Comprehensive analysis of the impact of endemic *Chlamydia pecorum* infection in cattle

by

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Abstract

Intracellular *Chlamydia* (*C.*) bacteria cause in cattle some acute but rare diseases such as abortion, sporadic bovine encephalomyelitis, kerato-conjunctivitis, pneumonia, enteritis and polyarthritis. More frequent, essentially ubiquitous worldwide, are low-level, asymptomatic chlamydial infections in cattle. In two independent prospective observational studies, we investigated the impact of naturally acquired endemic asymptomatic *C. pecorum* infections on neonatal health, fertility and milk production in dairy cows.

In calves, we analyzed in biweekly sampling the association of blood/plasma markers of health and infection with clinical appearance and growth in dependence of chlamydial infection intensity determined by mucosal chlamydial burden or contemporaneous anti-chlamydial plasma IgM. High chlamydial infection associated with reduction of body weight gains by up to 48% and increased conjunctival reddening ($P<10^{-4}$). Simultaneously decreased plasma albumin and increased globulin ($P<10^{-4}$) suggested liver injury by inflammatory mediators as mechanisms for the growth inhibition. This was confirmed by the reduction of plasma insulin like growth factor-1 at high chlamydial infection intensity ($P<10^{-4}$). High anti-*C. pecorum* IgM associated eight weeks later with a 66% increase in growth ($P=0.027$), indicating a potential for immune protection from *C. pecorum*-mediated growth depression

In first lactation dairy cows, we examined the association of cervical chlamydial burden and anti- *C. pecorum* immune trend (day 100/day 65 anti *C. pecorum* IgM) at the time of first

insemination with reproductive performance and milk production. *C. pecorum* cervical infection and low plasma luteinizing hormone levels associated highly significantly with reduction of fertility by up to 28% (P=0.007), suggesting that *C. pecorum*-induced inflammatory endometrial damage and dysregulation of the neuroendocrine axis of reproduction suppress fertility. In contrast, a declining anti-*C. pecorum* IgM response after first service and low plasma cholesterol and albumin, but not cervical chlamydial burdens, associated with reduction of milk production by up to 7% ($P<10^{-4}$), suggesting that declining anti-*C. pecorum* immunity and low liver health suppress milk production.

Our findings of very high plasma anti-*C. pecorum* IgM antibody concentrations in all calves and cows enrolled in the studies, and the simultaneous prevalence of multiple strains of *C. pecorum* suggest a high, steady-state endemic *C. pecorum* infection. These studies confirm the enormous economic impact of low-level asymptomatic *C. pecorum* infection and the need for a protective vaccine against *C. pecorum*.

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DIM	Days In Milk
EB	Elementary Body
ELISA	Enzyme Linked Immunosorbent Assay
IGF	Insulin-like Growth Factor
IACUC	Institutional Animal Care and Use Committee
IL	Interleukin
IFN	Interferon
LH	Luteinizing Hormone
LPS	Lipopolysaccharide
NO	Nitric Oxide
PCA	Principal Component Analysis
RB	Reticulate Body
TAI	Timed Artificial Insemination
TH	Helper T Cell
TNF	Tumor Necrosis Factor

Chapter 1 Literature Review

BIOLOGY OF CHLAMYDIAE

Developmental cycle. Bacteria of the phylum *Chlamydiae* are 0.2-1.5 μm in diameter, coccoid, nonmotile, obligate intracellular organisms, that parasitize virtually every eukaryotic organism and multiply in the cytoplasm of the host cells within membrane bound vacuoles, termed inclusions, by a unique developmental cycle (Kuo et al., 2011).

The unique intracellular developmental cycle of *Chlamydia* first described by Bedson et al. (1932) at the level of light microscopy is characterized at one extreme by a small electron-dense, 0.2-0.4 μm in diameter infectious elementary body (EB) and at the other by the larger, less dense, 0.6-1.5 μm in diameter, innocuous, dividing reticulate body (RB). Several electron microscopic studies thereafter have more finely characterized the developmental cycle showing also the transitional form, intermediate body. The structure, cellular composition and biology of the developmental forms are stage-specific and regulated by intracellular milieu which is not completely understood (Hatch, 1999). Elementary bodies have DNA condensed with protein and a few ribosomes. They are adapted for extracellular survival. The rigid trilaminar cell wall provides resistance to the physical and chemical barriers present in the extracellular environment (Hatch, 1999; Kuo et al., 2011). They are metabolically inert, presumably to prevent the extracellular initiation of the growth cycle. In contrast, RBs are metabolically active, have DNA condensed with more ribosomes, and surrounded by plastic trilaminar walls that are sensitive to sonic lysis (Hatch, 1999; Kuo et al., 2011). Cell walls of primary developmental forms; EB and

RB, contain lipopolysaccharide and resemble in many aspects, in structure and composition, including type III secretion apparatus of the cell walls of typical Gram-negative bacteria. The intermediate bodies are variable in shape and size, and their metabolic activity and infectivity is not known (Kuo et al., 2011).

The highlights of the chlamydial intracellular life cycle are: (a) attachment and entry of elementary bodies into host cells and their conversion to reticulate bodies, (b) logarithmic division of reticulate bodies, and (3) reorganization of a large fraction of the reticulate body population into a new generation of elementary bodies that is released from the host cell (Hatch, 1999; Kuo et al., 2011). The life cycle is complete when the reticulate bodies reorganize into the elementary bodies that survive extracellularly to infect new host cells upon their release from the host cells. The chlamydial developmental cycle, however, is not synchronized and each of the first two developmental stages overlaps into the next succeeding one (Fig 1; Kuo et al., 2011). In a young inclusion, only RBs are present. In a mature inclusion, all three forms (RB, EB, and intermediate bodies) are present (Fig 1; Kuo et al., 2011).

The initial attachment of the EB to host cells is facilitated by electrostatic interaction and secured by binding of ligands such as heat shock protein 70 (hsp 70) molecular chaperones, the outer membrane complex B (OmcB) protein, and major outer membrane protein (MOMP) followed by a receptor mediated endocytosis of the EB into host cells (Hatch, 1999; Kuo et al., 2011). Although the mechanisms are not completely understood, roles for mannose receptor, mannose-6 phosphate/insulin-like growth factor-2 receptor, and estrogen receptors have been suggested (Hatch, 1999; Kuo et al., 2011).

Inside host cells, EBs prevent fusion of the phagosomes with lysosomes and evade lysis by bactericidal enzymes, a survival strategy exhibited exclusively by live EBs (Eissenberg et al.,

1981). Within a few hours of internalization, the elementary bodies are quickly transported to a perinuclear region where they are fused with Golgi-derived sphingomyelin-containing vesicles initiating EB to RB transition (Majeed et al., 1991; Hackstadt et al., 1995). The dense nucleoid in RBs is dispersed into more evenly distributed fibrillar DNA, ribosomes increase in number, and the cell wall becomes thinner, more flexible, and more fragile preparing for the division by binary fission (Kuo et al., 2011). The first division of RB can be seen 8-10 hours post infection. The logarithmic multiplication of RB is achieved by 10-16 hours after infection. The doubling time during the exponential phase of growth is 2-3 hours. The proportion of dividing forms decreases after that, but division occurs throughout the developmental cycle. Within 24 hours after infection, the inclusion contains only reticulate bodies and the cycle comes to its end around 30-42 hours post infection when host cell can no longer support the nutrients requirement of dividing RBs (Kuo et al., 2011).

Nutrient and growth requirements. *Chlamydia* relies on its hosts for its entire nutrient requirements. Chlamydiae can freely access some of the constituents of metabolic pools of their hosts but not the exogenous substrate required for ATP synthesis (Reed et al., 1981). Therefore, *Chlamydia* needs to transport and use exogenous ATP for its own biosynthetic needs. The nutrient and growth requirement of *Chlamydia* is poorly understood because cell-free propagation is not possible. However, culture in embryonated eggs or in cell culture has shown that chlamydiae require several amino acids depending on the species, the biovars within the species, and the cell types used for culture (Allan et al., 1983).

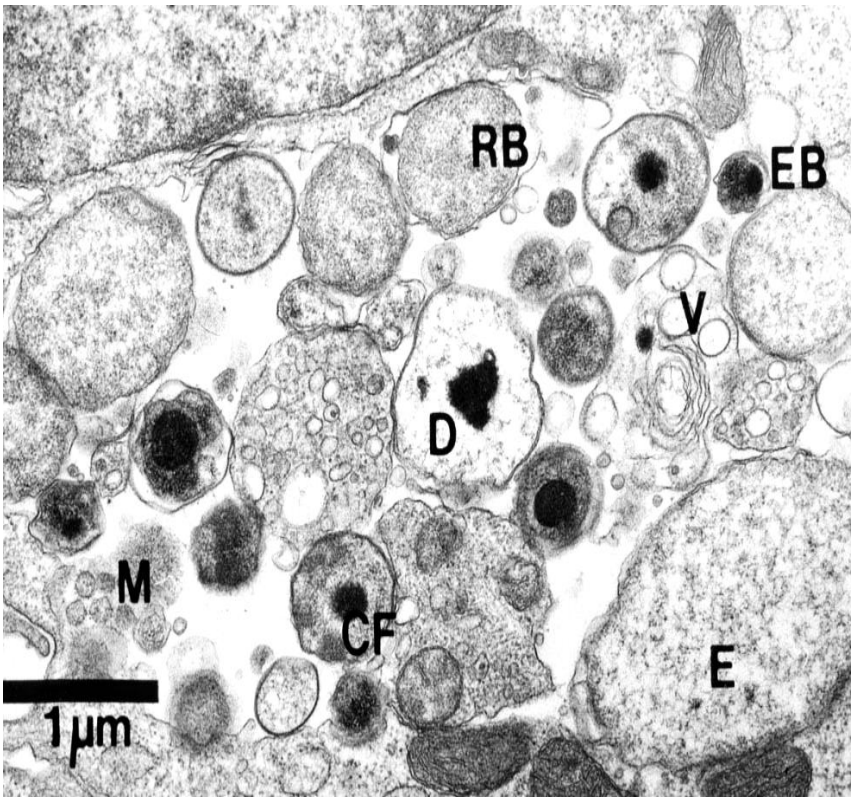

Figure 1. Transmission electron micrograph of a thin section of a mouse fibroblast L929 cells infected with *Chlamydia psittaci* showing different developmental forms of the chlamydial lifecycle. As shown in the figure, at any time the mature inclusion can have all three forms; elementary body (EB), reticulated body (RB), and different appearances of intermediate bodies such as condensing forms (CF), reticulate bodies with developmental arrest due to nutrient deprivation such as enlarged (E), degenerate (D), vacuolar (V), and microvacuolar (M) reticulate bodies. Image courtesy B. Kaltenboeck.

