk production traits of these cows. Because of the complex multivariate interactions between such chlamydial infections, the host, and the environment, it is unlikely that single-factor associations with disease or production traits can be identified. Rather, specific combinations of multiple factors will likely determine disease susceptibility and production impact.

VACCINATION AGAINST *CHLAMYDIA* SPP.

Vaccine candidate antigens. There is considerable economic and health interest in improved anti-chlamydial vaccines with higher efficacy in livestock. While attenuated live and inactivated *C. abortus* vaccines for prevention of sheep/goat abortion are available, little is known about their efficacy and consistency. Furthermore, such vaccines are expensive because of the difficulty of propagating these agents. Future anti-*Chlamydia* spp. vaccines will therefore likely be subunit vaccines composed of protective proteins and adjuvant for optimal targeting of the immune response for prophylaxis as well as immunotherapy. Mouse models of chlamydial diseases have been extensively used to identify protective vaccine candidates and effective delivery system. Vanrompay *et al.* (2001) used genetic immunization to vaccinate turkeys with *C. psittaci ompA*, and

achieved significant reduction in chlamydial shedding, and virtually complete protection from disease after respiratory challenge with *C. psittaci.* Stemke-Hale et al. (2005) used genome wide expression library immunization (ELI) with pools of plasmids to identify vaccine candidate genes. They identified *dnaX*$_2$*, gatA, gatC, pbp3* as best candidates, and observed complete protection in mice from *C. abortus* induced diseases after genetic immunization with these antigens.

RESEARCH OBJECTIVES

1. **Post-abortion effects of *Chlamydia abortus* infection on the ovine mammary gland**. Analyze the data obtained from a sheep flock by repeated sampling during a natural chlamydial abortion episode. Correlate these findings with the current literature to better understand the temporal relationship of the chlamydial infections with pregnancy outcome in sheep, immune response, and subsequent health status of the mammary gland.

2. **Quantitatively determine the influence of *Chlamydia* spp. infection on the health of the mammary gland as determined by milk quality and production of dairy cows.** To achieve this objective, monitor 23 dairy cows from the Auburn University Large Animal Clinic and EV Smith Research Center, Shorter, Alabama, for milk quality and quantity for 20 weeks at 2-week intervals by analysis of each mammary gland quarter milk production, and milk SCC, protein, and fat. Relate quarter milk and mucous membrane *Chlamydia* spp. numbers as detected by quantitative PCR,

other bacteria as detected by standard bacterial culture, and serum anti-*Chlamydia* IgM antibodies to inter-cow and inter-quarter mammary gland health and production.

Chapter 2: Post-Abortion Effects of *Chlamydia abortus* Infection on the Ovine Mammary Gland

INTRODUCTION

Chlamydiae are obligate intracellular bacteria with a biphasic life cycle (Barron, 1988). The metabolically active forms of these bacteria replicate within cytoplasmic vacuoles of eukaryotic host cells and are termed reticulate bodies (RB). Driven by poorly understood regulatory mechanisms, these RB reorganize to spore-like, metabolically inactive chlamydial forms termed elementary bodies (EB). EBs are the infectious form of chlamydiae that are released from the host cell and survive for a long time in the environment (Stephens, 1999). Infections with the nine chlamydial species (Stephens et al., 2009) are ubiquitous in the animal kingdom. In sheep, the most prominent chlamydial disease is abortion caused by *C. abortus* [formerly *C. psittaci* serogroup 1 or *C. psittaci omp*1 type B577]. The first report of chlamydial abortion in sheep [also known as ovine enzootic abortion (OEA) or enzootic abortion of ewes (EAE)] was a description of the disease in Scotland by Greig (1936). The aetiological agent was identified later by Stamp *et al.* in 1950 (Stamp et al., 1950) and is now recognized worldwide (Aitken et al., 1990, Borel et al., 2004) as one of the most important and

dramatic disease manifestations of animal chlamydial infection (Aitken, 1993, Storz and QLOC VETM, 1971).

C. abortus is excreted by infected and aborting ewes in huge numbers in diseased placenta, uterine discharges, and feces, and *C. abortus* infection is most likely contracted by uninfected sheep at lambing time (Aitken I.D., 2000, Papp et al., 1994). Thus, in flocks with naturally protracted lambing season an abortion amongst the earliest lambing ewes can lead to *C. abortus* infection of cohort animals. During the hematogenous phases of the infection, the organisms invade the fetus older than 60 days of gestation (Aitken, 1993). Fetal infection subsequently proceeds independently of maternal immunity, leading in many cases to late-term abortion (Storz and QLOC VETM, 1971). A second phase of chlamydial generalized infection during the abortion process boosts complement-fixing antibody titers of the post-abortion (pa) peak. The latter can be used as an indication of chlamydial abortion (Perez-Martinez et al., 1986, Storz and QLOC VETM, 1971). Despite the importance of chlamydial abortion, some of the mechanisms of *C. abortus* infection leading to abortion, as well as the consequences of the systemic dissemination of *C. abortus* during abortion are poorly understood (Jones, 1995).

The present study was conducted as a prospective investigation after *C. abortus*-induced abortion had been diagnosed at the onset of lambing season in a flock of 49 milk sheep. This allowed repeated and extensive sampling while a natural chlamydial herd abortion episode unfolded. Virtually all ewes and live-born as well aborted lambs were infected with *C. abortus*. This indicated that other factors in addition to chlamydial infection determined the abortion outcome. The time point of chlamydial infection relative to gestation was the key determinant of abortion outcome of *C., abortus*

infection. Furthermore, chlamydial infection of the mammary gland in connection with abortion or parturition was found, as well as clinical signs of mastitis. Our results indicate that natural chlamydial infection of the ovine mammary gland resulted in subtle mastitis. However, anti-chlamydial immunity after abortion, but not after delivery of a live lamb, associated with protection from mastitis caused by *Streptococcus agalactiae* or *Staphylococcus aureus*.

MATERIALS AND METHODS

Experimental animals. The study was initiated when a case of chlamydial abortion had been diagnosed in early winter at the onset of the lambing season in a flock of 49 Swiss White Alpine ewes. The sheep were maintained in a single-stall closed barn and milked manually twice daily. The feed consisted of ad libitum hay and two daily rations of grain and compound feed supplemented with minerals. Lambs were weaned at 6 weeks of age. Ewes had a mean age of 3.3 (1-8) years (range) and 2.1 lactations (0-7). Replacement ewes were selected from offspring and raised on the farm.

Specimen collection. The early detection of the chlamydial abortion enabled a prospective study design. Beginning 35 days after the first chlamydial abortion, specimens were collected from the herd. Blood samples for serum were obtained by puncture of the jugular vein in 4-week intervals for 100 days. Tissue specimens were collected from all placentae that could be retrieved, and from livers of aborted lambs. Nasal cytobrush swabs were collected from live lambs, the brush was cut off, and stored

in 0.4 ml sterile saline in microcentrifuge tubes. Milk samples, combined from both halves of the mammary gland, were obtained on days 1, 3, 6, 14, and 40 - 60 post partum (pp) or post abortus (pa). One to 3 mammary gland tissue biopsies of each ewe were collected between day 1 and 100 pp/pa with a single-use biopsy device (Tru-Cut® Biopsy Needle, 18 Ga, Baxter Healthcare Corp., Valencia, CA, USA). Milk specimens were immediately used for determination of somatic cell counts (SCC) and plated out for standard bacterial culture. The remaining milk samples and all other specimens were stored at -20°C for ELISA or PCR analyses. All animal procedures were approved by an institutional animal experimentation control committee.

Serology. Sera were analyzed for antibodies against chlamydiae as described before by complement-fixation test (CFT), and by enzyme-immunoassay (EIA) using partially purified *C. abortus* strain B577 elementary bodies as antigen (Kaltenboeck et al., 1997). Causes of abortion other than chlamydiae were examined by analyses of all sera for the presence of antibodies against i) *Coxiella burnetti* by CFT (Field et al., 1983, Hunt et al., 1983), ii) *Toxoplasma gondii* by direct agglutination test (Desmonts and Remington, 1980, Fulton and Turk, 1959), iii) *Leptospira* spp. by microagglutination test (Faine, 1982), iv) Maedi-Visna virus by agarose gel immunodiffusion test (Kurstak et al., 1981), and v) Border Disease virus by serum neutralization test (Bolin et al., 1991). For examination of a *Mycoplasma agalactiae* etiology of mastitis, serum antibodies against *M. agalactiae* were determined by EIA (Schaeren and Nicolet, 1982).

Milk analyses. Milk SCC were determined by fluoro-opto-electronic cell counting by use of a Fossomatic FC (Foss A/S, Hillerød, Denmark) somatic cell counter (Schmidt-Madsen, 1975). All milk samples were analyzed by standard bacterial cultures (Dinsmore et al., 1992)

***Chlamydia* spp. *ompA* PCR.** *Chlamydia* spp. infection status was assessed in tissue and milk specimens by nested *Chlamydia omp*A PCR as described before (Kaltenbock et al., 1997). Briefly, sample DNA was extracted by the CTAB method, a segment of the chlamydial *ompA* gene was amplified with degenerate, *Chlamydia* genus-specific primers. Subsequently, a small aliquot of this PCR was re-amplified with nested internal *Chlamydia* genus-specific primers, and the reaction analyzed by agarose gel electrophoresis. The primary PCR of positive reactions was typed by a set of *Chlamydia* species-specific PCRs.

Statistical analysis. All statistical analyses were performed with the Statistica 7.0 software package (StatSoft, Inc., Tulsa, OK). The day when the first chlamydial abortion (diagnosed in fetus and placenta) occurred in the herd was considered the day of high-level bolus infection of the herd due to direct contact with fetus and secundinae. Based on day of infection and day of parturition or abortion, ewes were classified as infected late ($\geq$ 99 days) or early ($<$ 99 days) in gestation. To identify confounding factors, the data were also stratified for age and parity of the ewes, and for culture detection of extracellular bacteria in milk and PCR-detection of *Chlamydia* spp. in association with the mammary gland. SCC and CFT data were logarithmically transformed. CFT and

ELISA samples were classified in relation of sampling day to parturition/abortion date into four groups (10 weeks ante partum - 1 week pp [week -10 to +1], week 2 pp [week +2], week 6 pp [week +6], and week 10 pp [week +10]). For analysis of the kinetics of the *Chlamydia*-specific complement-fixing antibody response, the serial correlations of CFT titers were calculated by subtracting log-transformed CTF titers of consecutive time points. Serial correlation 1 (SC 1) is the difference between log CFT [week +2] and log CFT [week -10 to +1], similarly serial correlation 2 (SC 2) is the difference between log CFT [week +6] and log CFT [week +2]. Normal distribution of data was confirmed by Shapiro-Wilk's W test, and homogeneity of variances by Levene's test. Abortion timeline data were analyzed by logistic regression (Fig. 1). ELISA, CFT, and SCC data were evaluated by repeated measures analysis of variance (ANOVA), and comparisons of means under the assumption of no *a priori* hypothesis were performed by the Tukey honest significant differences (HSD) test (Figs. 2, 3). Non-normally distributed SCC data were examined by the more conservative and distribution independent non-parametric Mann-Whitney U-test (Figs. 4, 5), and frequency data were analyzed by Fisher's exact test (Tabs. I, II). Differences at $p \leq 0.05$ in all tests were considered significant.

RESULTS

Frequency of infection. Of the total 39 ewes retained for analysis, 17 (44%) aborted and 22 (56%) delivered clinically healthy lambs (Tab. I). However, as indicated by Table I, chlamydiae were found by nested *ompA* PCR in all specimens of placentae of all ewes, and in livers of aborted fetuses (except for one) as well as in nasal swabs of live lambs.

In all PCR analyses, only *C. abortus* was identified throughout the study. Evidence for an additional microbial cause of the abortions was not found in serological analyses. The incidence of chlamydial infection of the mammary gland during the first 3 weeks pa/pp, as determined by mammary gland biopsy, was significantly higher in abortion ewes (88%) than in live-lambing ewes (38%) (Tab. II). However, over the span of the total 48-day observation period, no significant difference was observed in chlamydial detection and prevalence of other mastitis-causing bacteria among abortion and live-lambing ewes (Tab. II). Thus, a single chlamydial abortion lead to chlamydial infection of entire flock, but the consequences of this infection differed for individual animals as evident in different pregnancy outcomes as well as in the early pa/pp colonization of the mammary gland by chlamydiae.

Table I. Abortion frequency, PCR detection of *Chlamydia*, and estimated time of bolus inoculation during gestation.

Abortion Status		Positive *Chlamydia* PCR		Time of Bolus Inoculation	
		Lamb	Placenta	Early < 99 days	Late ≥ 99 days
Live Lamb	22 (56%)	14/14	15/15	16 (41%)	6 (15%)*
Abortion	17 (44%)	9/10	10/10	3 (8%)	14 (36%)*
TOTAL	39 (100%)	23/24	25/25	19 (49%)	20 (51%)

* p=0.0011

Table II. Abortion status and PCR detection of *C. abortus* in mammary gland biopsy specimens and milk, and culture detection of *S. aureus* and *S. agalactiae* in milk.

Abortion Status	Mammary gland biopsy Day 1-21 *C. abortus* PCRs		All mammary gland & milk *C. abortus* PCRs		Total milk *S. aureus* & *S. agalactiae*	
	Positive[a]	Negative	Positive[b]	Negative	Positive[b]	Negative
Live Lamb	6 (38%)	10 (62%)	13 (59%)	9 (41%)	5 (24%)	16 (76%)
Abortion	7 (88%)	1 (12%)	11 (65%)	6 (35%)	3 (19%)	13 (81%)

[a] p=0.034, difference between live-lambing and abortion ewes

[b] p<0.05, difference between live-lambing and abortion ewes

Relationship between time of infection and pregnancy outcome. Based on the PCR analyses, all animals were *Chlamydia*-infected, and other factors interacted with the infection to determine the outcome abortion or delivery of a live lamb. Typically, sporadic chlamydial abortions occur in young ewes during the first gestation period, but not in older multiparous ewes (McCauley and Tieken, 1968, McKercher et al., 1966, Young et al., 1958). In contrast, in this investigation mean age and parity of both abortion and live-lambing ewes did not differ (3.3 years; 2.3 parities), and we did not find any age and parity predisposition for abortion. However, it was obvious that abortions occurred primarily in the early phase of the lambing season. Since chlamydial abortions typically occur at or near term and all aborted fetuses in this investigation were fully developed on visual inspection, we assumed a gestation time of 150 days for all ewes. Under this assumption, 14 of the 17 abortion ewes (82%) were infected late in pregnancy at a time of 99 or more gestation days (Tab. I), estimated by the time between first chlamydial abortion in the herd and the abortion dates of the ewes. To further analyze the effect of time of chlamydial infection during gestation, the data was examined by the logistic regression procedure for the probability of live lambing or abortion as predicted by the time point of bolus inoculation relative to gestation. The logistic regression model for this data predicts with very high probability ($p<10^{-4}$) an abortion if the chlamydial infection occurred during the last third of pregnancy (Fig. 1).

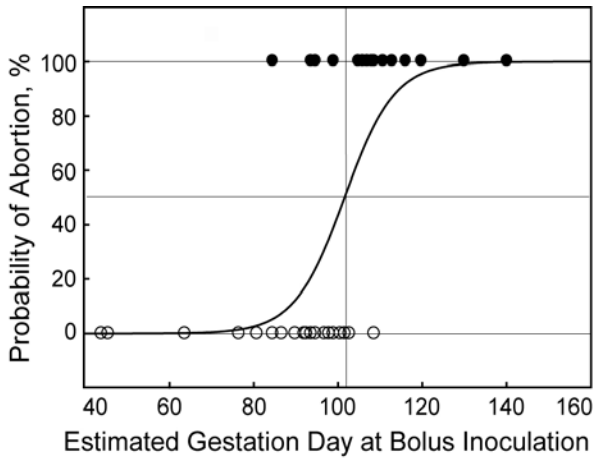

Figure 1. Logistic regression model predicting pregnancy outcome as a function of time of infection. The logistic regression model predicts very high probability of abortion if the *C. abortus* bolus infection of a pregnant ewe occurs in the last third of the 150-day pregnancy, with a 50% probability of abortion if infected on day 101 of gestation ($p<10^{-4}$).

Serum antibody responses in live lambing and abortion ewes. Since virtually all ewes were infected (Tab. 1) and time of infection rather than presence of chlamydial infection (Fig. 1) was the predictor of pregnancy outcome, we sought to identify immune correlates for the outcome by evaluating the differences in immune responses between abortion and live lambing ewes. The *Chlamydia* immune status of the herd prior to the bolus infection of the flock by the first chlamydial abortion is unknown. Two weeks prior to abortion/partus, all ewes had antibody responses against *Chlamydia* spp. detectable by ELISA and CFT, and responses of ewes that subsequently aborted were not different from ewes that delivered live lambs (Fig. 2A and 2B). As expected, the subsequent antibody response as measured by ELISA showed a trend of increase in both abortion and live lambing ewes, but a significant increase only in abortion ewes between day -14 and day 72, suggestive of strong humoral immune response by abortion ewes (Fig. 2A)**.** In contrast CFT titers differed markedly in both amplitude and trend over time between abortion and live lambing ewes. Between day -14 and day 14 of parturion/abortion the CFT titers tended to be higher in abortion ewes but lower in live lambing ewes. Following abortion there was a highly significant decrease in CFT titer in abortion ewes between day 14 and day 42 ($p<0.01$; Fig. 2B) whereas CFT titers remained largely constant during this period in live lambing ewes. After day 42, CFT titers in all ewes trended higher, but not significantly so. The behavior of CFT titers can be better visualized as serial correlation between consecutive time points (Fig. 2C). The increase of complement fixing antibodies against *C. abortus* from pre-abortion (day -14) to early pa (day 14) as compared to the decrease from pre-parturition to early pp is highly significantly evident in the 4-fold increased CFT serial correlation 1 ($p<0.01$; CFT-SC1;

Fig. 2C). Conversely, the subsequent rapid pa decrease between days 14 and 42 versus the largely constant CFT titers in live-lambing ewes is highly significantly evident in the 4-fold lower CFT serial correlation 2 of abortion ewes ($p<0.01$; CFT-SC2; Fig. 2C).

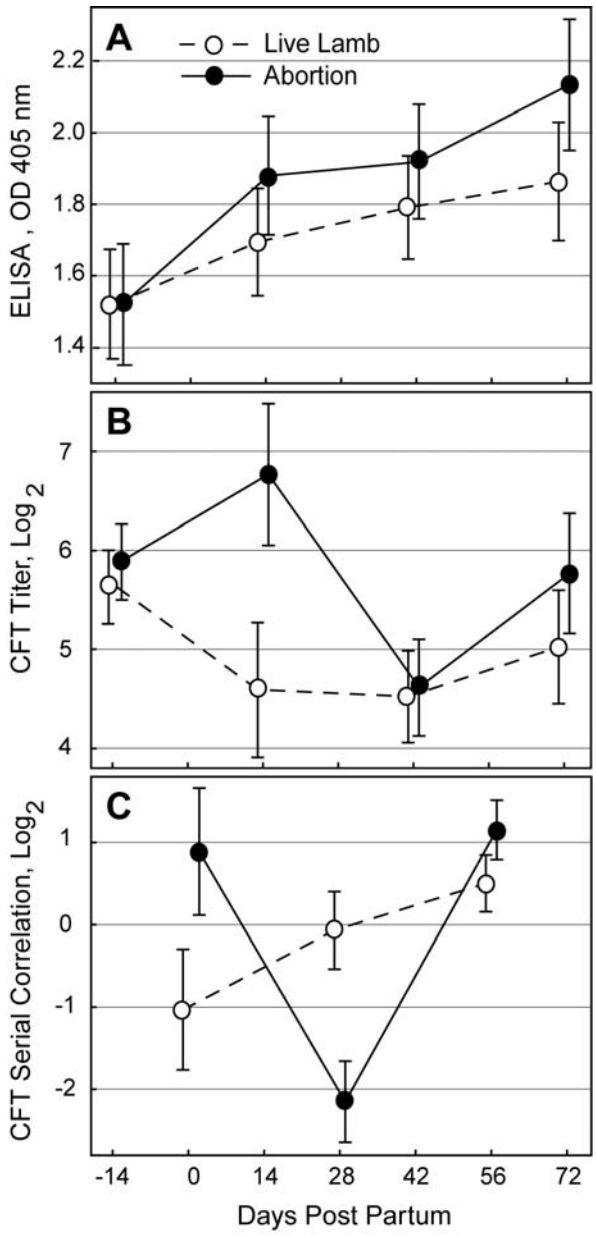

Figure 2. Anti-*C. abortus* antibody responses of ewes. Data are shown as means ±SEM and were analyzed by repeated measures ANOVA and the Tukey HSD test. A: ELISA titers increased significantly between day -14 and day 72 in abortion ewes ($p<0.001$), but not in live-lambing ewes. B: CFT titers tended to decrease in lambing ewes between day -14 and day 14, but to increase in abortion ewes, and then dropped highly significantly in abortion ewes between day 14 and day 42 ($p<0.01$). C: The serial correlation of CFT

between day -14 and day 14 (CFT-SC1) was highly significantly higher, and between day 14 and day 42 (CFT-SC2) highly significantly lower in abortion sheep than in live-lambing sheep ($p<0.01$).

Factors affecting milk somatic cell counts of the whole flock. During the initial phase of the study we found clear evidence of pa/pp mastitis such as occasional clots in milk and milk somatic cell counts several-fold higher than 100,000 cells per ml, indicating inflammation of the mammary gland. This prompted a detailed examination of the factors influencing SCC, and consequently the health of the mammary gland. Serology excluded *M. agalactiae* as cause of mastitis.

Effect of abortion. As shown in Figure 3A**,** abortion ewes, but not live-lambing ewes, showed a highly significant rapid decrease in SCC between day 1 and day 6 pa/pp to 100,000 cells per ml or less ($p<0.0001$). Subsequently SCC did not differ significantly between abortion and live-lambing ewes (Fig. 3A).

Effect of mammary gland infection by extracellular bacteria. Ewes negative for extracellular bacteria showed a significant decrease in SCC within two weeks pp from more than 10^6 cells/ml to approximately 10^5 cells/ml ($p=0.002$; Fig. 3B). In contrast, ewes positive for extracellular bacteria showed only a marginally significant reduction in SCC two weeks pa/pp ($p=0.03$; Fig. 3B). In all ewes, SCC on day 48 trended again higher such that they were not significantly different from earlier SCC numbers.

Effect of mammary gland infection by C. abortus. Ewes positive for any association of chlamydial infection with the mammary gland (*C. abortus* DNA in mammary gland biopsy or milk) showed a significant reduction in SCC within one week pp ($p<0.0002$; Fig. 3C), and SCC remained at approximately 100,000 cells/ml for two weeks pp ($p<0.0002$). Mammary gland *Chlamydia*-negative ewes also showed significant reduction in SCC, but only at 14 days pp ($p=0.03$; Fig. 3C). Thus *Chlamydia*-positive as well as -negative ewes largely trended in a similar fashion towards decline in

SCC over the period of two weeks pp followed by a trend towards increase in SCC for the remaining observation period. However, *Chlamydia*-positive ewes showed a rapid and significant decline in SCC within the first week while *Chlamydia*-negative ones did not.

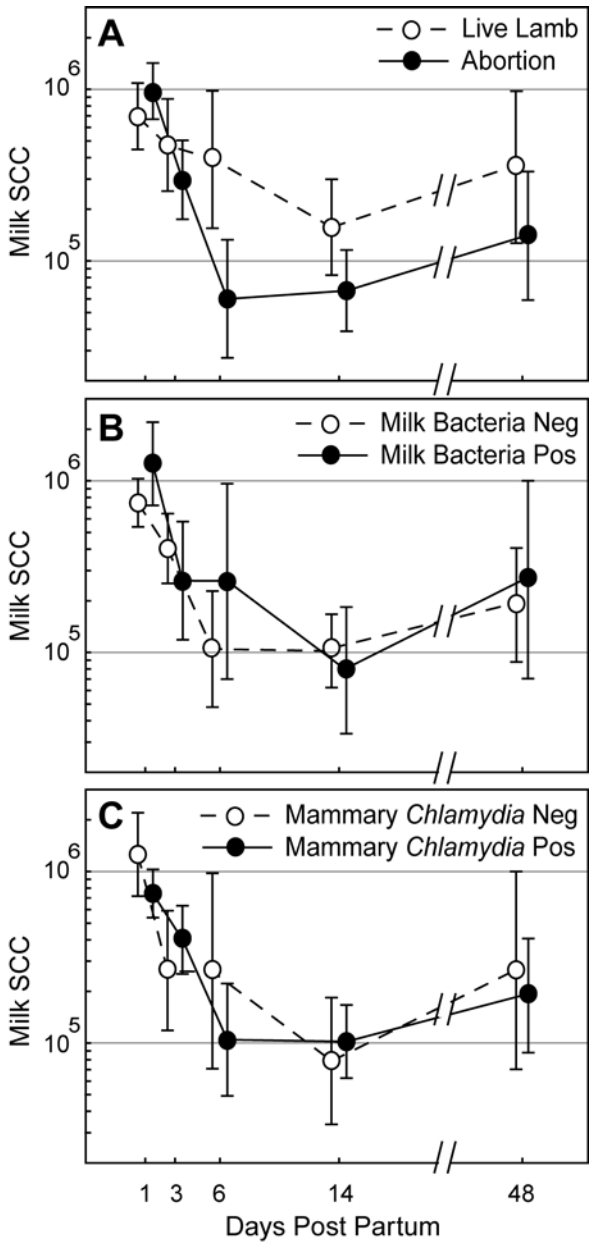

Figure 3. Effect of abortion and concurrent *Staphylococcus aureus*/*Streptococcus agalactiae* or *C. abortus* infection on milk SCC of ewes. Data are shown as antilog of mean log SCC ± SEM and were analyzed by repeated measures ANOVA and the Tukey HSD test. A: From day 1 to day 6, SCC in abortion ewes decreased rapidly to reach physiological values below 100,000 cells/ml milk (p<0.0001), but not in live-lambing ewes. Over the 48-day observation period, milk SCC tend to be lower in abortion sheep

than in live-lambing sheep but the difference does not reach statistical significance (p=0.056). B: From day 1 to days 6 and 14, SCC decreased highly significantly in ewes that had no concurrent bacterial infection (p=0.002). In ewes with bacterial infection, SCC decreased only between day 1 and day 14 (p=0.03). C: Mammary gland *Chlamydia*-positive sheep showed a significant and rapid decrease in SCC from day 1 to days 6 and 14 (p<0.002).