Taxonomic classification of *Chlamydia.* The tree methods using 16S rRNA are widely used for the evaluation of evolutionary relationships among *Chlamydia* and *Chlamydia*-related organisms and have shown very similar relationships irrespective of methods used for tree construction (Petersen et al., 1997; Everett et al., 1999; Corsaro et al., 2002; Kuo et al., 2011). The 16S rRNA sequence similarity proposed by Everett et al. (1999) is currently accepted for the classification of members of the order *Chlamydiales* at the family and genus level. In accord, members at the family level share ≥ 90% 16S rRNA similarities while at genus level they generally share ≥ 95% 16S rRNA similarity Everett et al. (1999). Therefore, the members sharing ≥ 90% 16S rRNA identity were retained in the family *Chlamydiaceae* and other *Chlamydia*-like organisms with 80-90 % 16S rRNA similarities to *Chlamydiaceae* are placed into new families. Everett et al. (1999) proposed classification of the order *Chlamydiales* into four families; *Chlamydiaceae, Parachlamydiaceae, Simkaniaceae* and an unnamed family which included strain WSU 86-1044. The members of the family *Chlamydiaceae* were separated into two genera; *Chlamydia* and *Chlamydophila* (Everett et al., 1999). Genus *Chlamydophila* comprised of six members; *Chlamydophila psittaci, Chlamydophila pecorum, Chlamydophila pneumoniae, Chlamydophila caviae, Chlamydophila felis,* and *Chlamydophila abortus* while genus *Chlamydia* had three members; *Chlamydia muridarum*, *Chlamydia suis* and *Chlamydia trachomatis.* However, the genus names *Chlamydia* and *Chlamydophila* were not used consistently in the literature, and recently it was proposed to keep only the genus name *Chlamydia* (*C.*) and transfer all *Chlamydophila* species to the genus *Chlamydia* (Fig 2; Kuo et al., 2011). The order *Chlamydiales* as well has been extended and currently comprises eight families of *Chlamydia* and distantly related *Chlamydia*-like organisms (Kuo et al., 2011).

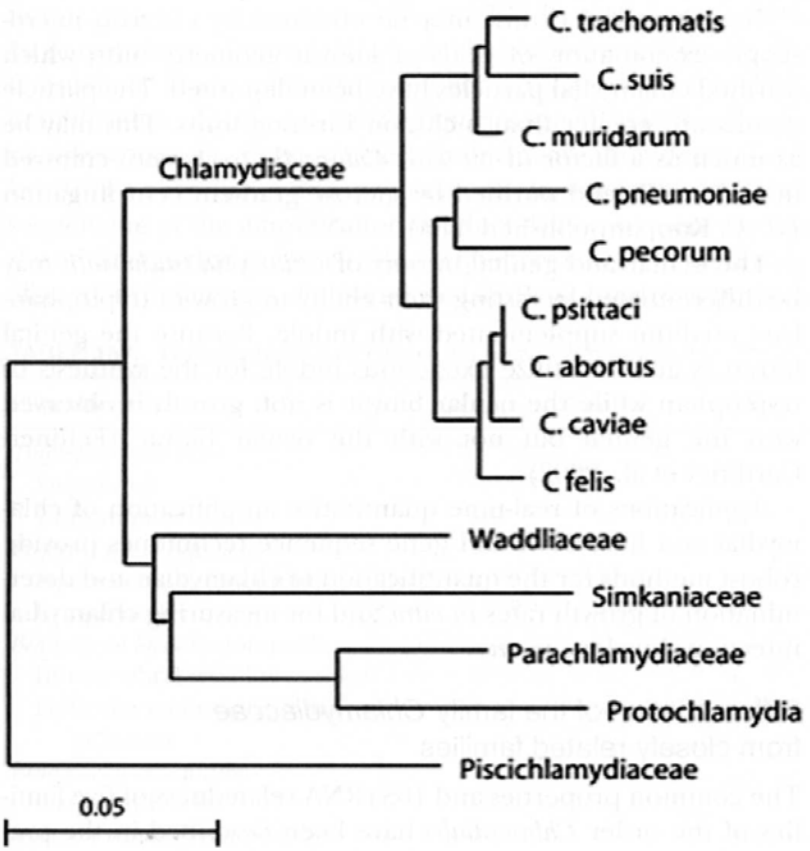

Figure 2. Phylogeny of the order *Chlamydiales* based on 16S rRNA similarities (Kuo et al. 2011). The tree was constructed using *Chlamydiaceae* and *Chlamydiaceae*-related organisms using neighbor joining with jukes-cantor distance corrections. Bar=0.05 substitutions/site (Kuo et al. 2011). Members of the family *Chlamydiaceae* share >90% sequence identity with each other while *Chlamydia*-like organisms with <80% relatedness to family *Chlamydiaceae* are grouped in separate families (Everett, 1999).

CHLAMYDIAL DISEASES

Disease manifestations. Chlamydial species cause various diseases unique to their hosts. The human pathogen, *C. trachomatis* (Grayston and Wang, 1975), causes ocular and genitourinary tract infection in humans often leading to blindness (trachoma), chronic salpingitis and tubal infertility (Kuo et al., 2011). *C. pneumoniae* infection is also common in humans and causes various respiratory manifestations such as pharyngitis, bronchitis, and pneumonia (Grayston et al., 1989). In humans, *C. pneumoniae* has also been linked to atherosclerosis and coronary heart disease (Kuo et al., 1993).

C. psittaci primarily infects birds and causes systemic, often latent respiratory and enteric infection widely known as psittacosis. *C. psittaci* is also infectious to humans (Kuo et al., 2011). Humans may contract *C. psittaci* infection by the airborne route, either by direct contact with infected birds or indirectly by inhalation of dust contaminated with infected bird's fecal material. *C. suis* causes conjunctivitis, pneumonia, enteritis and polyarthritis in swine (Kuo et al., 2011). *Chlamydia felis* causes conjunctivitis in cats which is often accompanied by rhinitis. In mice and hamsters, *C. muridarum* infection causes pneumonitis and less frequently interstitial keratitis. Guinea pigs are infected with *C. caviae* which causes follicular conjunctivitis and interstitial keratitis (Kuo et al., 2011).

C. abortus and *C. pecorum* commonly infect ruminants. In sheep, goats and cattle they cause abortion, vaginitis, endometritis, seminal vesiculitis, mastitis, polyarthritis, pneumonia, enteritis, encephalomyelitis and several other latent infections (Shewen, 1980: Reinhold et al., 2011). *C. pecorum* also causes similar infections in swine and koalas (Kuo et al., 2011).

Pathogenesis. *Chlamydia* spp. disease potential is determined by a complex array of infectious dose and host response factors such as host cell nutrient availability to chlamydial growth, host genetic susceptibility, host immune status which is further based on host age, concurrent diseases, reinfection, superinfection, and stresses.(Ramsey, 2006, Ward, 1999, Wang et al., 2009). Chlamydial lipopolysacchardide, a gram-negative endotoxin, plays an essential role in common disease conditions that are manifested as chronic granulomatous lesions of mononuclear cell aggregates and fibrosis. $CD4^+$ lymphocytes are crucial for host protection by restricting chlamydial replication via a Th1-type immune response (Morrison et al., 1995; Wang et al., 2009)

Chlamydia spp. normally infect mucosal epithelium, single cell-columnar layer of the epithelium, where they undergo a biphasic developmental cycle that produces infective forms, EBs, which then infect neighboring cells once RBs reorganize to EBs and are released (Brunham et al., 2005). At the site of mucosal infection *Chlamydia* induces inflammation that is characterized by redness, edema and often mucopurulent discharge (Peipert, 2003). However, despite initiating local inflammation, most infected animals remain asymptomatic.