The above results indicate an influence of lambing status on SCC, with abortion resulting in pronounced reduction of SCC as compared to normal lambing. Contrary to expectations, the presence of extracellular bacteria as well as of *C. abortus* in the mammary gland do not influence SCC when analyzed on the whole flock level. To dissect the interaction between extracellular bacterial infection with chlamydial detection in the mammary gland and abortion, ewes were stratified for presence or absence of extracellular bacteria in milk, and these strata were analyzed separately.

Factors influencing milk somatic cell counts in absence or presence of extracellular bacteria in milk

Chlamydial infection of the mammary gland. Figure 4A shows that in the absence of other extracellular bacterial infections the effect of chlamydial mammary gland infection on udder health and milk quality is much more evident. Ewes positive in the *C. abortus* PCR of a mammary gland biopsy collected during the 3-week period pa/pp showed significantly higher SCC on day 1 (Fig. 4A). This trend continued on days 3 and 6 with higher median SCC in *Chlamydia*-positive ewes, but the low number of animals prevented conclusive statistical analysis. In the presence of *S. agalactiae* and *S. aureus* infection, the overall SCC were higher than in ewes without extracelluar bacterial infection , but chlamydial infection of the mammary gland did not show an influence on SCC (Fig. 4B).

Pregnancy outcome. Abortion as well as live lambing ewes with sterile milk showed no significant differences in SCC pa/pp (Fig. 5A). Both abortion and live lambing ewes tended towards lower SCC within the first week pa/pp and showed median

values of 100,000 cells per ml of milk or less on days 6 and 14 pa/pp. In the presence of extracellular bacterial infection, SCC were significantly lower in abortion ewes on days 3 and 14 pa as compared to live lambing ewes (Fig. 5B). This effect was also evident on days 1 and 6, but did not reach significance due to the limited number of animals available for analyses.

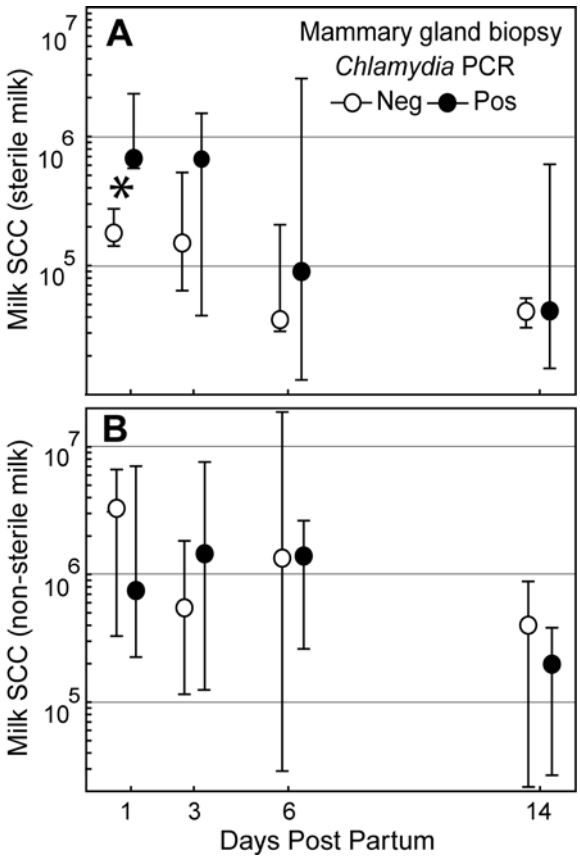

Figure 4. Effect of chlamydial infection of the mammary gland on milk SCC in the first two weeks of lactation. *C. abortus* was detected by PCR analysis of biopsies obtained within 3 weeks after abortion/parturition. Median data are shown, error bars indicate minimum/maximum, and the asterisk indicates significant differences. A: Ewes without *S. agalactiae* or *S. aureus* infection (sterile milk): milk SCC were significantly higher on day 1 in *C. abortus*-positive ewes than in ewes without chlamydial infection of the mammary gland (p=0.02; Mann-Whitney U test; *C. abortus*-positive ewes n = 7; *C. abortus*-negative ewes n = 3). B: Ewes with *S. agalactiae* or *S. aureus* infection (non-sterile milk): no significant differences in milk SCC of *C. abortus*-positive and negative ewes were observed, but SCC in both groups were higher than in ewes without extracellular bacterial infection.

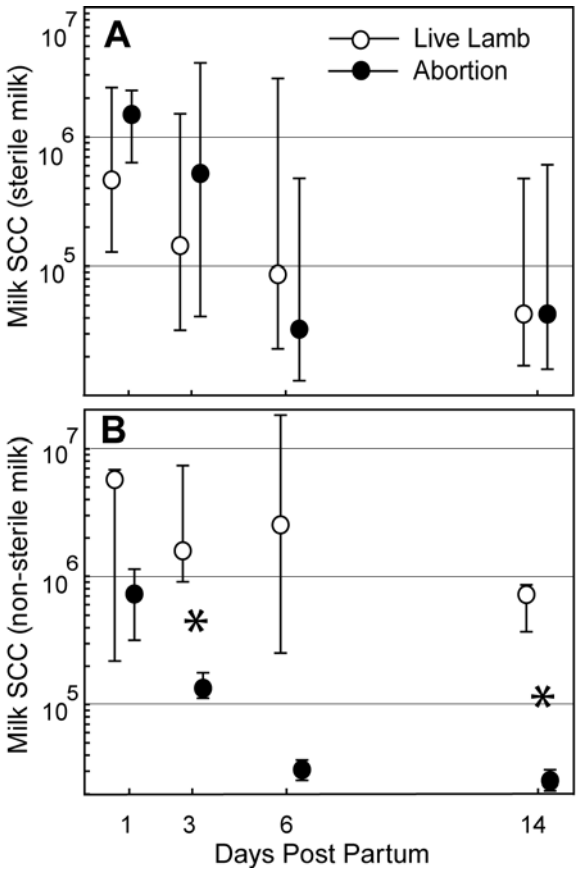

Figure 5. Effect of abortion on milk SCC in the first two weeks of lactation. Median data are shown, error bars indicate minimum/maximum, and the asterisk indicates significant differences. A: Ewes without *S. agalactiae* or *S. aureus* infection (sterile milk): milk SCC did not differ between abortion and live-lambing ewes. B: Ewes with *S. agalactiae* or *S. aureus* infection (non-sterile milk): abortion ewes had significantly lower milk SCC on day 3 and day 14 than live-lambing ewes positive for *S. agalactiae* or *S. aureus* in milk ($p \leq 0.045$; Mann-Whitney U test; abortion ewes n = 4; live lambing ewes n = 3).

DISCUSSION

Chlamydia abortus is the most common abortion agent in sheep, but gaps in our knowledge on these infections still exist, and functional consequences of these infections after abortion have rarely been analyzed. The present study was conducted after *C. abortus*-induced abortion had been diagnosed in a flock of 49 milk sheep in a dead lamb delivered at the onset of lambing season. This incidental finding of chlamydial abortion provided a rare opportunity to study the disease progression of ovine chlamydial abortion in a natural setting by repeated and extensive sampling. In addition, pa/pp chlamydial infection of the ovine mammary gland was identified. This allowed the use of milk somatic cell counts as sensitive indicator of the functional inflammatory consequences of these infections, and its association with the immune response following extensive exposure to *C. abortus*.

An immediately surprising finding was the very high abortion rate of 44% without any age or parity predisposition. In flocks experiencing *C. abortus* infection for the first time, typically up to 30% of pregnant ewes abort, primarily young animals, although sheep of all ages are susceptible to infection (Rodolakis et al., 1998, Shewen, 1980, Young et al., 1958)}. As the infection becomes enzootic, between 5-10% of largely primiparous ewes abort annually (McCauley and Tieken, 1968, McKercher et al., 1966). The *Chlamydia* immune status of the flock before the abortion episode remains unknown because samples of the herd collected prior to the initial abortion were not available. However, the high percentage of abortions without age/parity predilection strongly suggests a first exposure of the herd to *C. abortus*.

Detection of *C. abortus* in virtually all ewes and lambs of the herd irrespective of the abortion status indicated that factors in addition to chlamydial infection determined the abortion outcome. Age, parity, and pre-abortion/parturition anti-chlamydial antibody responses did not differ between ewes that lambed or aborted, thus were not determinants of pregnancy outcome. Identification of the initial chlamydial abortion case allowed precise timing of the bolus infection of each ewe relative to gestation time. This analysis showed unambiguously that the likelihood of abortion was very high if *C. abortus* bolus inoculation occurred in the last third of pregnancy. A possible explanation for this observation may be placental dynamics and fetal immune response during ovine gestation. The synepitheliochorial placenta in sheep is characterized by the absence of fusion between maternal uterine epithelium and the fetal chorionic membrane, with placental structures called placentomes serving as exclusive sites of limited infiltration by maternal blood vessels (Wooding and Flint, 1984). At around 60 days of gestation, maternal haematomas begin to develop at the maternal-fetal interface in the hilus of each placentome. These haematomas allow direct contact between maternal blood and fetal trophoblast, and thus provide the first opportunity for placental infiltration by circulating *C. abortus* (Buxton et al., 1990). In experimental inoculations, the earliest placental lesions can be observed at 90 days of gestation, in the hilus of the placentome where *C. abortus* can be demonstrated within chorionic epithelial cells (Aitken I.D. , 2007, Buxton et al., 1990). Simultaneously, antichlamydial maternal inflammatory cytokines such as interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) are down regulated at the trophoblast junction to avoid rejection of the fetus by the maternal immune system (Entrican, 2002). These factors potentially enhance chlamydial replication and invasion

of the placenta and fetus during a time when the fetus becomes increasingly immunocompetent after approximately 80 days of gestation (Buxton et al., 2002, Nettleton and Entrican, 1995) and responds with increased inflammation to *C. abortus*. The pathogenesis of OEA has been linked to the ability of the ewe to control the levels of infectious EBs in blood, suggesting that both timing and dose of placental infection are important factors (Sammin et al., 2005, Sammin et al., 2006). These data are consistent with the findings in this study. Collectively, data of this and other investigations suggest that the susceptibility of ewes to chlamydial abortion increases with gestation time due to enhanced chlamydial replication and lesions at the maternal-fetal interface, enhanced access of chlamydiae to the fetus, as well as increased fetal immunopathology in response to the chlamydial infection. Overall, this convergence of susceptibility factors results in a sharp temporal delineation between relative resistance and relative susceptibility to chlamydial abortion in dependence of ovine gestation phase.

In addition to abortion, chlamydiae may also cause mastitis in ruminants, and thus add to losses by potentially reducing quality and quantity of milk. For instance, Papadopoulos and Leontides (1972), Gupta (August 1988), and Corner (Corner et al., 1968), Mecklinger (Mecklinger et al., 1980), and Ronsholt (Rønsholt and Basse, 1981) experimentally produced mastitis in ewes, does, and cows, respectively, by intramammary inoculation of chlamydiae. While these studies examined localized chlamydial infection of the mammary gland, they did not investigate mammary gland inflammation or did not find culture evidence for chlamydial colonization following natural chlamydial abortion or experimental parenteral (but not via teat canal) chlamydial inoculation (Jones and Anderson, 1989). During the initial phase of our study we found

clear evidence of pa/pp clinical mastitis such as occasional milk clots and detected chlamydial DNA in milk, but even more frequently in (88%) mammary gland biopsies of abortion ewes (Tab. II). These data clearly establish the potential of chlamydiae to colonize the mammary gland after infection of the genital tract. Unexpectedly, the infection of all ewes and lambs with *Chlamydia*, irrespective of pregnancy outcome (Tab. I), confounded the analysis of the effect of *Chlamydia* on the mammary gland because *Chlamydia*-free controls were unavailable. Further confounding were differences in immune responses to the chlamydial infection after abortion and normal lambing, and infections of the mammary gland by extracellular mastitis bacterial pathogens.

We quantified the pathogenic potential of the chlamydial infection by enumerating SCC, a direct correlate of the inflammatory status of the mammary gland. Overall SCC in ewes are thought to be higher than in cows (Gonzalo et al., 1994) but the discrimination between healthy udders, subclinical mastitis, and clinical mastitis based on SCC in ewes is not well established (Fthenakis et al., 1991, González-Rodríguez and Cármenes, 1996, Green, 1984, Las Heras et al., 1999). We used the widely accepted value of 100,000 cells/ml as the physiological limit for a healthy ewe. Analysis of the whole-flock data revealed that SCC in abortion ewes dropped below this physiological limit within one week pa, while live lambing ewes remained above this limit during the entire pp observation period (Fig. 3A), thus showed signs of subclinical mastitis. This suggests that the nature of the maternal adaptive anti-chlamydial immunity generated in response to abortion, but not to live lambing (Fig. 2), protected against chlamydial mastitis, akin to vaccination against *Chlamydia* that reduced SCC of dairy cows to physiological levels (Biesenkamp-Uhe et al., 2007).

Surprisingly, neither mammary gland presence nor absence of extracellular bacterial mastitis agents or of chlamydiae resulted in SCC differences when analyzed on the whole herd level (Fig. 3B and 3C). Nevertheless, important inferences were possible by stratifying ewes for the presence or absence of extracellular mastitis pathogens in milk, and by analyzing these strata in dependence of chlamydial dissemination, indicated by presence or absence of chlamydiae in early pa/pp mammary gland biopsies, and of anti-chlamydial immunity, indicated by abortion or live lambing. The presence of chlamydiae in otherwise sterile mammary glands resulted in significant early inflammation that subsided by 2 weeks after abortion/parturition (Fig. 4A). In bacterially-infected mammary glands SCC were much higher, but a discernible influence of chlamydiae was not evident (Fig. 4B). Pregnancy outcome did not associate with differential SCC in sterile mammary glands (Fig. 5A). In sharp contrast, in ewes with bacterially-infected mammary glands, abortion associated with significantly lower SCC during the first two weeks of lactation than in live lambing ewes (Fig. 5B). Thus, in the presence of *C. abortus* herd infection, abortion or its correlated differential anti-chlamydial immune response strongly protected against streptococcal and staphylococcal mastitis.

Chapter 3: Health Effect of Natural *Chlamydia* spp. Infection on the Bovine Mammary Gland

INTRODUCTION

Exposure to infection with obligate intracellular *Chlamydia* spp. bacteria is probably ubiquitous in cattle worldwide, with high seroprevalence rates approaching 100% in some investigations (Cavirani et al., 2001, Kaltenboeck et al., 1997, Wang et al., 2001). Two *Chlamydia* species are routinely detected in cattle, *C. abortus* and *C. pecorum* (Everett et al., 1999, Schachter et al., 1975). Acute infections with these bacteria have been associated with numerous distinct clinical disease entities in cattle, most prominently abortion and fertility disorders, sporadic encephalomyelitis, kerato-conjunctivitis, pneumonia, enteritis, and polyarthritis (Bowen et al., 1978, DeGraves et al., 2004, Griffiths et al., 1995, McKercher et al., 1966, McNutt and Waller, 1940, Otter et al., 2003, Storz et al., 1968, Twomey et al., 2003, White, 1965, Wilson and Thomson, 1968, Wittenbrink et al., 1993). However, the vast majority of *Chlamydia* spp. infections in cattle, particularly of low-level infections frequently detected after introduction of sensitive PCR methods, are not associated with obvious clinical disease (DeGraves et al., 2003a, Jee et al., 2004). A well balanced host-parasite relationship appears to represent

the common nature of chlamydial infection (Shewen, 1980). Thus, while it is clear that high-dose experimental inoculations and natural infections with *Chlamydia* spp. result in defined disease manifestations, the health impact of the ubiquitous subclinical infections remains unknown.

Mastitis, the inflammation of the mammary gland, is the most prevalent production disease in dairy cows and is among the livestock diseases that cause the greatest economic losses in animal agriculture (Seegers et al., 2003). In the United States, mastitis is estimated to cause an annual loss approaching 2 billion dollars (Schroeder, 1997). Losses are mainly due to reduction in milk quantity and in quality. Classically, infections with bacteria such as *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Escherichia coli* have been the main cause of bovine mastitis (Schukken et al., 2003). Intensive husbandry practices have been associated with an increased incidence of mastitis caused by atypical bacterial agents such as *Streptococcus dysgalactiae* and *Mycoplasma bovis* (Pfützner and Sachse, 1996, Schukken et al., 2003). Despite decades of intensive research in bovine mastitis and extensive prophylactic and therapeutic measures, bovine mastitis remains a major problem in the dairy industry, and causal agents remain undiagnosed in a large proportion of cases ("sterile mastitis").

Experimental inoculation of *C. abortus* via the teat canal produces a severe acute mastitis of the inoculated mammary glands accompanied by fever and anorexia (Corner et al., 1968, Mecklinger et al., 1980, Rønsholt and Basse, 1981). After initial fibrinous and serous secretion and pronounced swelling of the udder in the first week, the disease appears self-limiting, leading to a state of reduced milk production and mammary gland

atrophy. *C. abortus* has also sporadically been associated with naturally occurring bovine mastitis (Kaltenboeck et al., 1992, McKercher et al., 1966, Wehnert et al., 1980).

DeGraves et al (2004) demonstrated that subclinical chlamydial infections may potentially have a profound negative effect on bovine fertility, and that these losses may be reduced with appropriate herd management practices. Jee et al. (2004) detected *C. abortus* in the milk of 15% of dams without any signs of disease. One-hundred microliter milk samples from a single udder quarter were tested per week for 12 weeks post partum. Thus sampling intensity was low, and higher prevalence of *Chlamydia* spp. in milk may be detected with higher sampling intensity. Nevertheless, these results indicate that low-level natural infection of the bovine mammary gland with *Chlamydia* spp. most likely is common.

Bovine mastitis has been intensely studied for a long time, and numerous parameters have been established for routine monitoring of udder health (Erskine, 2001, Green et al., 2004, Pyorala, 2003). Uniformly accepted is the number of somatic cells in milk. Milk from a healthy bovine mammary gland contains less than 100,000 somatic cells per milliliter, and there is consensus that more than 10^5 SCC per ml bovine milk indicate inflammation of the mammary gland. Milk with 100,000 or more cells per ml is of reduced value because manufacturing properties are compromised, particularly for cheese production (Dohoo and Leslie, 1991, Schukken et al., 2003). Clinically manifest mastitis is typically associated with SCC above 10^6 (Green et al., 2004). Milk SCCs of individual dairy cows are routinely monitored as one of several determinants of raw milk quality and cost. This well established parameter for continuous, non-invasive monitoring of inflammation of the mammary gland offers an intriguing potential for the

study of the effects of clinically inapparent chlamydial infections. Even at low levels of SCC, i.e., just above 50,000 cells/ml, milk composition is changed in separate udder quarters (Tolle et al., 1971). Interestingly, it has been observed that in cow composite milk samples with SCC, 100 000 cells/ml, more than 10% included individual udder quarters with a California Mastitis Test (CMT) score >3; 50% of these quarters were infected with bacteria (Berglund et al., 2004). Continuous simultaneous detection of chlamydial infection and inflammatory status of the mammary gland by PCR and SCC, respectively, allow for long-term assessment of the impact of chlamydial infection on the health of an isolated organ. This is important not only for cattle, but also for the understanding of human chronic inflammatory diseases such as pelvic inflammatory disease and reactive arthritis, or coronary heart disease, for which a strong association with *C. trachomatis* and *C. pneumoniae* infection, respectively, has been found (Danesh et al., 1997, Saikku et al., 1988, Stephens, 1999).

In the post-partum period of highest milk production by dairy cows, many physiological changes occur, such as uterine involution and reconstitution of the non-pregnant immune status. These changes may also affect susceptibility to subclinical systemic or localized disease. The present study was conducted as an observational study to investigate in detail the magnitude, type, kinetics, and cyclical characteristics of the health effects of low-level *Chlamydia* infections on bovine milk and serum parameters. In this observational study, we aimed at studying the yield and composition of milk at the udder quarter level in early lactation (from week 2 to 22 post-calving), to determine the incidence of systemic and localized chlamydial infection, and the changes in milk quality and quantity associated with these infections. It is well known that milk composition has

daily variation hence repeated sampling at the same time of the day was done to minimize the effect of daily variation. Quantitative data for both exposure and outcome measures from each mammary gland quarter allowed for multiple stratification strategies in statistical analysis. The results indicate a profound impact of endemic chlamydial infection of dairy cows on milk production, surprisingly caused largely by systemic effects of chlamydial infections outside the mammary gland.

MATERIALS AND METHODS

Dairy herd. The study was performed in a 140-head dairy herd of 75% Holstein and 25% Jersey cows at the EV Smith Dairy Unit of the Alabama Agricultural Experiment Station in Shorter, AL, USA. Animals were housed in free-stall housing with mattresses, fed a total mixed ration on corn silage base, and spent a minimum of 6 hours per day in pasture. Cows were milked twice daily in double-four herring bone milking parlors with Westfalia Surge milkers using standard hygiene and teat-dipping practices. Lactating cows had mean age of 41 months and comprised 89% (125/140) of the total herd. The average daily milk yield of lactating cows was 53 lbs containing 3.2% protein and 4.0% fat. Mean milk SCC of the herd on test days ranged from 1.9×10^5/ml to 4.6×10^5/ml with an average of 3.3×10^5/ml (Tab. I). Routine herd health monitoring for bovine pathogens and herd health maintenance procedures were provided by the Auburn University Large Animal Clinic. All animal procedures were approved by Auburn University's Institutional Animal Care and Use Committee (IUCAC).

Table I. Summary of annual production data of the study herd.

Test day production[a]	Mean (min, max)		
No. of cows	140 (131, 151)		
No. of cows in milk	125 (103, 146)		
Percent cows in milk	89 (77, 98)		
Milk yield, lb.	53 (34.9, 66.4)		
Fat percent in milk	4.0 (3.5, 4.4)		
Protein percent in milk	3.2 (3.1, 3.3)		
Days in milk	182 (127, 261)		
Actual SCC (1000)	327 (193, 463)		
Lactation comparison	**Lactation**		
	1st	**2nd**	**3rd +**
Number of cows	58	40	49
Age (months)	25	38	62
Days to first service	77	78	74

[a] Data are compiled for the year preceding the last sample collection from study animals.

Experimental design. The investigation was designed as a prospective cohort observational study that examined in detail the effect of natural infection with *Chlamydia* spp. on milk quality and health of the mammary gland in dairy cows, thus as characterization of subclinical chlamydial mastitis. Cows were continuously enrolled in the study. Beginning 2 weeks post-calving until week 20 of lactation, individual animals were sampled in a 2-week interval. In total, 17 Holstein cows were sampled over the study duration of 40 weeks.

Specimens. At each sampling time point, the following samples were collected from each cow:

1) Milk samples from each mammary gland quarter were collected for total nucleic acid extraction and for bacterial culture. Milk samples were obtained before milking to avoid any influence of disinfectants and detergents used during wash cycle. Single service paper towels were used to clean and dry the udder, followed by thorough disinfection with 70% ethanol. After discarding the first few beams of milk, approximately 10 ml milk from each quarter was collected aseptically into a 15 ml sterile tube and kept on ice until further processing in laboratory:

    a) For nucleic acid extraction, 600 µl of the aseptically collected milk was mixed with 600 µl 6 M guanidine-HCL, 10 mM urea, 10 mM Tris-HCl, 20% Trition X-100 (v/v), pH 4.4 in a 2.0 ml microcentrifuge tube with screw cap and stored at -80ºC.

    b) For standard bacterial culture, collected milk samples were processed immediately.

2) After initial sampling, the cows were milked, and milk meters connected with each of the mammary gland quarters quantified milk yield per quarter and collected individual representative quarter milk samples for determination of milk SCC, protein, and fat. For this purpose, four Tru-test WB Ezi-test milk meters were assembled into a single unit that was attached to a metal bracket and then mounted on a rolling tripod (Fig. 1). The WB Ezi-Test Milk Meter is a general purpose compact, ICAR (International Committee for Animal Recording) approved meter with a built-in sampling valve that diverts a portion of the milk flow. Milking cups were connected via a quarter claw and independent hoses to milk meters, allowing milk yield measurement and milk sample collection for each quarter (Fig. 1). The quarter milk sampler was designed to use the vacuum and pulsator from the milking parlor and allow the flow of milk back into the bulk tank. The quarter milk sampling unit was cleaned in place during the wash cycle of milking parlor before and after each sampling. Milk samples from each quarter were collected in DHIA-supplied collection tubes containing 1 g of potassium dichromate as preservative.

Figure 1. Quarter milk sampler designed to utilize the vacuum and pulsator from the m0ilk parlor. A. Four Tru-test meters (red arrows) attached to a metal bracket and mounted on a rolling tripod. The unit was designed to facilitate the flow of milk back into the bulk tank (blue arrows), following the by-pass of the measurement/sampling valves. B. Close-up view of the sampling meters attached to the mammary gland via the quarter claw (red arrows). A milk sample for analog yield estimate and analysis is continuously collected in the meter column and can be seen in the Figure (blue arrows).

3) Vaginal swab samples were collected by 10 sec rotation of the cytobrush (Puritan®, Hardwood Products Company LP, Guilford, ME, USA), the cytobrush handle was clipped, and the swab was immediately transferred into 400 µl RNA/DNA Stabilization Reagent for Blood/Bone Marrow (Roche Applied Science, Indianapolis, IN, USA) in a 1.5 ml microcentrifuge tube with screw cap. Swab samples were centrifuged at 250×g for 1 min and stored at -80 ºC without the cytobrush.

4) Blood was collected from coccygeal blood vessels of the cows with a 10 ml blood collection tube (13x100mm Vacutainer® Brand Tubes with Hemogard™ Closure, Becton Dickinson and Company, Franklin Lakes, NJ, USA). Serum was separated by centrifugation at 1300×g for 15 min, and stored in 2 ml microcentrifuge tubes with screw cap at -80 ºC. In the vaccine study blood samples were collected only once in 4weeks for 5 months.

Mammary gland biopsies - For collection of mammary gland biopsies, cows were sedated by intravenous administration of Xylazine. For the biopsy procedure, the mammary gland was disinfected and biopsied with Tru-Cut® biopsy needles (Becton Dickinson and Company, Franklin Lakes, NJ, USA).