In epithelial cells, *Chlamydia* spp. infection initiates production of various pro-inflammatory mediators, including CXC-chemokine ligand 1(CXCL1), CXCL8 (also known as Interleukin-8), CXCL16, granulocyte/monocyte colony stimulating factor (GM-CSF), IL-1α, IL-6 and tumor necrosis factor (TNF) (Rasmussen et al., 1997; Johnson, 2004). Expression of CC-chemokine ligand 5 (CCL5) and CXCL10 is also upregulated in epithelial cells which in turn increases the expression of chemokines that upregulate production of interferon (IFN)-γ, IFN-α, IFN-β, and IL-12 (Maxion et al., 2002; Johnson, 2004). Most of these cytokines are T helper 1

(Th1) cell cytokines which polarize the immune response to a protective Th1 response (Brunham et al., 2005). In contrast, TNF-α, IL-1α and IL-6 are involved in pathology associated with *Chlamydia* spp. infection (Darville et al., 2003).

Th1 cells are the inducers of a protective delayed type hypersensitivity (DTH). The DTH response features tissue infiltration of $CD4^+$ T cells and macrophages, and release of pro-inflammatory Th1 cytokines such as IL-1, IL-2, IL-12, IFN-γ, or TNF-α. The protective role of the Th1 response is centered on the role of interferon gamma (IFN-γ), a potent stimulator of macrophages which Th1 cells produce (Perry et al., 1997, Rottenberg et al., 2000). IFN-γ induces production of the enzyme indoleamine-2, 3-dioxygenase (IDO) that degrades crucial amino acid tryptophan in host cells which ultimately results in *Chlamydia* death by starvation. Th1 cytokines including IFN-γ increase the production of free radical molecules such as reactive oxygen species (ROS) and reactive nitrogen oxide species (RNOS), that can induce apoptotic cell death (Swindle et al., 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).

***Chlamydia* spp infection in cattle.** In cattle, the two species, *C. abortus* and *C. pecorum*, are routinely detected in acute infections with distinct clinical symptoms such as fertility disorders and abortion, mastitis, sporadic encephalomyelitis, kerato-conjunctivitis, pneumonia, enteritis and polyarthritis (McNutt et al., 1940; Storz et al., 1960; 1966; McKercher et al., 1966; Wilson et al., 1968; Storz et al.,1971; Rønsholt et al., 1981; Wittenbrink 1988; Griffiths et al., 1995; Kaltenboeck et al., 1997; Cox et al., 1998; Otter et al., 2003; DeGraves et al., 2004). A causal relationship of *Chlamydia* spp. with genital infection and reproductive disorders such as

endometritis, late-term abortion and still births has also been established through experimental studies (Papp et al., 1993; Papp et al., 1996; Jones et al., 1998).

However, besides these infrequent acute infections many more asymptomatic chlamydial infections can be detected in livestock (Reinhold et al., 2011), particularly after introduction of PCR diagnostics and commercially available ELISA assays (Livingstone et al., 2006; Sachse et al., 2009). It has been understood for a long time (Shewen, 1980) that a large percentage of cattle cohorts are *Chlamydia*-positive when just a few animals of these cohorts are randomly sampled (Godin et al., 2008; Petit et al., 2008;), although at different prevalence rates in individual animals ranging from quite low (<5%) to high (50-100%) (Reinhold et al., 2011). Higher herd prevalence typically associates with frequent sampling of many animals and high assay quality (Perez-Martinez et al., 1986; Smieja et al., 2001; Amin et al., 2003; DeGraves et al., 2003; Kauffold et al., 2007; Sachse et al., 2009), large cohort size and population density (Jee et al., 2004), open and low-quality herd management, poor hygiene, natural siring (Kemmerling et al., 2009), and nutritional deficiencies resulting in metabolic disorders (Wehrend et al., 2005). Thus, high prevalence in essence associates with all factors that favor susceptibility to, and transmission of, chlamydial infections, and with effective methods to detect them.

The few cases of severe or fatal chlamydial disease, in particular circumstances that favor transmission such as herd abortions, have always been considered the “tip of the iceberg” (Kaltenboeck et al., 2005), but these rare diseases are of little economic consequence. However, the health impact and economic consequences of the ubiquitous asymptomatic chlamydial infections in cattle have largely remained unknown. Only few studies have addressed this question, but they all significantly associate clinically asymptomatic chlamydial infections with decreased herd health and performance. Wehrend et al. (2005) found a high risk for ovarian

cysts and reproductive disorders if chlamydial antigen was present in the uterus of dairy cows, and Jaeger et al. (2007) and Reinhold et al. (2008) showed that latent chlamydial respiratory infection associated with airway obstruction and pulmonary inflammation in calves aged 2-7 months. Kemmerling et al. (2009) found lower milk yield and reproductive performance in dairy herds that tested positive for chlamydial infection.

While these studies demonstrated an association between asymptomatic chlamydial infection and health, they could not elucidate the cause-effect relationship, i.e. if chlamydial infection caused the health disorder or if the health disorder made the animals more susceptible to chlamydial infection. However, in an interventional field study using vaccination against *C. abortus* and *C. pecorum*, Biesenkamp et al. (2007) unequivocally demonstrated causality of latent chlamydial infection when *Chlamydia*-vaccinated animals showed a highly significant decline in milk somatic cell counts over mock-vaccinated animals. In an experimental study, DeGraves et al. (2004) showed a causal effect of chlamydial infection on reduction of fertility in heifers after *C. abortus* challenge without any clinical disease symptoms.

Similar to adult cattle, little is known about the health effects of widespread clinically asymptomatic chlamydial infections in calves. Calves are typically born free of chlamydiae (Jee et al., 2004), but may also be born infected after in utero infection (Reggiardo et al., 1989), and become again infected within the first weeks of life. Jee at al. (Jee et al., 2004) reported a 61% prevalence of chlamydial infection in apparently healthy young calves.

In comprehensive analyses, this dissertation will investigate the association of low level endemic chlamydial infection with neonatal health, growth, fertility and milk production in dairy cattle taking into consideration chlamydial infection intensity as well as the host systemic response to chlamydial infection. Given the multi-factorial nature of chlamydial disease, it is

likely that the host immune and metabolic status in addition to infection intensity may very well contribute to the observed influence in performance of the cattle.

Vaccination against *Chlamydia* spp infection. Increasing reports of high prevalence of asymptomatic chlamydial infection in a wide range of livestock species indicated the need for efficient prophylactic vaccines against *Chlamydia* spp. infection. The live-attenuated and inactivated vaccines currently in use especially against *C. abortus* in ruminants and *C. felis* in cats often carry the risk of being pathogenic. Moreover, little is known about their efficacy and consistency (Schautteet, et al., 2011). The high cost of such vaccines also makes them unsuitable as prophylactic vaccines in commercial dairy and other livestock industries. A subunit vaccine composed of protective antigens and adjuvant for optimal targeting of the immune response for prophylaxis as well as immunotherapy may efficiently overcome these concerns.

Recent advances in chlamydial immunobiology, cell biology, molecular pathogenesis, genomics, availability of complete genome sequences of *Chlamydia* spp. and animal models of infection have provided the information and techniques required to identify immunodominant chlamydial proteins. Chlamydial proteins such as the major outer membrane protein (MOMP), outer membrane complex (Omc) proteins, heat shock proteins (Hsp), members of the polymorphic membrane protein family (Pmp), proteins of the type III secretion system, chlamydial outer protein N (CopN), OmpH-like protein, enolases (Eno) and several other proteins have been identified as possible vaccine candidates by screening a genomic DNA expression library and assessing the immune response of mice immunized with those candidates after challenge infection with live *Chlamydia* (Murdin et al., 2000; Goodall et al., 2001; Finco et

al., 2005; Stemke-Hale et al.,2005; Li et al., 2006; Rodríguez et al 2006; McNeilly et al., 2007; Tammiruusu et al., 2007; Li et al., 2010). However, efficacious vaccines are not yet commercially available. Mouse model anti-*Chlamydia* vaccine studies are often confronted with a failure to elicit or reproduce a strong Th-1 response which is crucial for protection against *Chlamydia* infection. Several factors are responsible for poor Th-1 response such as dosing (concentration/frequency/interval), adjuvant, age of mice and several other limiting factors. A current trend in anti-*Chlamydia* vaccine research is focused on vaccine delivery approaches that can efficiently boost the Th-1 response (He et al., 2007; Sykes, 2008) and vaccines for commercial use can be expected soon.

RESEARCH OBJECTIVES

1. **Impact of endemic *C. pecorum* infection on growth rates of calves.** Observe 51 female calves at 2-week intervals from birth until 15 weeks of age at the EV Smith Research Center, Shorter Alabama, for health, growth, and development. Correlate the surrogate plasma markers of liver health, albumin and globulin, with biweekly growth rates and the plasma marker of growth, insulin like growth factor-1 (IGF-1) in dependence of vaginal and conjunctival chlamydial load and anti-*C. pecorum* IgM.

2. **Impact of endemic *C. pecorum* infection on fertility and milk production in dairy cattle.** Analyze in 451 first lactation dairy cattle at Barrington Dairy, Montezuma,

Georgia, the association of cervical *C. pecorum* infection at first timed artificial insemination with reproductive performance and milk production. Correlate cervical chlamydial infection intensity, anti-*C. pecorum* IgM immune trend, circulating luteinizing hormone, the plasma markers of liver health, albumin and cholesterol, with performance parameters such as conception and full-term pregnancies after first insemination, confirmed pregnancies at 205 days in milk, and milk yield.