Milk analyses. Each quarter milk sample was examined for standard mastitis pathogens such as *Streptococcus agalactiae, Staphylococcus aureus*, or *Escherichia coli* by augmented culture techniques on Macconkey's lactose agar (MLA) and 5% sheep blood agar plates. After streaking, the plates were incubated at 37°C under microaerophilic

conditions and observed for growth for 72 hrs. All distinct colony types from positive cultures were submitted for precise species identification to the Diagnostic Bacteriology Laboratory at the Department of Pathobiology, College of Veterinary Medicine, Auburn University.

Samples for SCC and chemical analyses were sent to the regional DHIA laboratory, at Baton Rouge, Louisiana. Somatic cell counts were determined by laser-based flow cytometry of ethidium bromide-stained DNA of nuclei of somatic cells (National MastitisCouncil, 2004). Milk protein and fat were determined by mid infrared (2-15 μm wavelength) absorption spectroscopy.

DNA extraction. Nucleic acid for PCR was isolated from quarter milk and vaginal cytobrush samples using the High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. The brush tip was removed from and 40 μl of Proteinase K (20 mg/ml in ddH_2O) added to the cytobrush sample, or 120 μl of Proteinase K was added to quarter milk samples. Both were then incubated at 600 rpm shaking at 72ºC for 30 min (Eppendorf Thermomixer). The milk sample then received three hundred microliters isopropanol and 300 μl chloroform (to extract fat); to the cytobrush sample 100 μl of isopropanol were added. After vortexing briefly, the aqueous sample solutions, but not the lipophilic chloroform phases of the milk samples, were transferred to the glass fiber filter device for DNA-binding. Samples were centrifuged at 3000×g for 3 min. Five-hundred microliters of inhibitor removal buffer were added and samples filtered again at 3000×g for 1 min, followed by two washes with 500 μl wash buffer. Centrifugation at 14,000×g for 10 sec

removed traces of wash buffer. Each sample filter was inserted into a fresh collection tube and 20 µl elution buffer (10 mM Tris-HCl, pH 8.4, 0.01 mM EDTA) pre-warmed to 72ºC was added. The filters were incubated at 600 rpm shaking at 72ºC for 2 min; centrifugation at 14,000×g for 1 min recovered the elution buffer. A second elution step was performed and the typical total amount of recovered specimen was 35 µl per sample. Samples were stored at -80ºC.

Real-time polymerase chain reaction (PCR). For detection and quantification of chlamydiae, the *Chlamydia* 23S rRNA gene was used as the target gene in Fluorescence Resonance Energy Transfer (FRET) real-time PCR (qPCR) on the LightCycler® platform (Roche Applied Science, Indianapolis, IN, USA). Melting curve analysis was used to differentiate *Chlamydia* spp. as described before (DeGraves et al., 2003a, DeGraves et al., 2003b). Samples were analyzed in duplicate in a glass capillary tube with 5 µl of extracted specimen DNA and 15 µl of master reaction mixture. Quantitative standards used were 10^4, 10^3, 10^2, 10 copies of *C. abortus* B577 and 10 copies of *C. pecorum* DNA extracted from purified elementary body preparations by the High Pure method, and quantified by the PicoGreen® DNA fluorescence assay (Molecular Probes, Eugene, OR, USA).

***Chlamydia* ELISA antigen.** Prototype *C. abortus* strain B577 (VR-656; American Type Culture Collection, Manassas, VA), isolated from the kidney of an aborted sheep fetus (38), were cultured in buffalo green monkey kidney (BGMK) cells (BioWhittaker, Walkersville, MD) as described (Huang et al., 2002). Chlamydial elementary bodies

(EB) from *C. abortus* strain B577 (VR-656; American Type Culture Collection, Manassas, VA), isolated from the kidney of an aborted sheep fetus (Storz, 1966), were harvested in cell culture medium and purified by step-gradient centrifugation and suspended in sucrose-phosphate-glutamate (SPG) buffer (DeGraves et al., 2004). Two hundred and fifty microliters of EB stock was added to 750 µl of protein denaturation buffer (0.5 M Tris-HCl, pH 7.0, 20% SDS (v/v), 20% Glycerol (v/v), 1 M Dithiothreitol) and boiled for 10 min. Chlamydial lysates were concentrated by centrifugal ultrafiltration in 3 kD molecular weight cut-off ultrafiltration devices (Microcon YM-3; Fisher Scientific Company, Newark, DE, USA), reconstituted in PBS-25 mM DTT, and stored -80°C. Protein content of EB lysate antigens was quantified by NanoOrange® Protein fluorescence assay (Molecular Probes, Eugene, OR, USA).

***C. abortus* ELISA**. Sera were tested by enzyme-linked immunoabsorbent assay (ELISA) for antibodies against chlamydiae. In previous studies, immunoglobulin M, the initial antibody isotype developed after encounter of an antigen, proved to be the best indicator for protective immune functions against chlamydial infections (DeGraves et al., 2004). For this reason, only anti-chlamydial IgM were analyzed in this study. Furthermore, the same study and unpublished data show a very high degree of cross-reactivity in whole-Chlamydia lysate ELISAs (~80%) between *C. abortus* and *C. pecorum*, the main chlamydial strains found in cattle. ELISA analyses using either antigen yielded similar quantitative and identical functional results. For that reason, IgM antibodies against *C. abortus* strain B577 elementary body lysate were determined, using a previously reported method that used chemiluminescent detection of the bound anti-bovine IgM (Jee et al.,

2004). Briefly, one hundred microliters lysate per well containing 1 µg protein/ml in bicarbonate coating buffer was coated onto white, high protein binding flat bottom microtiter plates. Plates were incubated overnight at 4 ºC, the coating solution aspirated, and wells washed 5 times with wash buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween-20). Wells were then blocked with 200 µl of assay diluent (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween-20, 10% normal rabbit serum) for 1 hour at room temperature followed by removing assay diluent from the wells and 1.5 hrs incubation with sera 1:100 diluted in assay diluent. Bound antibody was detected with alkaline phosphatase-conjugated polyclonal goat antiserum against bovine IgM 1:600 diluted in assay diluent followed by incubation with chemiluminescent substrate. The assay was read in a Tecan Spectrafluor Plus reader and results reported as relative light units per second (rlu/sec). The background signal for negative control serum from a gnotobiotic calf (0-100 rlu/sec) was subtracted from the data, and results were normalized between assay plates by a factor adjusting for differences between rlu of the positive control serum.

Bovine serum amyloid A ELISA. The Tridelta Phase^{TM} range SAA kit (solid phase sandwich ELISA) was used to determine bovine SAA serum concentration as per manufacturer's instructions. Briefly, manufacturer-provided microtitre strips were pre-coated with monoclonal antibody specific for SAA. Standards of known SAA content or diluted serum samples were added into microwells along with biotinylated anti-SAA monoclonal antibody. Plates were then washed with wash buffer to remove all unbound material followed by addition of streptavidin-horse radish peroxidase conjugate.

Following the incubation, TMB substrate solution was added and the intensity of the color produced was proportional to the concentration of SAA present in the original specimen.

Statistical analysis. All statistical analyses were done with the Statistical Analysis Software SAS/STAT 9.1® (SAS Institute Inc., Cary , NC) and the Statistica 7.0 software package (StatSoft, Inc., Tulsa, OK). Samples collected from 5 cows of the dairy herd at the Auburn University Large Animal Clinic were not included in the analysis to avoid any confounding variables due to vastly different management practice, herd health and production parameters. During the study one cow from the EV Smith Research Center was diagnosed in one of the mammary gland quarters with severe *Prototheca* spp. infection with poor prognosis and was excluded from the study. Normal distribution of data was confirmed by Shapiro-Wilk's W test and normal quantile plots, and homogeneity of variances by Levene's test.

Principal component analysis (PCA) based on the correlation matrix was used to reduce the dimensionality of the data set and to identify new meaningful underlying variables. PCA involves a mathematical procedure that transforms a number of (possibly) correlated variables into a (smaller) number of uncorrelated variables called principal components.(Rao, 1964) The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. The principal components are thus a linear combination of optimally-weighted observed variables (Hotelling, 1933, Johnson and Wichern, 1998)

The principal components were then used to perform a disjoint cluster analysis to delineate natural clusters present in the cases (Hartigan, 1985). The cluster analysis procedure uses Euclidean distances, hence the cluster centers are based on least-squares estimation. The observations were divided into clusters such that every observation belonged to one and only one cluster. The principal components were then used as predictor or criterion variables in subsequent analyses.

Probability of binary outcome of total chlamydial detection and milk chlamydial detection (negative/positive) was modeled as a function of milk production and composition, immune parameters, and principal components using multiple logistic regression (Allison, 2001). Significant differences in production parameters, immune parameters and principal components amongst clusters were determined using Tukey's unequal HSD test. The Fisher Exact test was used to test for significant differences in frequency and prevalence among different groups and clusters.

RESULTS

Prevalence of chlamydial infection

Seroprevalence. All cows had high concentrations of IgM antibodies against the *C. abortus* lysate antigen in all serum samples tested. The ELISA signals exceeded the signal of the negative control serum by 4- to 76-fold. These antibodies persisted in all animals, and fluctuated substantially over the whole study period in most animals, indicating an ongoing herd infection with chlamydiae. These infections were asymptomatic because none of the animals showed obvious clinical signs of disease. Since other chlamydial species found in cattle, in particular *C. pecorum*, have a high degree of antibody cross-reactivity, these antibodies indicate the existence of infection with bovine chlamydiae in all animals rather than specifically with only *C. abortus*.

Chlamydial detection in milk and vagina. Of the total 17 cows retained for analysis, 12 (70%) were positive in any one of the specimens for *Chlamydia* spp by 23S rRNA gene FRET real-time PCR detection (Tab. II). All positive specimens showed very low copy numbers of chlamydiae per PCR, with most PCRs indicating 1-2 copies per 5 µl sample volume (out of 35 µl total volume), and 5 samples in the range of 3-40 *Chlamydia* spp. target copies per PCR. Melting curve analysis unambiguously identified *C. pecorum* in all positive specimens throughout the study.

In 11/12 (92%) of the positive cows, *C. pecorum* was detected in the vaginal mucous membrane, and 2 of these 11 cows (18%) were also positive in milk; however only one out of the 12 *Chlamydia*-positive cows (7%) was positive only in the milk sample from one mammary gland quarter (Tab. II). As shown in Table II, chlamydiae were detected in 12% of the total vaginal mucous membrane swabs, and only in 0.7% of all quarter milk samples.

Table II. Prevalence of *Chlamydia* spp. as detected by 23S rRNA gene PCR in vagina and milk.

Sample	*Chlamydia* detection in cows		*Chlamydia* detection in samples	
	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE
Vagina	11	6	20	150
Milk	3	14	5	675
Total	12	5	25	825

Chlamydial detection in mammary gland tissue. Even with repeated extensive milk sampling from each quarter of the mammary gland (10×4 = 40 milk samples/cow), the frequency of *Chlamydia* spp. detection in milk samples was substantially lower than expected. In order to investigate the possibility of chlamydial colonization in mammary gland without concurrent shedding/detection in milk, mammary gland tissue biopsies from seven cows were analyzed by *Chlamydia* 23S rRNA gene FRET real-time PCR. Biopsies were obtained from all four udder quarters of seven cows from the study herd that had been designated for culling. None of the 28 mammary tissue samples showed evidence of mammary gland colonization by *Chlamydia* spp.

Overall, the prevalence data for *Chlamydia* spp. indicate an asymptomatic endemic infection of the study herd with *C. pecorum*. In this situation, relatively infrequent and seemingly random detection of very low numbers of *C. pecorum* genomes, primarily at vaginal epithelium, is combined with persistently high and fluctuating IgM antibody levels against *Chlamydia* spp. This suggests frequent episodes of re-infection by *C. pecorum*.

Composite milk sample of all quarters versus quarter milk sample. The working hypothesis of this investigation was that the main effect of chlamydial infection on milk would be due to interstitial colonization of the mammary gland. Given the high milk sampling frequency, detection of the majority of chlamydial mammary gland infections was anticipated even if chlamydial presence in milk would be substantially lower than in mammary gland tissue. Repeated milk sampling, supposedly, could have helped to compare and contrast milk parameters in chronically infected quarters, recurrent or

intermittently infected quarters and *Chlamydia*-free quarters from the same or different individuals. However, the limited detection of chlamydiae in milk samples strongly indicates that chlamydial colonization of the bovine mammary gland under conditions of an endemic *C. pecorum* infection of a herd is rare (<1% of udder quarter samples). The corollary of this finding is that the influence of chlamydial infection on the mammary gland, if any, would primarily result from systemic effects of mucosal infections rather than from local effects of direct udder colonization by *Chlamydia* spp. To elucidate the effects of systemic, and occasional localized chlamydial infections, analyses and comparisons of total milk yield and milk composition per cow will be more informative. Thus, milk yield and milk components across the mammary gland quarters were combined in a weighted approach to represent the total milk production of the cow.

Effect of chlamydial infection on milk and serum parameters

Chlamydial infection of cows. Since all cows were seropositive for anti-*Chlamydia* IgM, we sought to identify, if any, association between chlamydial presence and milk production and immune parameters. *Chlamydia* detection (negative/positive) in any specimen (vaginal swab or milk; total *Chlamydia* detection) was regressed in a multiple logistic regression model against milk yield, milk SCC, milk fat, milk protein, serum anti-*Chlamydia* IgM, and the acute phase protein serum amyloid A (SAA). Of all parameters in this model, milk yield, milk fat and milk protein predicted highly significantly chlamydial presence or absence in cows (Tab. III). Chlamydial presence significantly associated with higher milk yield (36.24 lb and 29.41 lb in *Chlamydia*

positive and negative cows, respectively, OR= 0.913, *P*=0.004), higher milk fat (2.81% and 2.66% in *Chlamydia* positive and negative cows, respectively, OR = 0.587, *P*=0.034), and lower milk protein (2.90% and 3.20% in *Chlamydia* positive and negative cows, respectively, OR = 14.79, *P*<0.001). Surprisingly, total chlamydial presence in cows was not significantly correlated with milk SCC.

Table III. Total *Chlamydia* detection (NEG/POS) in milk and vagina combined analyzed by logistic regression on production and immune parameters.

Variable[a]	Mean		Odds ratio[b]	95% Wald Confidence Limits	Pr > ChiSq
	NEG	POS			
Milk yield	29.41	36.24	0.913	0.858 - 0.972	0.0043
Fat %	2.66	2.81	0.587	0.359 - 0.961	0.0341
Protein %	3.20	2.90	14.795	3.476 - 62.970	0.0003

[a] Only significant variables are shown.

[b] Odds Ratio Estimates: probability modeled for chlamydial detection = Negative.

Chlamydial infection of the mammary gland. In order to isolate the effect of colonization of mammary gland by *C. pecorum*, chlamydial detection in milk only (negative/positive) was regressed against milk yield, milk SCC, milk fat, milk protein, serum anti-*Chlamydia* IgM, and the acute phase protein serum amyloid A. In this multiple regression model milk SCC and milk protein predicted, with high significance, presence of mammary gland chlamydial infection. Chlamydial detection in milk associated with higher milk SCC (5.31 and 5.09 in *Chlamydia* positive and negative respectively, OR= 0.12, $P<0.0001$) and lower milk protein (2.73 and 3.05 in *Chlamydia* positive and negative respectively, OR= 999, $P<0.0001$). Thus, chlamydial infection of the mammary gland had a significant added effect on the inflammatory status of the mammary gland as compared to the systemic effect of chlamydial infection that did not increase, on its own, milk SCC.

Table IV. *Chlamydia* detection (NEG/POS) in milk only analyzed by logistic regression on production and immune parameters.

Variable[a]	Mean		Odds ratio[b]	95% Wald Confidence Limits	Pr>ChiSq
	NEG	POS			
Log SCC	5.09	5.31	0.115	0.041 - 0.322	<.0001
Protein %	3.05	2.73	999	224 - 999	<.0001

[a] Only significant variables are shown.

[b] Odds Ratio Estimates: probability modeled for chlamydial detection = Negative.

Hypothesis-free data exploration by principal component analysis.

Subclinical natural *Chlamydia* spp. infections are influenced by, and have effects on, multiple production and health factors (Livingstone and Longbottom, 2006). Principal components were created to help in understanding the cumulative effect of infection on these factors. These components are linear combinations of optimally-weighted observed variables. Each successive principal component (PC) explains a smaller fraction of the total observed variance. Thus, it is important to balance the total explanatory power of PCs against the high complexity of interactions between original variables in numerous PCs required to achieve maximum explanatory power. As shown in the scree plot for this PCA (Fig. 2), the first three PCs explain 69% of the data variance and the major weights of the original variables in these PCs are amenable to relatively simple, biologically relevant explanations. For this reason, the first 3 PCs were considered primary components and were used in subsequent analyses that are open to meaningful interpretation.

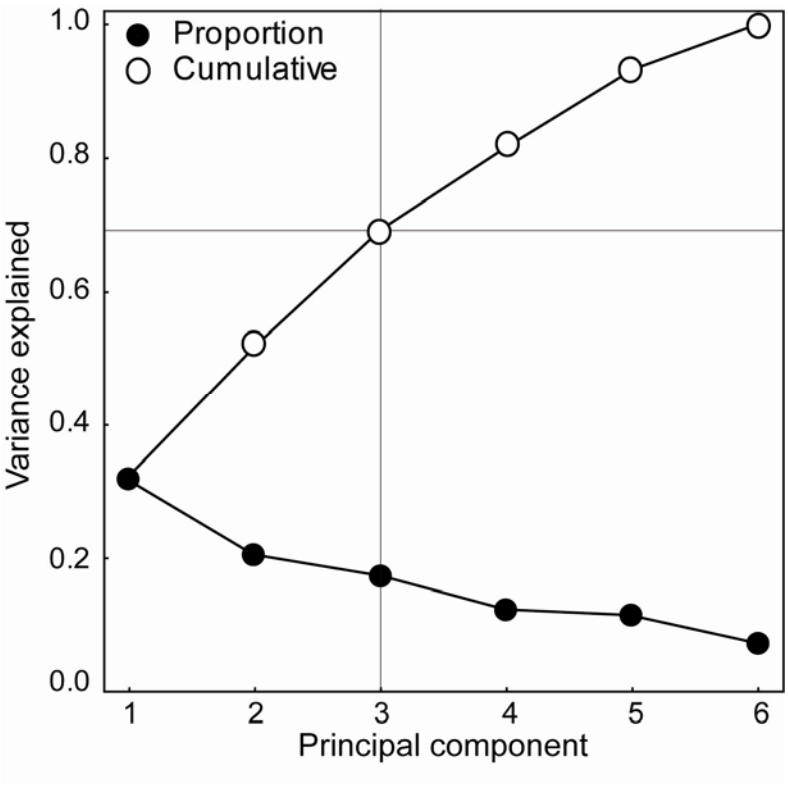

Figure 2. Scree plot depicting the fraction of total variance in the data explained by each principal component (PC). The first three PCs combined represent 69% of the total variance.

Table V. Weighted coefficients for the linear combination of original variables to three primary principal components.

Variable	PC 1[a] Production loss index	PC 2 Anti-*Chlamydia* immune index	PC 3 Inflammation index
Milk yield	-0.51	0.27	0.13
Log SCC	0.46	-0.09	0.46
Fat %	0.31	0.36	-0.59
Protein %	0.54	-0.38	-0.20
SAA	0.30	0.42	0.61
***Chlamydia* IgM**	0.21	0.69	-0.17

[a] Highly correlated coefficients are shown in red. PCs are named to express their biological significance based on the dominantly contributing variables.

The primary principal components PC 1-3 received practically meaningful names that attempt to explain the biological effect associated with each PC as deduced from the main weighted contributions of the original variables:

Production loss index (Prod loss). This first principal component explains 32% of variability in the dataset (Fig. 2). The linear combination of the original variables is largely a contrast of milk yield with SCC and protein content (Tab. V).

PC 1 = -0.51×Milk yield, lb + 0.46×Log SCC + 0.31×Milk fat, % + 0.54×Milk protein, % + 0.21×Anti-chlamydial IgM, rlu +0.30×Serum amyloid A, ng/ml

This formula implies that low milk yield and high milk SCC and protein increase PC 1, which therefore can be termed 'Production Loss Index'.

Anti-*Chlamydia* immune index (*Chl* immun). The second principal component explains 21% of the data variability (Fig. 2) and is primarily weighted on the anti-chlamydial immune response (Tab. V).

PC 2 = 0.27×Milk yield, lb - 0.09×Log SCC + 0.36×Milk fat, % - 0.38×Milk protein, % + 0.69×Anti-chlamydial IgM, rlu +0.42×Serum amyloid A, ng/ml

Cases with high immune response as measured by anti-chlamydial IgM antibodies will be represented with high PC 2. Thus, this component can be termed 'Anti-*Chlamydia* Immune Index'.

Inflammation index (Inflam). The third and final principal component explains 17% variability in the data (Fig. 2). The linear combination in this PC is positively weighted

on the acute phase protein Serum Amyloid A (SAA) and negatively weighted on milk fat, and can therefore be termed 'Inflammation index' (Tab. V).

PC 3 = 0.13×Milk yield, lb + 0.46×Log SCC - 0.59×Milk fat, % - 0.20×Milk protein, % - 0.17×Anti-chlamydial IgM, rlu +0.62×Serum amyloid A, ng/ml

Effect of chlamydial infection on principal components

A multiple logistic regression model was used to investigate the relevance of production loss index, anti-*Chlamydia* immune index and inflammation index in predicting total and mammary gland chlamydial infection of the cows.

Chlamydial infection of cows. The anti-*Chlamydia* immune index and production loss index predicted with high significance presence of total chlamydial infection. Chlamydial presence significantly associated with higher *Chl* immun index (0.25 and -0.60 in *Chlamydia* positive and negative cows, respectively, OR= 0.378, $P<0.0001$; Tab. VI) and lower production loss (-0.25 and 0.60 in *Chlamydia* positive and negative respectively, OR= 1.715, $P=0.0002$; Tab. VI).

Table VI. Total *Chlamydia* detection (NEG/POS) in milk and vagina combined analyzed by logistic regression on principal components.

Principal component	Mean		Odds ratio[b]	95% Wald Confidence Limits	Pr > ChiSq
	NEG	POS			
***Chl* immun**	-0.60	0.25	0.378	0.244 - 0.586	<.0001
Prod loss	0.60	-0.25	1.715	1.286 - 2.287	0.0002

[a] Only significant variables are shown.

[b] Odds Ratio Estimates: probability modeled for chlamydial detection = Negative.

Chlamydial infection of the mammary gland. *Chlamydia* detection in the mammary gland (negative/positive) was regressed against production loss index, anti-*Chlamydia* immune index and inflammation index. Of all the principal components, only the anti-*Chlamydia* immune index predicted with high significance the presence of chlamydial infection. Chlamydial presence associated significantly with higher anti-*Chlamydia* immune index (0.66 and -0.14 in *Chlamydia* positive and negative respectively, OR= 0.53, *P*=0.0009; Tab. VII).

Table VII. *Chlamydia* detection (NEG/POS) in milk only analyzed by logistic regression on principal components.

Principal component	Mean		Odds ratio[b]	95% Wald Confidence Limits	Pr > ChiSq
	NEG	POS			
***Chl* immun**	-0.14	0.66	0.526	0.361 - 0.768	0.0009

[a] Only significant variables are shown.

[b] Odds Ratio Estimates: probability modeled for chlamydial detection = Negative.

Natural clustering

In PCA, each case (10 cases for the 10 sample collection data per cow) receives discrete values for the PCs. These values, by their combination of original outcome variables, reduce the dimensionality of the data while still explaining a large fraction of the variance. Associations of cases in the 3-dimensional PC space might dissect functional biological outcomes that are not obvious in the original data. Hypothesis-free associations can be obtained by cluster analysis which associates cases by their least Euclidean distances in the PC dimensions, and the biological meaning of the PCs may aid in interpreting such clusters by juxtaposing low and high responder cases in clusters separated well along one PC dimension.

Exploratory cluster analysis based on the first three principal components delineated the observed cases into four natural groups (Fig. 3, Tab VIII). Out of the 3 possible reductions of the 3-dimensional clusters into 2-dimensional graphs, visualizing production loss index (PC 1) and inflammation index (PC 2) against anti-*Chlamydia* immune index (PC 2) revealed clearly separated clusters (Fig. 3). Clusters 1 and 3 were the largest with 68 and 87 observations, respectively. Cluster 1 cases had significantly higher average milk yield and lower average milk SCC when compared with cluster 3, thus less production loss (Tab. VIII; Tukey's studentized HSD $P<.05$). The feature distinguishing these two clusters were high anti-*Chlamydia* IgM antibodies in cluster 1 (Tab. VIII; Tukey's studentized HSD $P<.05$). The above features were also reflected by significantly lower Prod loss index and higher *Chl* immun index in cluster 1 as compared to cluster 3 (Tab. IX; Tukey's studentized HSD $P<.05$). Thus cases in cluster 1 were

high producers with strong immune response. Cluster analysis is also an effective procedure for detecting outliers which often appear as clusters with few members. Clusters 2 and 4 were small clusters with only 10 and 5 observations, respectively. These clusters represented high production loss and high anti-*Chlamydia* immune index and were delineated from each other by inflammation index. Observations in these clusters were characterized by bacterial mastitis in the acute (cluster 4) or healing phase (cluster 2) and chlamydial infection. The limited number of observations in clusters 2 and 4 prevent meaningful statistical inference from these clusters. Therefore, only clusters 1 and 3 will be discussed further.