Chapter 2: Impact of endemic *C. pecorum* infection on growth of neonatal calves

INTRODUCTION

Obligate intracellular bacteria of the phylum *Chlamydiae* infect virtually every eukaryotic organism, from single-celled amoebae to multicellular hosts including vertebrates (Horn et al., 2011). The family *Chlamydiaceae* comprises the single genus *Chlamydia* (*C.*) which encompasses nine species that cause the majority of chlamydial diseases in mammals and birds (Kuo et al., 2011). In cattle, the two species, *C. abortus* and *C. pecorum*, are routinely detected in acute infections with distinct clinical symptoms such as fertility disorders and abortion, mastitis, sporadic encephalomyelitis, kerato-conjunctivitis, pneumonia, enteritis and polyarthritis (McNutt et al., 1940; Storz et al., 1960; 1966; Wilson et al., 1968; Storz et al.,1971; Rønsholt et al., 1981; Wittenbrink 1988; Otter et al., 2003; DeGraves et al., 2004). However, besides these infrequent acute infections many more asymptomatic chlamydial infections can be detected in livestock (Reinhold et al., 2011), particularly after introduction of PCR diagnostics and commercially available ELISA assays (Livingstone et al., 2006; Sachse et al., 2009). It has been understood for a long time (Shewen, 1980) that a large percentage of cattle cohorts are *Chlamydia*-positive when just a few animals of these cohorts are randomly sampled (Godin et al., 2008; Petit et al., 2008;), albeit at different prevalence rates in individual animals ranging from quite low (<5%) to high (50-100%) (Reinhold et al., 2011). Even higher herd prevalence typically associates with frequent sampling of many animals and high assay quality (Perez-Martinez et al., 1986; Smieja et al., 2001; Amin, 2003; DeGraves et al., 2003; Kauffold et al.,

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The objective of the present investigation was to quantify the impact of these infections in a comprehensive prospective study. We followed a cohort of female calves from birth to 15 weeks of age, and found that they all became asymptomatically infected with *C. pecorum*. Thus, rather than associating presence or absence of chlamydial infection with changed health, we analyzed if changed intensity of chlamydial infection resulted in different health outcomes. In addition to parameters of chlamydial infection and clinical appearance, whole blood and plasma markers for health and growth were evaluated. In the absence of overt disease, we focused on physiological parameters that would be negatively affected by responses to chronic inflammation, in particular body weight and its change over time, the economically most important health parameter in juvenile cattle. We show here that subclinical infection by *Chlamydia pecorum* reduces growth rates and body weight, and find strong support that anti-*C. pecorum* immunity after peak infection protects calves from subsequent losses in body weight.

MATERIAL AND METHODS

Calves and calf husbandry. The study was performed at the EV Smith Dairy Unit of the Alabama Agricultural Experiment Station in Shorter, AL, USA. Dams were maintained in free-stall housing with mattresses, fed a 17% protein total mixed ration on corn silage base, and spent 6 hours per day on a grass lot. They were vaccinated once annually with a multivalent vaccine against bovine viral and bacterial diseases (Vira Shield 6+VL5 HB, Novartis Animal Health) and dewormed with doramectin (Dectomax, Pfizer Animal Health). Immediately after birth, calves were kept with the dam in a calving pen and fed colostrum of their dam or a colostrum pool. The next day, calves were separated from dams and housed in individual pens until they were weaned at 7 to 8 weeks of age. They received per day 4 kg bulk milk and water and 24% protein custom-mixed grain starter feed ad libitum containing 0.01% of the coccidiostatic lasalocid. Only healthy female calves born to dams that were free of any clinically apparent disease were enrolled in the study because male calves were removed from the herd at 2 weeks of age. After weaning, calves were raised in a common pasture with freely accessible hutches together with herd replacement heifers. In addition to seasonal grazing, they were provided hay ad libitum and 3 kg per day of starter calf compound feed containing 20% crude protein and 0.005% lasalocid. Throughout the study period, calves did not receive any antibiotics. Calf-dam 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 in protocol # 2010-1714 by the Auburn University Institutional Animal Care and Use Committee.

Experimental design. The investigation was a prospective cohort study that examined the effect of natural infection with *Chlamydia* spp. on neonatal health and growth rates. In total, 26 Jersey and 25 Holstein female calves were continuously enrolled over a 25-week period and sampled for 39 weeks. Individual animals were sampled in 2-week intervals starting as early as on day 0 immediately after birth until 15 weeks of age. At each sampling time point, body weight was recorded and calves were scored for clinical parameters including alertness, muzzle dryness, conjunctival and vaginal mucosal color prior to swab sampling, lacrimal secretion, gait, and any signs of lameness. Mucosal color was recorded as an inflammation score ranging from 1-4 (1 = white, anemic; 2 = normal pink; 3 = mild to moderate redness; 4 = pronounced redness). In addition, EDTA-blood, heparin plasma, and conjunctival and vaginal cytobrush specimens were collected from each calf.

Hematological analyses. Blood was collected by venipuncture of the jugular vein in EDTA- and heparin-treated blood collection tubes (BD Vacutainer, Becton Dickinson and Company). Plasma was obtained from heparin blood by centrifugation at 1300×g for 15 min. Complete blood counts were performed using an Advia 120 automated hematology analyzer following manufacturer's instructions (Siemens Medical Solutions). Complete blood counts and differential blood counts were recorded as absolute values and percentages, as well as morphologies of white blood cells (WBC), red blood cells (RBC) and platelets including left shift, atypical lymphocytes, blast cells, immature granulocytes, nucleated RBC, RBC fragments, RBC ghosts, platelet clumps, and large platelets.

Plasma analyses. Plasma colorimetric chemistry analyses (iron, albumin, total protein) were performed by use of an automated cobas c 311 systems analyzer following manufacturer's instruction (Roche/Hitachi). Globulin was calculated as total plasma protein minus albumin. Plasma concentrations of IGF-1 were measured using a validated double antibody precipitation method as originally described (Elsasser et al., 1988). Modifications to the assay as presently performed included the use of both human (100% homology to bovine) IGF-1 and rabbit antihuman IGF-1 serum (GroPep) as used for construction of the standard curve and as used as the primary antibody, respectively. Radioactive tracer for the assay was human ^{125}I-IGF-1 (Perkin Elmer).

Anti-*C. pecorum* immunoglobulin M (IgM) ELISA. *C. pecorum* type strain E58 (ATCC VR-628; McNutt et al., 1940) was propagated and elementary bodies (EB) purified as described (Li et al., 2005), and EB lysates for use as antigen were prepared as described earlier (Jee et al., 2004). Lysate antigen equivalent to 0.1 µg protein/well in bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) was coated onto white, high protein binding flat-bottom microtiter plates. Plates were incubated overnight at 4°C, the coating solution aspirated, and wells washed 5 times with wash buffer (0.1 M Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1% (v/v) Tween-20). Wells were then blocked with 200 µl of assay diluent (0.1 M Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1% Tween-20, 10% (v/v) normal rabbit serum) for 1 hour at room temperature followed by 30 min incubation of 100 µl calf plasma diluted 1:25 in assay diluent. After 5x washing, bound bovine IgM was detected with horseradish peroxidase-conjugated polyclonal sheep antibody against bovine IgM (Bethyl Laboratories), 1:300 diluted in assay diluent, followed after 5x washing by incubation with chemiluminescent substrate (Roche Applied Science). 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 the negative control serum from a gnotobiotic calf (<500 rlu/sec) was subtracted from the data, and results were normalized between assay plates by a factor adjusting for differences between the signal of the positive control serum (McNutt et al., 1940,: Jee et al., 2004).

Quantitative detection of chlamydiae, *ompA* typing, and DNA sequencing. Conjunctival cytobrush swab samples were collected with a single swab from both eyes by extroverting the lower eyelid with thumb and index finger to form a pocket in which the cytobrush (Puritan, Hardwood Products) was rotated 5 times. 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) in microcentrifuge tubes. Swab samples were centrifuged at 250×g for 1 min and stored at -80°C without the brush. Vaginal swab cytobrush samples were collected similarly after cleaning and alcohol disinfection of the perivaginal area.

Prior to nucleic acid extraction, all samples were homogenized (Precellys 24 tissue homogenizer/grinder; Bertin). After total nucleic acid extraction by glass fiber binding and elution in 2×20 µl Tris-EDTA buffer (DeGraves et al., 2003), chlamydial DNA was quantitatively detected and differentiated by modified real-time fluorescence resonance energy transfer (FRET) PCR targeting the *Chlamydia* spp. 23S rRNA gene (Jee et al., 2004;, DeGraves et al., 2003). Primer CHL23SUP was replaced by primers CPEC23SUP (GGGGTTGTAGGGTCGATAACGTGAGATC) and CTR23SUP (GGGGTTGTAGGRTTGRGGAWAAAGGATC) which were each used at 0.5 µM final concentration. New probes used were genus-specific CHL23SFLU

(GRAYGAHACAGGGTGATAGTCCCGTA-6FAM; 0.1 µM), *C. abortus*-, *C. psittaci*-, *C. pecorum*-, *C. pneumoniae*-, *C. felis*-, and *C. caviae*-specific CP23LCR (LightCycler Red 640-ACGAAARAACAARAGACKCTAWTCGAT-Phosphate; 0.2 µM) and *C. trachomatis*-, *C. suis*-, and *C. muridarum*-specific CTR23CY5.5 (Cy5.5-ACGAAAGGAGAKMAAGACYGACCTCAAC-Phosphate; 0.2 µM). This probe composition allowed detection of the CP23LCRF FRET signal at 640 nm and of the CTRCY5.5 signal at 705 nm. All other parameters remained unchanged. Confirmatory typing of chlamydial species was performed by chlamydial *ompA* real-time FRET PCR as described (DeGraves et al., 2003). DNA sequencing of filter-purified *ompA* amplification products was performed with both primers by a fluorescent Sanger method (Eurofins MWG Operon). The DNA sequence of the novel *ompA* genotype Smith3v8 identified in this study has been deposited to GenBank with the accession # JX272924.