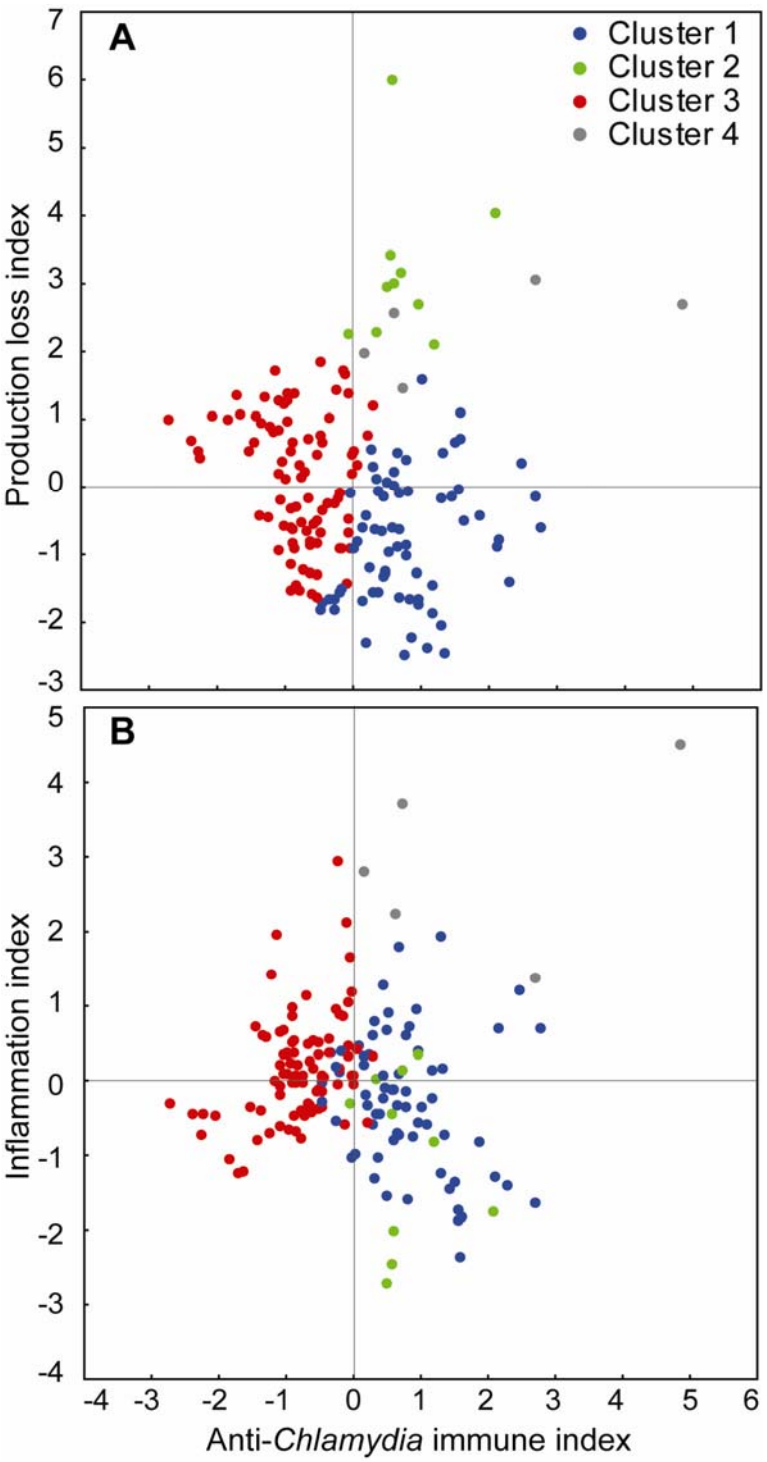

Figure 3. Natural clusters of cases identified by least Euclidean distances based on the three primary principal components. An increase of the production loss index is primarily associated with a decrease in milk yield and high milk SCC, an increase of the

anti-*Chlamydia* immune index represents mainly elevated anti-*Chlamydia* IgM, and an increase of the inflammation index largely reflects high acute phase protein serum amyloid A (SAA). Clusters 1 and 3 are the dominant and relevant clusters with 68 and 87 observations, respectively, that are delineated by the anti-*Chlamydia* immune index (A, B). Thus, cluster 1 is characterized by high, and cluster 3 by low anti-*Chlamydia* IgM. In contrast, clusters 2 and 4 each contain 10 and 5 observations, respectively, and represent cases of bacterial mastitis that consistently associate with high production loss (A) and high anti-*Chlamydia* immune index (A, B). They are separated by their inflammation index (B) into the acute (cluster 4) and recovery phase (cluster 2) of bacterial mastitis.

Table VIII. Mean values of original variables and three principal components of the four natural clusters of cases.

Cluster	N	Variable[a]	Mean	95% CI	
		Milk lb	39.29	37.19	41.40
		Log SCC	4.81	4.67	4.95
		Fat %	2.92	2.75	3.10
		Protein %	2.77	2.72	2.81
1	68	SAA, µg/ml	58.81	36.21	81.41
		Chl IgM	2547	2276	2818
		Prod loss	-0.79	1.02	-0.57
		Chl immun	0.78	0.60	0.96
		Inflamm	-0.27	-0.49	-0.05
		Milk lb	24.80	22.89	26.71
		Log SCC	5.97	5.59	6.35
		Fat %	4.49	3.52	5.46
		Protein %	3.58	3.28	3.88
2	10	SAA, µg/ml	147.91	64.00	231.81
		Chl IgM	3647	2452	4842
		Prod loss	3.19	2.37	4.01
		Chl immun	0.75	0.33	1.16
		Inflamm	-1.00	-1.82	-0.18
		Milk lb	31.71	30.26	33.15
		Log SCC	5.23	5.11	5.36
		Fat %	2.47	2.35	2.60
		Protein %	3.10	3.05	3.16
3	87	SAA, µg/ml	53.65	38.01	69.30
		Chl IgM	1226	1089	1362
		Prod loss	0.12	-0.08	0.32
		Chl immun	-0.80	-0.93	-0.68
		Inflamm	0.16	0.01	0.31
		Milk lb	28.30	19.81	36.79
		Log SCC	6.02	5.40	6.64
		Fat %	2.32	1.54	3.10
		Protein %	3.08	2.86	3.31
4	5	SAA, µg/ml	586.40	236.54	936.27
		Chl IgM	3424	-351.01	7199
		Prod loss	2.35	1.56	3.14
		Chl immun	1.81	-0.62	4.24
		Inflamm	2.93	1.41	4.45

[a] Median SAA level from all clusters was 26.27 µg/ml with a range of 2.9 µg/ml to 484.12 µg/ml.

Chlamydial infection and extracellular bacterial mastitis

In epidemic chlamydial abortion in sheep flock, as discussed in chapter 2, the presence of chlamydial infection and high serum concentrations of complement-binding anti-*Chlamydia* immunoglobulins was associated with reduced severity of extracellular bacterial infection, and thus chlamydial infection protected against extracellular bacterial mastitis. Clusters 1 and 3 are remarkably different in milk production, principal components, and anti-*Chlamydia* IgM (Fig. 3; Tab. VIII). In order to investigate the presence of a similar relationship between chlamydial presence and extracellular bacterial mastitis in dairy cows we further investigated the differences in milk and serum parameters and principal components between and within the dominant clusters 1 and 3 with respect to presence or absence of chlamydial and extracellular bacterial infection.

Of the total 68 observation in cluster 1, 88% were from *Chlamydia*-positive cows. This is in sharp contrast to cluster 3 where 57% observations were from *Chlamydia*-positive cows (Fig. 4, Tab. IX; $P<0.01$, two-tailed Fisher Exact Test). However, 75% of observations from *Chlamydia*-positive cows in cluster 1 were negative for extracellular bacterial mastitis. This is again in sharp contrast to cluster 3 where only 48 % of observation are bacteria-negative (Fig. 4, Tab. IX; $P<0.01$, two-tailed Fisher Exact Test). Therefore, the frequency distribution of bacterial infections in *Chlamydia*-positive cows of clusters 1 and 3 associates with high anti-*Chlamydia* IgM, and thus suggests a highly significant protective effect of high anti-*Chlamydia* IgM from bacterial mastitis.

In addition to the frequency distribution of infections, observations from *Chlamydia*-positive cows in clusters 1 and 3 were significantly different in milk and

serum characteristics (Tab. X). Cluster-1 observations from *Chlamydia*-positive cows that are negative for extracellular mastitis are significantly higher in milk production and milk fat, but significantly lower in SCC as compared to corresponding observations in cluster 3 (Tab. X, Tukey's studentized HSD $P<.05$). These observations are largely comparable in serum concentration of acute phase protein SAA (Tab. X). The differences among these observations are also reflected as higher anti-*Chlamydia* immune index in cluster 1 (Tab. IX; Tukey's studentized HSD $P<.05$). Thus, the significantly higher anti-*Chlamydia* IgM in cluster 1 (Tukey's studentized HSD $P<.05$) correlate with better milk yield and quality (Fig. 4, Tab. X).

In conclusion, a strong immune response against *Chlamydia* spp. is clearly associated with protection against extracellular bacterial infection. In the absence of bacterial infection of the mammary gland, high anti-*Chlamydia* immunity associates with reduction of the negative effect of chlamydial infection, be it in the mammary gland or elsewhere in the host, as evidenced by increased milk yield and milk fat, and reduced milk somatic cell counts.

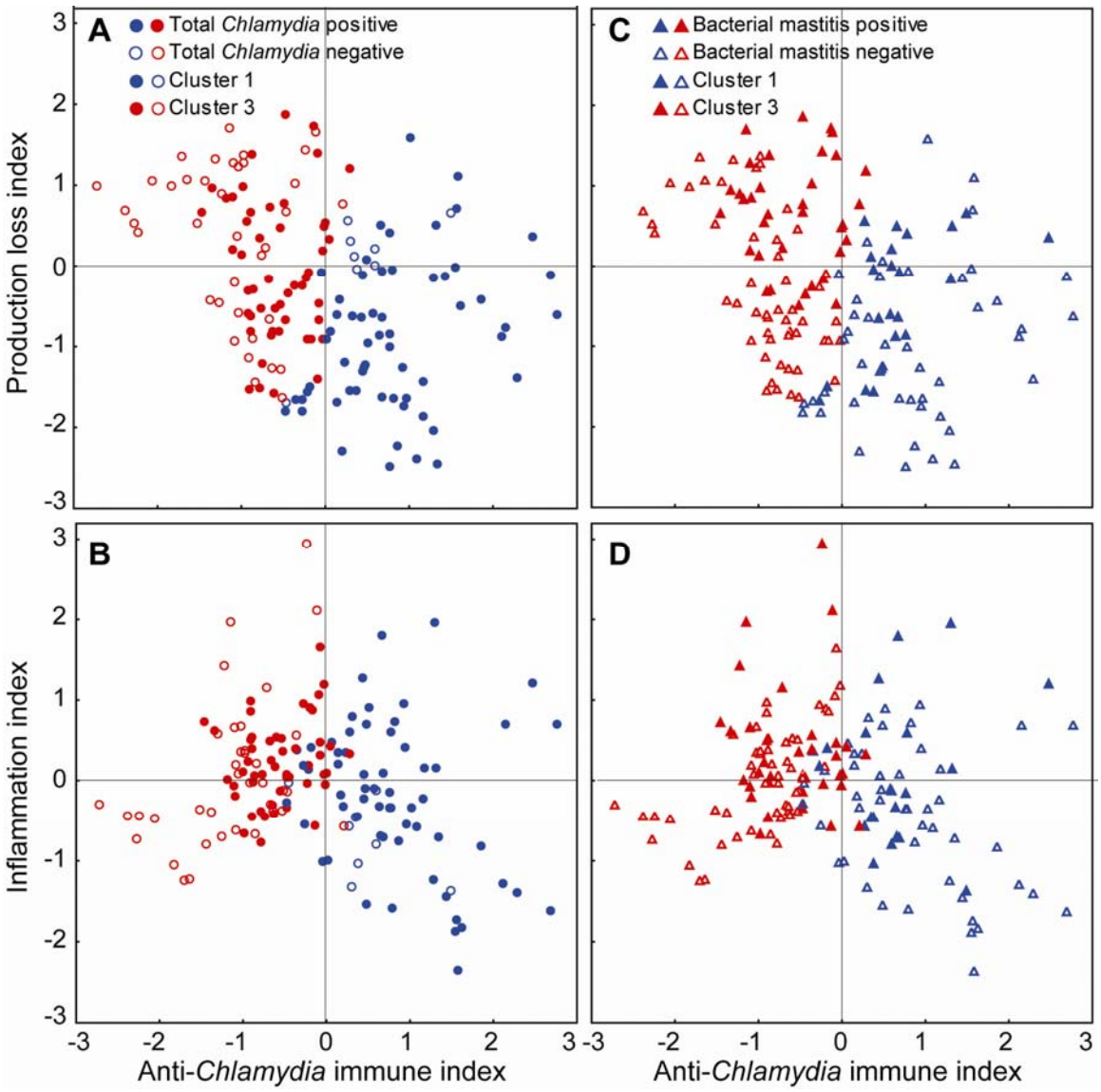

Figure 4. Stratification of cluster 1 and 3 cases based on *Chlamydia* detection and bacterial mastitis. Cluster 1 cases trend towards higher anti-*Chlamydia* immune index (A-D) and lower production loss index (A, C) than cluster 3 cases. Inflammation index does not delineate cluster 1 and 3 cases (B, D). *Chlamydia* positive cows trend towards lower production loss index (A) and higher anti-*Chlamydia* immune index (A, B) than *Chlamydia* negative cows but do not differ in inflammation index (B). Cows with bacterial mastitis tend to have higher production loss index than cows without bacterial

mastitis (C) but do not differ in anti-*Chlamydia* immune (C, D) and inflammation index (D).

Table IX. Principal components of clusters 1 and 3 contrasted for chlamydial detection and bacterial mastitis.