Statistical analyses. All statistical analyses were performed with the Statistica 7.0 software package (StatSoft). Chlamydial load data were logarithmically transformed after addition of 1 to the linear data. Negative results were treated as 0 in the log-transformed data. The average chlamydial load per calf at any sampling time point was calculated as the mean logarithm of vaginal load plus conjunctival load multiplied by the ratio of vaginal: conjunctival mean logarithm. The difference in PCR positivity of conjunctival and vaginal samples was evaluated by Chi square test.

Cases were separated by median values or 33.3 and 66.6 percentiles into ordinal categories of chlamydial loads or anti-*C. pecorum* IgM, or into clusters by cluster analysis of principal components. Principal component analysis (PCA) based on the correlation matrix was

used to reduce the dimensionality of the data set and to identify principal components as linear combinations of optimally-weighted underlying original variables. The principal components were then used to perform a disjoint cluster analysis to delineate natural clusters present in the cases based on least-squares estimation of Euclidean distances. Effects between groups were measured using Student's t-test for normally distributed data as determined by Shapiro-Wilk's W test. Comparison of means of ordinal data such as the conjunctival inflammation score was performed by the non-parametric Mann-Whitney test.

RESULTS

Development of calves. Over a 6 month period, a total of 26 Jersey and 25 Holstein female calves were enrolled in the study immediately after birth. If possible, plasma and swab samples were collected prior to the first colostrum feeding and calves were further sampled in two-week intervals from one week to fifteen weeks of age. The average body weight in the first week of life was 29.97 ± 0.94 (SEM) kg (Jersey 26.14 ± 0.79 kg, Holstein 33.81 ± 1.34 kg; $P<10^{-4}$), and at 15 weeks 82.86 ± 2.57 kg (Jersey 70.66 ± 2.37 kg, Holstein 95.55 ± 2.99 kg; $P<10^{-4}$) (Fig. 1A). The average growth rate expressed as percent body weight difference relative to the body weight two weeks earlier was 15.22 ± 0.48 %, without significant differences between the breeds. As evident in Fig. 1A, biweekly body weight gain declined from the maximum of 21.7% in week 7 to a minimum of 7.5 % in week 13, presumably due in part to abrupt weaning at 8 weeks of age and the switch to roughage feeding with low nutrient density (Khan et al., 2011).

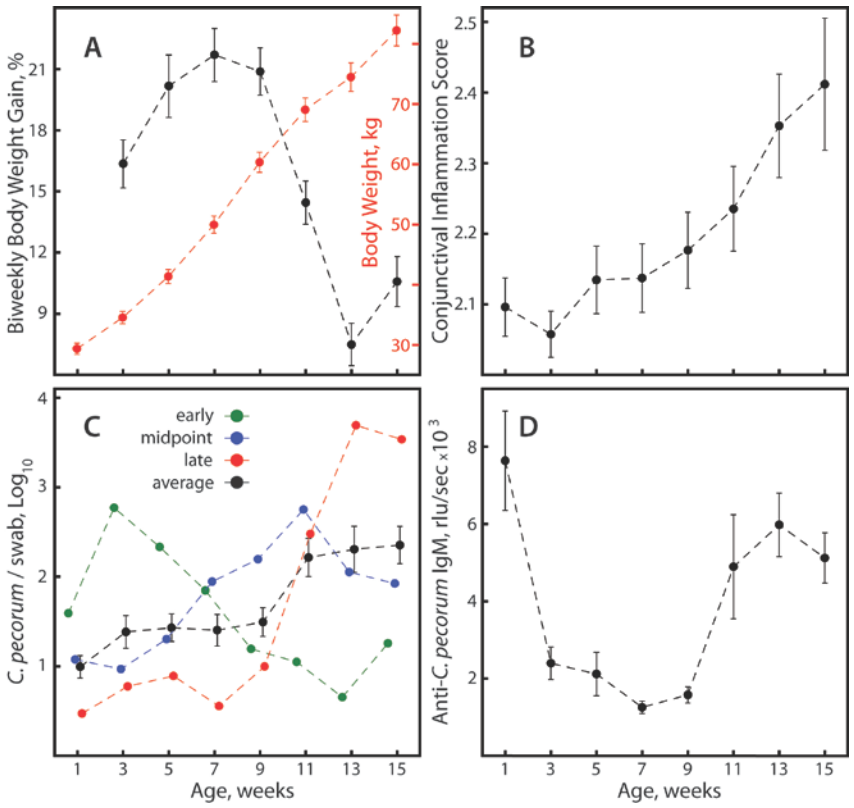

Figure 1. Development of calves and chlamydial infection. The progression over the sampling period is shown. (A) Body weight gain over successive 2-week periods and absolute body weight. (B) Conjunctival inflammation as expressed by an arbitrary score from 1-4 for redness, with 2 for normal pink coloration of the conjunctiva. (C) Average *C. pecorum* genomes per cytobrush swab detected by *Chlamydia* spp. 23S rRNA gene real-time FRET PCR. Early, midpoint, or late indicates peak *C. pecorum* infection before week 9, in week 9 or 11, or in week 13 or 15. For clarity, only the mean of all calves is shown with error bars. (D) Anti-*C. pecorum* IgM antibodies as determined by chemiluminescent ELISA using a lysate antigen of *C. pecorum* elementary bodies. Data are shown as means ± SEM.

The clinical appearance of all calves during the entire study period was normal, without obvious signs of clinical disease. Similarly, complete and differential blood cell counts as well as plasma albumin, globulin, iron, and IGF-1 did not exceed normal ranges. However, as shown in Fig 1B, the arbitrary score for redness and inflammation of the conjunctiva (1 = anemic, 2 = normal pink, 3 = pronounced red, 4 = deep red) continuously and highly significantly increased from less than 2.1 in weeks 1 and 3 to 2.4 in week 15 ($P<0.005$).

Out of 43 precolostral conjunctival and vaginal cytobrush swab samples obtained immediately after birth from the total of 51 calves in the study, 27 were negative for chlamydial DNA in the *Chlamydia* spp. 23S rRNA gene real-time PCR, but 16 calves were positive at low copy number below 10 chlamydial genomes in either conjunctival or vaginal swab, or both (n=8, 5, and 3, respectively). Some calves (n=16, 37%) at birth were free of PCR or serological evidence (anti-*C. pecorum* IgM) of chlamydial infection, while all others either showed only PCR (n=8, 19%) or serological evidence (n=11, 28%) of chlamydial exposure, or both (n=8, 19%). Eventually, all calves in the study became *Chlamydia* spp. PCR-positive as well as developed anti-*C. pecorum* IgM. The data from pre-colostrum sampling were used to establish pre- or postnatal chlamydial infection, but were not used in subsequent repetitive analyses of the calves.

From all 51 calves included in the study, a total of 816 conjunctival and vaginal specimens were collected in 2-week intervals between 1 to 15 weeks of age, and 606 (74.3%) of these specimens were positive in the *Chlamydia* spp. 23S rRNA gene PCR. The average chlamydial load per positive conjunctival swab was 43 genomes (range 1-65,800; antilog), and of vaginal swabs 227 (range 1-1,771,600; antilog). The only chlamydial species detected was *C. pecorum* in the *Chlamydia* spp. 23S rRNA gene PCR.

Of the 408 paired conjunctival-vaginal calf specimens, 64 were negative in both swabs, 50 were positive only in the conjunctival swab, 32 only in the vaginal swab, and 262 positive in both, with no breed difference. Based on the statistically identical positivity of both sampling sites (Chi square test), but the lower conjunctival load ($P<10^{-4}$), we calculated a mean-adjusted overall chlamydial burden per calf and sampling time as described in methods, referred thereafter as *C. pecorum*/swab. Typically, both conjunctival and vaginal specimens became very low positive at one sampling time point, remained low for two to four weeks, then increased to peak positivity and declined again to negativity over two to four weeks. Based on the infection kinetics, we categorized the calves into three approximately equal groups as early (1-5 weeks: n=13), midpoint (7-11 weeks: n=20) or late (13-15 weeks: n=18) peak *C. pecorum*-infected (Fig. 1C). Average infection intensity was independent of breed and increased significantly with the age of the calves (e.g., week 7 vs. week 15 = 24 vs. 225 genomes/swab; $P<0.001$) particularly after weaning at 8 weeks when they were transferred from individual hutches to a common pasture. Some of the calves with an early peak infection before week 5 had a recurrence or increase of the infection towards the end of sampling in week 15.

C. pecorum ompA real-time PCR of 31 selected specimens from 19 calves distributed along the complete sampling period confirmed *C. pecorum* (Kaltenboeck et al., 1993, 1997, Mohamad et al., 2008, 2010; Kaltenboeck et al., 2009). However, sequencing of the amplification products identified three distinct strains with the *ompA* genotype 1710S (GenBank Accession # M73033.1), Maeda (GenBank Accession # AB512085.1), and the novel *ompA* genotype Smith3v8 (GenBank Accession # JX272924). Over the first eight months of specimen collection, only genotype Smith3v8 was identified in *C. pecorum*-positive swabs.

This strain disappeared within a two-week interval and was replaced by strains 1710S and Maeda in the last 3 sampling months. In the strain transition period, mixed infections with combinations of Smith3v8 and Maeda and/or 1710S were identified in four calves by dual peaks at polymorphic positions in the *ompA* amplicon sequences. Three calves also were infected with different strains in conjunctiva and vagina, and three calves over time showed repeated infection peaks with different strains before and after the 2-week strain transition period, while such repeated peaks prior to transition were only caused by strain Smith3v8.

Anti-*C. pecorum* IgM. From 40 calves sampled that had presumably received no colostrum, a complete absence of plasma IgM antibodies against *C. pecorum* indicated that only 21 truly had been sampled immediately after birth before first suckling, while 19 showed variable levels of anti-*C. pecorum* IgM. Mean plasma anti-*C. pecorum* IgM were highest with 7569 relative light unit (rlu)/sec at 1 week of age, then dropped precipitously to a minimum of 1,256 rlu/sec early sampling time points (Fig 1D, $P<10^{-4}$), suggesting a rapid disappearance of passively acquired IgM antibodies (Husband et al., 1972; Porter, 1972; Smith et al., 1976). After week 7, IgM antibody levels rose again, presumably due to an emerging antibody response to chlamydial infection, with a secondary peak of 5,978 rlu/sec anti-*C. pecorum* IgM in week 13. Similar to chlamydial infection, the kinetics of antichlamydial antibody levels also varied among calves. While in some calves, antibody levels remained at or dropped to zero for several weeks after week 1 (n=15), others showed only a minor decline followed by steady or increasing antibody levels (n=36). Based on the week-7 antibody minimum, to lessen the potential influence of passive immunity, we performed subsequent analyses with data only from week 7 to 15, and utilized the week 1-to-week 5 dataset for confirmation of results.

Physiological effects of *C. pecorum* infection. To determine if *C. pecorum* infection influenced health and weight gain of the calves, we categorized the data of all calves from week 7 through week 15 into 2 or 3 ordinal groups based on parameters of contemporaneous chlamydial exposure, i.e. *C. pecorum* load (*C. pecorum*/swab) or anti-*C. pecorum* IgM, the primary and early response antibody isotype (Jee et al., 2004). These 255 observations were separated into 2 or 3 equal groups of low or high, or low, intermediate, or high parameter values. Analysis of these groups revealed significant differences in biweekly weight gains, conjunctival inflammation, and several plasma markers that correlated with *C. pecorum* load or anti-*C. pecorum* IgM (Table 1, 2). Most prominently, high chlamydial loads or high antichlamydial antibodies highly significantly associated with reduced weight gain and increased conjunctival inflammation (Table 1). Animals with high chlamydial load showed 13.77% biweekly weight gain as compared to animals with low load with a 16.26% weight gain, a 15% reduction in the growth of calves with high chlamydial infection (P=0.047). This association was even more pronounced in 3-category analysis of chlamydial load groups (P=0.003), or when animals were categorized by antibody levels. At the highest difference at 3 antibody categories, animals with low anti-*C. pecorum* plasma antibodies showed an 18.90% biweekly weight gain, while calves with high antibody levels gained only 10.91% body weight over 2 weeks, a 42% growth rate reduction ($P<10^{-4}$). Conjunctival inflammation scores followed a similarly significant, but reverse pattern in which high chlamydial load or anti-chlamydial antibodies associated with high inflammation. This suggests a strong association between inflammatory response to chlamydial infection and the rate of weight gain in which high inflammation correlates with low weight gains in these calves.

Table 1. Physiological and chlamydial infection markers categorized by *C. pecorum* load and plasma anti- *C. pecorum* IgM.

Grouping Parameter	Category[#]	N	Biweekly Body Weight Gain, %		Conjunctival Inflammation Score		*C. pecorum* / swab, Log_{10}		Anti-*C. pecorum* IgM, rlu/sec	
			Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
C. pecorum load	Low	128	16.26[a]	14.57 - 17.95	2.15[A]	2.09 - 2.21	0.69[A]	0.59 - 0.80	2,151[A]	1,765 - 2,538
	High	127	13.77	11.98 - 15.56	2.39	2.28 - 2.49	3.24	3.07 - 3.41	5,408	4,073 - 6,742
	Low	85	16.46[A]	14.51 - 18.41	2.14[A]	2.07 - 2.22	0.34[A,B]	0.26 - 0.42	2,034[A]	1,619 - 2,450
	Intermediate	85	16.35[A]	13.98 - 18.71	2.21[A]	2.12 - 2.31	1.79[A]	1.69 - 1.89	2,657[A]	1,974 - 3,339
	High	85	12.25	10.23 - 14.27	2.45	2.32 - 2.58	3.75	3.57 - 3.92	6,629	4,751 - 8,506
Anti-*C. pecorum* IgM	Low	128	17.53[A]	15.78 - 19.28	2.14[A]	2.08 - 2.20	1.46[A]	1.26 - 1.66	878[A]	753 - 1,003
	High	127	12.49	10.85 - 14.13	2.39	2.29 - 2.50	2.45	2.16 - 2.75	6,691	5,443 - 7,938
	Low	85	18.90[A,b]	16.68 - 21.13	2.11[A]	2.04 - 2.17	1.52[A]	1.26 - 1.78	463[A, B]	362 - 563
	Intermediate	85	15.24[A]	13.40 - 17.08	2.20[A]	2.11 - 2.29	1.47[A]	1.21 - 1.73	2,186[A]	2,055 - 2,317
	High	85	10.91	8.87 - 12.96	2.49	2.36 - 2.63	2.88	2.52 - 3.24	8,670	6,950 -10,390

[#] *C. pecorum* load: low <1.79, high ≥1.79; low <1.02, intermediate <2.62, high ≥2.62; anti-*C. pecorum* IgM: low <2173, high ≥2173; low <1270, intermediate <3636, high ≥3636.

Significant differences are indicated by [a] from "High" at $P<0.05$; [A]from "High" at $P<0.01$; [b] from "Intermediate" at $P<0.05$; [B] from "Intermediate" at $P<0.01$.

To further identify possible physiological mechanisms that mediate the effect of chlamydial infection on growth rates, we analyzed a set of hematological parameters that could potentially also serve as surrogate markers for such mechanisms. While complete and differential blood counts and plasma iron did not show consistent differences, plasma albumin and globulin showed highly significant and consistent differences in dependence of chlamydial infection intensity (Table 2). Plasma albumin followed the pattern of weight gains and was highly significantly reduced at high chlamydial infection intensity. In contrast, plasma globulin showed the reverse pattern and followed the changes in conjunctival inflammation, with high values highly significantly associated with high chlamydial infection intensity.

These data support the notion that clinically inapparent chlamydial infection elicits an inflammatory response that is detrimental to growth in calves, consistent with the growth stunting effects of bacterial infections or LPS of gram-negative bacteria that is mediated by circulating inflammatory cytokines such as IL-1, IL-6, and TNF-α (Lang et al., 2002; Elsasser et al., 2008). Another possibility, however, would be that confounding factors that are collinear with the parameters of chlamydial infection simulate the observed effects. While controlling for breed, time of enrollment, or cohort size does not change the patterns of result, controlling for age or body weight of the calves reduces many effects substantially and eliminates some. In fact, chlamydial loads, anti-chlamydial IgM levels, and conjunctival inflammation increased with age, and concomitantly with body weight, of the calves (Fig. 1B, C, D). Simultaneously the body weight gains of the calves decreased with age (Fig. 1A), at a time when their nutrition was converted to roughage, and the calves had more frequent cohort contacts on the free-range pasture.

Table 2. Plasma markers categorized by *C. pecorum* load and anti-*C. pecorum* plasma IgM category.

Grouping Parameter	Category[#]	N	Albumin, g/dL		Globulin, g/dL		IGF-1, ng/mL	
			Mean	95% CI	Mean	95% CI	Mean	95% CI
C. pecorum load	Low	128	3.31^{A}	3.27 - 3.34	3.00^{A}	2.91 - 3.09	105.32^{a}	93.07 - 117.57
	High	127	3.17	3.13 - 3.22	3.32	3.22 - 3.42	87.57	77.11 - 98.03
	Low	85	3.30^{A}	3.26 - 3.35	2.98^{A}	2.88 - 3.09	107.46^{A}	92.22 - 122.70
	Intermediate	85	3.29^{A}	3.24 - 3.34	3.08^{A}	2.98 - 3.19	101.36^{a}	87.24 - 115.48
	High	85	3.13	3.07 - 3.19	3.41	3.29 - 3.54	80.63	68.24 - 93.01
Anti-*C. pecorum* IgM	Low	128	3.30^{A}	3.27 - 3.33	2.92^{A}	2.84 - 2.99	107.43^{A}	95.20 - 119.66
	High	127	3.18	3.13 - 3.23	3.41	3.31 - 3.51	85.45	75.09 - 95.81
	Low	85	3.31^{A}	3.27 - 3.35	2.80^{A,B}	2.72 - 2.89	116.06^{A,b}	101.18 - 130.94
	Intermediate	85	3.29^{A}	3.24 - 3.34	3.15^{A}	3.05 - 3.26	95.85	82.03 - 109.67
	High	85	3.13	3.07 - 3.19	3.53	3.42 - 3.63	77.54	65.03 - 90.04

[#] *C. pecorum* load: low <1.79, high ≥1.79; low <1.02, intermediate <2.62, high ≥2.62; anti-*C. pecorum* IgM: low <2173, high ≥2173; low <1270, intermediate <3636, high ≥3636.