Total *Chlamydia*	Bacterial Mastitis	Principal component	Cluster 1			Cluster 3		
			N	Mean	95% CI[a]	N	Mean	95% CI
Negative	Negative	Prod loss	2	-0.70	-13.44 - 12.03	28	0.12	-0.26 - 0.51
		Chl immun		-0.08	-4.94 - 4.78		-1.31	-1.54 - -1.08
		Inflamm		-0.67	-8.87 - 7.53		-0.28	-0.48 - -0.08
	Positive	Prod loss	6	0.25	-0.05 - 0.56	9	1.08	0.70 - 1.45
		Chl immun[a]		0.61	0.14 - 1.09		-0.57	-0.96 - -0.18
		Inflamm[a]		-0.72	-1.19 - 0.26		1.13	0.26 - 1.99
Positive	Negative	Prod loss	45	-1.02	-1.30 - -0.74	24	-0.67	-0.92 - -0.41
		Chl immun[a]		0.87	0.63 - 1.11		-0.54	-0.66 - -0.42
		Inflamm[a]		-0.41	-0.67 - -0.15		0.38	0.12 - 0.64
	Positive	Prod loss[a]	15	-0.56	-0.99 - -0.12	26	0.50	0.24 - 0.78
		Chl immun[a]		0.70	0.34 - 1.07		-0.58	-0.78 - -0.38
		Inflamm		0.38	-0.09 - 0.85		0.09	-0.05 - 0.23

[a] The differences between corresponding variables in clusters 1 and 3 are significant ($P<0.05$; Tukey's HSD test).

Table X. Production and immune parameters of clusters 1 and 3 contrasted for chlamydial detection and bacterial mastitis.

Total *Chlamydia*	Bacterial Mastitis	Variable	Cluster 1			Cluster 3		
			N	Mean	95% CI[a]	N	Mean	95% CI
Negative	Negative	Milk lb	2	34.75	-31.96 - 101.46	28	28.31	25.37 - 31.26
		Log SCC		4.73	1.35 - 8.12		4.90	4.65 - 5.14
		Fat %		3.28	-7.66 - 14.21		2.40	2.23 - 2.57
		Protein %		2.80	1.99 - 3.61		3.22	3.12 - 3.32
		SAA, µg/ml		20.24	-70.27 - 110.75		16.69	10.21 - 23.17
		Chl IgM		1179	-6583 - 8941		926	722 - 1130
	Positive	Milk lb	6	27.00	24.49 - 29.51	9	34.14	31.52 - 36.77
		Log SCC[a]		4.95	4.73 - 5.16		6.05	5.87 - 6.24
		Fat %		2.85	2.29 - 3.42		2.71	1.98 - 3.44
		Protein %[a]		2.88	2.82 - 2.94		3.22	3.08 - 3.35
		SAA, µg/ml[a]		13.60	-3.10 - 30.31		178.45	66.71 - 290.18
		Chl IgM[a]		3588	3033 - 4144		1021	712 - 1331
Positive	Negative	Milk lb[a]	45	42.21	39.88 - 44.54	24	36.35	34.35 - 38.34
		Log SCC[a]		4.71	4.52 - 4.90		5.13	4.95 - 5.31
		Fat %[a]		3.05	2.85 - 3.26		2.40	2.18 - 2.61
		Protein %		2.77	2.71 - 2.83		2.88	2.81 - 2.95
		SAA, µg/ml		51.70	25.95 - 77.45		55.25	33.80 - 76.71
		Chl IgM[a]		2453	2082 - 2824		973	753 - 1193
	Positive	Milk lb	15	36.08	31.79 - 40.37	26	30.23	27.87 - 32.59
		Log SCC		5.07	4.79 - 5.36		5.41	5.27 - 5.56
		Fat %		2.51	2.13 - 2.89		2.54	2.31 - 2.78
		Protein %[a]		2.71	2.61 - 2.81		3.14	3.05 - 3.23
		SAA, µg/ml		103.37	35.17 - 171.57		48.77	31.00 - 66.54
		Chl IgM		2595	2298 - 2891		1852	1659 - 2045

[a] The differences between corresponding variables in clusters 1 and 3 are significant ($P<0.05$; Tukey's HSD test).

DISCUSSION

Chlamydial infections in cattle are of substantial economic significance worldwide. The subclinical, and thus inapparent, nature of diseases resulting from these chlamydial infections, such as reproductive failure, makes it difficult to accurately assess the economic and health impact of these infections (Livingstone and Longbottom, 2006).

In the present study, we identified endemic infection of a dairy herd by *C. pecorum.* Cows exhibited very low, intermittent chlamydial shedding. Chlamydial detection in milk was restricted to only one mammary gland quarter at any given sampling time point. These findings underscore the importance of intensive sampling and highly sensitive detection methods such as PCR to uncover the true chlamydial prevalence and shedding in animals.

The functional outcome of this low level endemic infection was different amongst individual animals as evident by variation in frequency of chlamydial shedding, mammary gland colonization, and difference in milk yield and composition. In previous studies, immunoglobulin M, the initial antibody isotype produced after initial encounter of an antigen, proved to be the best indicator for protective immune functions against chlamydial infections (DeGraves et al., 2004). For this reason, only anti-chlamydial IgM were analyzed in this study. Furthermore, the same study and unpublished data from our laboratory show a very high degree of cross-reactivity (~80%) between ELISAs using whole *C. abortus* and *C. pecorum* organism lysate antigens, the two main chlamydial species found in cattle. ELISA analyses using either antigen yielded similar quantitative and identical functional results. For that reason, IgM antibodies against *C. abortus* strain

B577 elementary body lysate were determined by using chemiluminescent detection of the bound anti-bovine IgM (Jee et al., 2004). Over the span of the total 140-day observation period all animals were seropositive for anti-chlamydial IgM antibodies, irrespective of chlamydial detection. While in observational studies such as this one any significant relationship does not qualify as cause and effect, these associations nevertheless contribute profoundly to the estimation of causal effects and future research designs.

The host response to an infection, especially to subclinical low level infection such as by *Chlamydia* spp., depends on a myriad of host-, pathogen-, and environmental factors. In this study, three principal components (simplified as biologically meaningful indices) better elucidated the host response in terms of milk- as well as serum parameters and clarified underlying trends in the multivariate data. Natural clustering based on these indices provided illuminating insight into the importance of the anti-chlamydial immune response in minimizing the production loss. Interestingly, in a chlamydial abortion episode in a flock of milk sheep (discussed in chapter 2), the presence of a strong anti-chlamydial immune response associated with lower incidence and severity of extracellular bacterial mastitis, similar to the main conclusion observed in this dairy herd with endemic chlamydial infection.

In order to evaluate the inflammatory status of the herd, the acute phase protein serum amyloid A was quantified in the sera. Median serum amyloid A observed in this study was 26.27 µg/ml with a range of 2.9 µg/ml to 484.12 µg/ml (based on Tab. VIII). These levels are considerably higher than reported SAA serum median levels of 1.4 µg/ml with a range 0.3 to 13.5 µg/ml in healthy cows obtained by use of the same ELISA

kit (Eiji Takahashi, 2007). These data indicate the existence of dairy herds that have clearly lower levels of inflammation than the herd in this study. While there are no other parameters that can be compared between these two herds, it may well be that the endemic chlamydial infection contributes to this difference in SAA concentrations.

This study observed strong evidence of changes in milk production with chlamydial infection. Total chlamydial presence was significantly associated with higher milk yield and fat percent, but reduced protein percent (Tab. III). The significant association of increased milk yield with presence of *Chlamydia* strongly suggests a heightened susceptibility of high-producing cows to chlamydial infection. However, milk yield, milk fat and protein contents varied significantly in different clusters when observations were categorized by chlamydial detection as well as extracellular bacterial mastitis. (Tab. X). For instance, the milk protein content in an inflamed udder quarter increases significantly since inflammation leads to increased influx of serum proteins such as albumin and immunoglobulins (Larsen et al., 2004, Merle et al., 2007).

Milk fat can be influenced by diet and nutrition in ruminants as fatty acids in milk fat, taken from circulation, are largely from intestinal absorption of microbial and dietary fatty acids (Barber et al., 1997, Bauman and Davis, 1974). However, in the presence of a negative energy balance, for example during the post-parturient period, lipolysis and mobilization of body fat increases in direct proportion to the energy deficit (Bauman and Griinari, 2001), thus bringing milk fat under control of liver metabolism. The major effect of cytokines on the liver is the stimulation of the acute phase response (Fleck, 2007), seen as an increase in APP and impairment in production of albumin and apolipoproteins (Bruss et al., 1997). Pro inflammatory cytokines can thus cause

metabolic diversion of nutrients into the inflammatory response away from growth and production (Bertoni et al., 2008, Elsasser et al., 2000). It has been shown that dairy cows that are highly affected by inflammation experience greater losses in body condition and have increased blood ketone bodies, even in the face of a reduced milk yield as compared to healthy cows. Thus, changes in milk composition can be due to the impairment of metabolic liver function and the anorexic effect of cytokines present during inflammation (Jenkins and McGuire, 2006).

The quarters of a mammary gland are anatomically and physiologically strictly separated (Akers, 2002). Since SCC rise in sterile quarters of a cow that is affected by bacterial mastitis in another quarter, it is clear that inflammation of one udder quarter affects the other quarters by means of circulating inflammatory mediators (Forsbäck et al., 2009). As a corollary to these findings, it may well be possible that extramammary chlamydial infection similarly affects milk production and composition via systemic pro-inflammatory cytokines which cause an impairment of metabolic liver function and reduced food uptake, and thus potentiate a negative post-partum energy balance (Jenkins and McGuire, 2006).

Many aspects of the bovine immune system change during the periparturient period, but uncontrolled inflammation is a dominant factor in several economically important disorders such as metritis and mastitis (Drackley et al., 2005, Grimble, 1990). Thus it is highly likely that systemic effects of chronic chlamydial diseases mediated by proinflammatory cytokines, particularly in the absence of a strong anti-chlamydial immune response, could be a common mechanism for reproductive impairment occurring in the context of infectious diseases. This hypothesis agrees with the strong improvement

of fertility observed when cows were treated with an anti-inflammatory drug (acetylsalicylate) in the first few days of lactation (Bertoni et al., 2004). Inflammation stimulates the immune system, resulting in the release of cytokines which may inhibit the action of FSH on LH receptor formation in cultured rat granulosa cells and inhibit FSH-induced cAMP production (Darbon et al., 1989, Schrick et al., 2001).

In medicine, metabolic syndrome is known to be caused by chronic low-grade inflammation, and to promote, in a positive feedback loop, more and uncontrolled systemic inflammation (Dandona et al., 2005). Altered lipid metabolism, increased circulating concentrations of non-esterified fatty acids and oxidative stress are significant contributing factors to systemic inflammation and to the development of inflammatory diseases in humans (Dandona et al., 2005). Dairy cows undergo many metabolic adaptations during the onset of lactation and thus are most susceptible to inflammatory effects in the post-parturient period. Our data suggest that low level chlamydial infections can precipitate the negative effect of metabolic adaptation by impacting magnitude and duration of inflammation, particularly in animals that do not control chlamydial infection well due to a low anti-chlamydial immune response. All the animals in this study were seropositive against chalmydial antigens, but titers differed between individuals. In addition, most animals exhibited cyclic variations in the antibody levels, indicating re-infection, and this cyclicity directly correlated to health and production status of animals.

Chapter 4: Overall Conclusions

In summary, several interesting and novel conclusions can be drawn from these investigations. The investigation of a *C. abortus* abortion episode in a sheep flock proves that age or genetic pre-disposition of the ewes and the fact of chlamydial infection itself are largely irrelevant for pregnancy outcome while a late time point of *C. abortus* infection during gestation is the main determinant of abortion. This time dependency suggests that the synepitheliochorial placentation type favors chlamydial invasion of the fetus if a chlamydial high-dose exposure occurs late in gestation. Furthermore, this study demonstrates the systemic dissemination of *C. abortus* during abortion and its subtle inflammatory effect on the mammary gland early in lactation. The higher exposure to chlamydiae during abortion as compared to parturition results in a qualitatively different immune response that apparently protects against streptococcal or staphylococcal mastitis. It is possible that the immune response against chlamydiae after abortion resembles a vaccine response (Biesenkamp-Uhe et al., 2007) which reduces milk somatic cells in cows. If so, then the endemic chlamydial infection must predispose ewes to higher milk SCC and reduce resistance against extracellular bacterial infections. Clearly, this result points towards a subtle but significant herd effect of chlamydial infection

This longitudinal post-partum study of 17 dairy cows gives yet another proof of ubiquitous nature of chlamydial infections in dairy cattle. In addition, strong evidence is

presented that localized presence of *Chlamydia* spp. in the mammary gland is not essential for an effect of chlamydial infection on milk production parameters but indicates that any chlamydial infection of a cow affects also the mammary gland. This effect of chlamydial infection on milk production is not restricted to SCC only, but extends also to other milk parameters such as yield, and fat and protein content. A strong immune response against *Chlamydia* spp. strongly associates with increased milk yield, improved milk composition, and resistance to extracellular bacterial infections.

However, the observational nature of these studies prevents establishment of causality. Therefore, interventional studies are required that specifically influence chlamydial infection to conclusively prove its effect on ruminant health and production. We anticipate that the vaccination against *Chlamydia* spp. can be one such means of an interventional approach. Vaccination will perturb existing anti-*Chlamydia* spp. immunity, and will allow to precisely deduce cause and effect between chlamydial infection and production traits, as has already been shown for milk somatic cell counts (Biesenkamp-Uhe et al., 2007). A corollary of such studies would be that they can also serve to test chlamydial vaccines. Overall, the findings in this study combined with previous data unequivocally advocate for anti-*Chlamydia* vaccination in livestock.

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