Significant differences are indicated by ^{a} from "High" at $P<0.05$; ^{A} from "High" at $P<0.01$; ^{b} from "Intermediate" at $P<0.05$; ^{B} from "Intermediate" at $P<0.01$.

To circumvent the problem of collinearity, we used two approaches: 1) analyze week 1-5 data as control dataset, and 2) analyze a physiological marker of growth. Use of the week 1-5 dataset highly significantly confirmed that calves with high anti-*C. pecorum* IgM had lower weight gains than those with low IgM (12.40 vs. 17.38%, P=0.003), despite the confounding influence of maternal antibodies and overall lower chlamydial infection intensity. Interestingly, in the week 1-5 dataset, age was highly significantly negatively correlated with anti-*C. pecorum* IgM ($P<10^{-4}$), but consequently, in contrast to the week 7-15 dataset, positively correlated with weight gain. Therefore, body weight gains were consistently higher in calves with low anti-*C. pecorum* IgM, but did not consistently change with age of the calves.

To confirm this observation, we analyzed as direct marker of growth the plasma levels of insulin-like growth factor-1 (IGF-1), the systemic mediator of somatic growth (Collet-Solberg et al., 2000). Again, IGF-1 completely followed the pattern of body weight gains and plasma albumin, and the reverse patterns of chlamydial infection intensity, conjunctival inflammation, and plasma globulin. These data confirm that chlamydial infection intensity itself associates with reduction in actual somatic growth, expressed as decreased plasma IGF-1, rather than confounding variables simulate this outcome while the propensity for body weight gains remains constant.

Multifactorial modeling of *C. pecorum* infection and host response. To analyze the complex interactions of *C. pecorum* infection and host response to it, we used a hypothesis-free modeling approach. Principal component analysis combined markers for chlamydial infection and host response to maximally explain the variance in the observations, subsequent cluster analysis grouped similar observations, and T-tests of clusters revealed the biological

significance. Anti-*C. pecorum* IgM, albumin, and globulin combined into two principal components (PC) resulted in the greatest explanatory power of all potential combinations of original variables ($r^2 = 0.813$; Fig. 2). PC 1 was composed in approximately equal parts by these 3 variables, with albumin negatively contributing, and explained 57% of the variance ($r^2 = 0.572$). We interpreted the strong contribution of anti-chlamydial IgM to PC 1, as well as the negative albumin and positive globulin contribution, as an indicator of the inflammation driven by chlamydial infection, and therefore termed PC 1 "Chlamydial Inflammation Index". PC 2 was positively affected by albumin and anti-*C. pecorum* IgM, but not globulin, and explained 24% of the variance ($r^2 = 0.241$). Our interpretation of the positive anti-chlamydial antibody contribution combined with the positive albumin contribution was that this reflected immune protection following chlamydial infection that improved liver function. Therefore we termed PC 2 "Metabolic Immune Protection Index".

Cluster analysis of these two principal components separated cases with high chlamydial inflammation index and low metabolic immune protection index as cluster 1 from cases with reverse index characteristics as cluster 2 (Fig. 2). Cluster 1 calves had a highly significantly lower biweekly weight gain of 9.31% as compared to cluster 2 calves with 17.82% weight gain, a profound 48% reduction of the growth rate (Table 3; $P<10^{-4}$). This reduced weight gain was highly significantly accompanied by decreased albumin, IGF-1, and metabolic immune protection index, and increased conjunctival inflammation, *C. pecorum* load, anti-*C. pecorum* IgM, globulin, and chlamydial inflammation index ($P<10^{-4}$). Interestingly, using these principal components to separate cases, plasma iron was also found highly significantly decreased in cluster 1 calves ($P=0.002$).

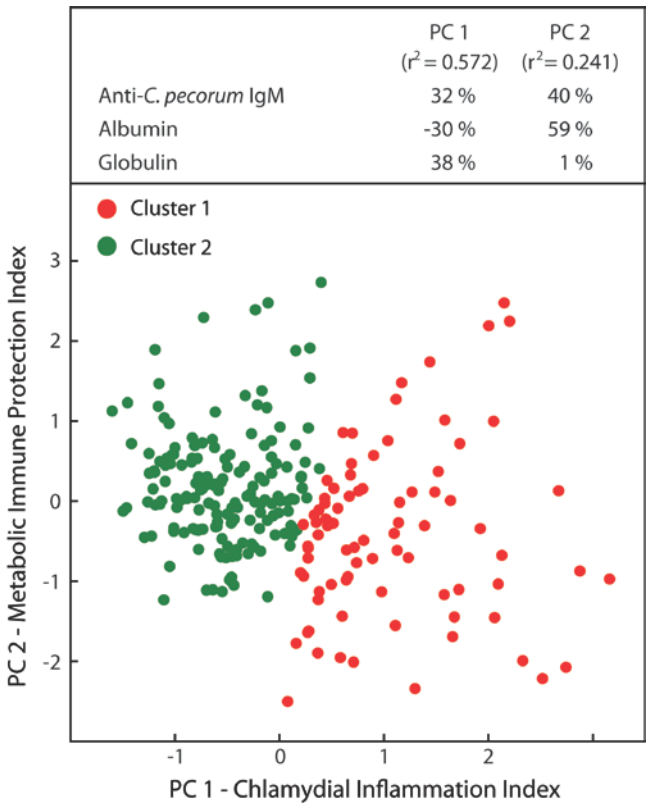

Figure 2. Multifactorial modeling of *C. pecorum* infection and host response by principal component and cluster analyses**.** Principal component analysis of the observations from week 7 to 15 used the three parameters that best accounted for data variance: plasma levels of anti-*C. pecorum* IgM antibody, albumin, and globulin. More than 81% of variance observed among animals and between sampling points were explained only by two principal components that were termed to reflect the biological significance of the combination of the original variables as shown with their partial r^2 in the upper panel. Cluster analysis of PCs for each data point separated all data into two clusters based on Euclidean distances between data points.

Table 3. Physiological and chlamydial infection parameters categorized by PCA cluster.

	Cluster 1 (N=84) [a]	Cluster 2 (N=171)
Biweekly Body Weight Gain, %	9.31 ± 2.04	17.82 ± 1.37
Conjunctival Inflammation Score	2.55 ± 0.14	2.13 ± 0.05
***C. pecorum* / swab, Log_{10}**	2.83 ± 0.36	1.53 ± 0.19
Anti-*C. pecorum* IgM, rlu/sec	7379.46 ± 1864.30	2001.70 ± 331.75
Iron, µg/dL	145.94 ± 9.82	168.37 ± 8.53
Albumin, g/dL	3.00 ± 0.05	3.36 ± 0.02
Globulin, g/dL	3.65 ± 0.10	2.92 ± 0.06
IGF-1, ng/mL	74.30 ± 13.18	107.38 ± 9.84
Chlamydial Inflammation Index	1.096 ± 0.17	-0.538 ± 0.09
Metabolic Immune Protection Index	-0.349 ± 0.25	0.172 ± 0.13

[a] Data are shown ± 95% confidence interval. Differences between cluster 1 and 2 are significant at $P=0.0017$ for plasma iron, and at $P<10^{-4}$ for all other parameters.

Anti-chlamydial IgM antibodies in our analyses thus far were used to indicate chlamydial infection intensity contemporaneously with health outcomes. In the next analysis, we asked if earlier chlamydial infection intensity could also act as a leading indicator of an anti-chlamydial immune response that protected against chlamydial infection and affected subsequent growth rates. To this end, we combined in principal component analysis anti-*C. pecorum* IgM in week 7 for each calf with plasma albumin and globulin data eight weeks later in week 15. Two PCs explained 82% of the variance (Fig. 3). Albumin contributed positively, and globulin negatively to PC 1, which was therefore termed "Metabolic Fitness Index" while PC2 was virtually exclusively positively driven by anti-*C. pecorum* IgM and was termed "Chlamydial Immune Protection Index". Cluster analysis separated all calves into two clusters, which we termed low and high responders on the basis of high or low values in the PCs (Fig. 3). Calves of the high responder cluster started out in week 7 with body weight gains similar to low responders, but gradually developed increasing weight gains that were significantly higher in weeks 13 and 15 (Fig. 4A; $P \leq 0.034$). These higher weight gains translated from similar starting body weights to 10.3 kg higher body weight of 89.7 kg of high responders compared to 79.4 kg of low responders, albeit statistical significance was not reached (Fig. 4B; $P=0.059$). IGF-1 followed a similar pattern and was significantly higher in week 13 for the high responder cluster (Fig. 4C; $P=0.014$). Clinically, the high responder cluster did not show conjunctival inflammation while the low responders developed increased inflammation over time, highly significantly so in week 13 (Fig. 4D; $P=0.003$). These physiological differences were accompanied by corresponding trends in markers of chlamydial infection intensity (Fig. 4E, F) and plasma markers of inflammation and metabolic health (Fig. G, H). These outcomes were consistent with previous results (Table 1, 2, 3; Fig. 2) in that the healthier high responder calves over time showed

reduced anti-*C. pecorum* IgM (Fig. 4E), *C. pecorum* loads (Fig. 4 F), plasma globulin (Fig. 4H), and increased plasma albumin (Fig. 4G). In summary, these data support the notion that *C. pecorum* infection leads to immune protection from disease or subclinical health consequences, but not to sterilizing immunity that immediately eliminates chlamydial infection.

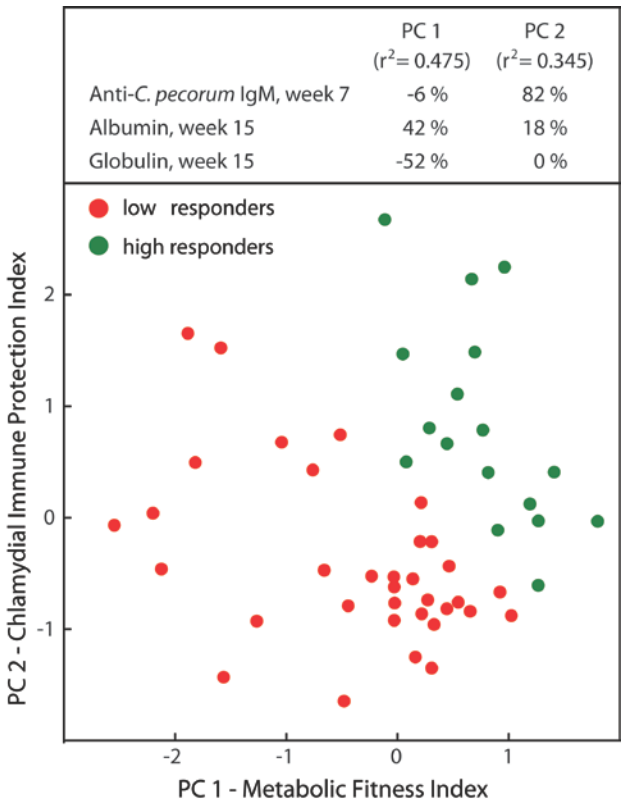

Figure 3. Modeling of metabolic health in dependence of earlier anti-*C. pecorum* immunity. Predictive modeling by principal component and cluster analysis of plasma albumin and globulin in week 15 of each animal combined with the corresponding anti-*C. pecorum* IgM 8 weeks earlier in week 7 generated two PCs with a combined r^2 of 0.82. Two clusters of calves were termed low (n=34) or high responders (n=17) based on the values for each PC.

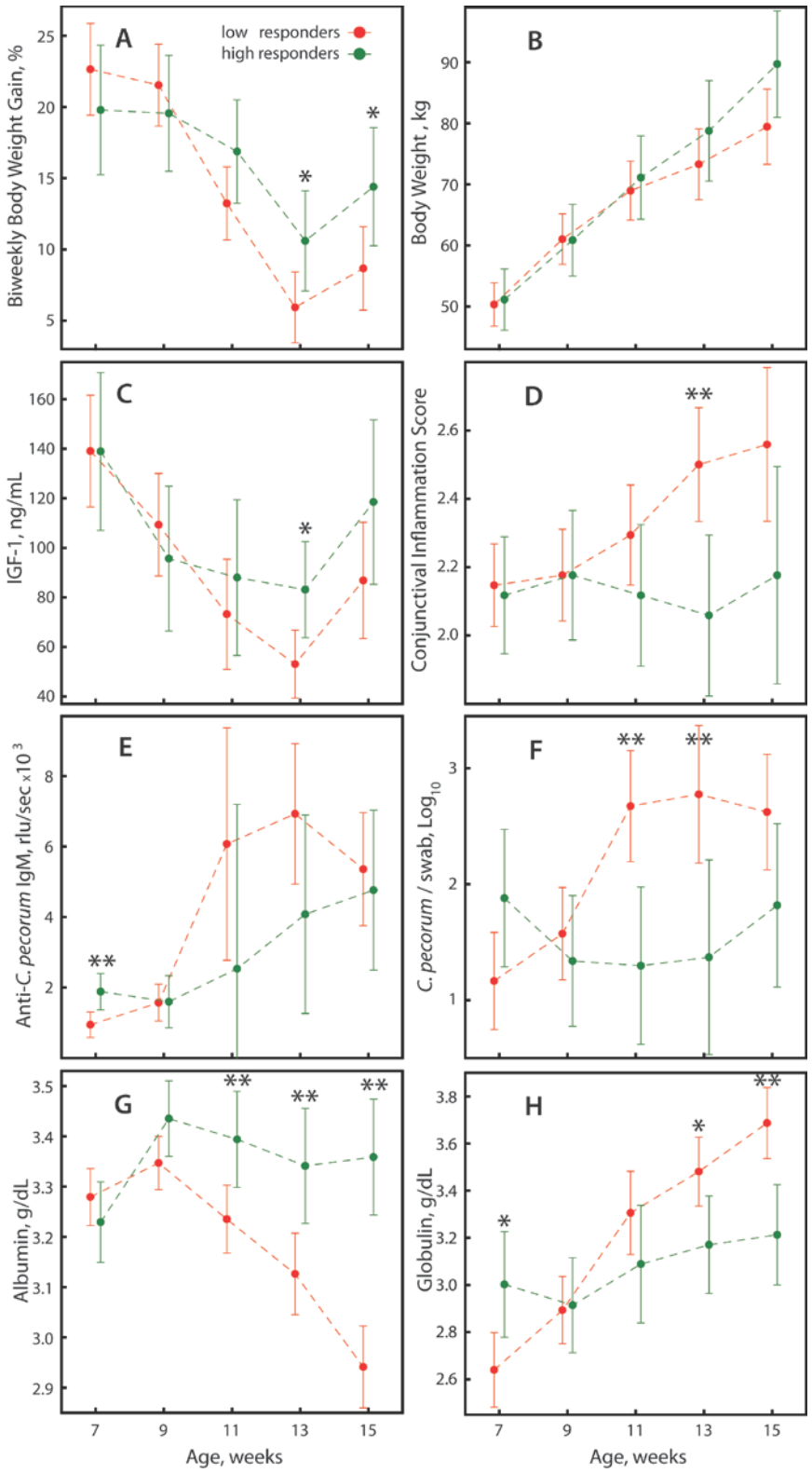
A
low responders
high responders
Biweekly Body Weight Gain, %
B
Body Weight , kg
C
IGF-1, ng/mL
D
Conjunctival Inflammation Score
E
Anti-C. pecorum IgM, rlu/sec x10 3
F
C. pecorum / swab, Log10
G
Albumin, g/dL
H
Globulin, g/dL
Age, weeks
Age, weeks

Figure 4. Growth, health, and chlamydial infection parameters based on modeling of metabolic health in dependence of earlier anti-*C. pecorum* immunity. The progression of low and high responder calves in Fig. 3 over the sampling period from week 7 to week 15 is shown. (A) Body weight gains. (B) Absolute body weight. (C) Plasma insulin-like growth factor-1. (D) Conjunctival inflammation. (E) Anti-*C. pecorum* IgM. (F) *C. pecorum* genomes per swab. (G) Plasma albumin. (H) Plasma globulin. For evaluation of statistical significances of differences between responders at sampling time points, data are shown ± 95% confidence interval. *, $P<0.05$; **, $P<0.01$.

DISCUSSION

The 100% prevalence of *C. pecorum* infection in individual calves over the study period, and the 74% prevalence at any given sampling time point, conform to other studies worldwide (Reinhold et al., 2011). The detection of multiple strains strongly indicates endemic infection in which several distinct *C. pecorum* strains circulate in the herd. Interestingly, our data do not show an obvious reason such as herd immunity for the circulation of multiple *C. pecorum* strains. We observed largely primary infections of the calves, and the switch to different strains was not immune-driven, because some calves did show repeated infection peaks about 10 weeks after primary infection with either the same or a different strain. In addition, some calves had mixed infections or simultaneous infection with two or more strains at different mucosal sites.

The prevalence of the infection was particularly high after weaning at 7-8 weeks of age when the calves were moved from individual hutches in which they had no direct contact with each other to a common pasture with free movement of all animals. The high sampling intensity was instrumental in proving that every single animal of the cohort experienced an acute phase of the chlamydial mucosal infection over a 4-6 week period, preceded and followed by occasional detection of low-level chlamydial infection that presumably was immune-controlled. The detection of IgM antibodies against *C. pecorum* in every single calf over the study period is consistent with the ubiquitous presence of *C. pecorum* in the herd and the highly efficient colostral transfer of maternal IgM antibodies to the calf, the short 4-day half-life of these antibodies in the calf, and the calf's own emerging IgM response to the chlamydial infection (Husband et al., 1972; Porter, 1972; Smith et al., 1976; Jee et al., 2004).

Despite the omnipresent *C. pecorum* infections, none of the study calves ever showed clinical signs of disease other than increased conjunctival redness that associated highly significantly with high chlamydial burdens or IgM antibody responses. In addition, none of the whole blood parameters included in the complete blood count as well as the plasma parameters analyzed deviated from accepted standard ranges (Knowles et al., 2000; Brun-Hansen et al., 2006; Mohri et al., 2007).

Thus, in the present study, we encountered a scenario different from the observational studies that addressed asymptomatic bovine chlamydial herd infection by scoring for presence or absence of chlamydial infection. Every animal in this study experienced a course of natural chlamydial infection. Thus, we did not ask the question if presence or absence of chlamydial infection associated with changed health, but if changed intensity of chlamydial infection resulted in different health outcomes. The results clearly support the notion that an increased chlamydial infection causes a significant reduction of the growth rate of calves (Table 1), and that increased immunity after peak infection protects from growth suppression for at least 8 weeks (Fig. 4). These results are consistent with those of Reinhold et al. (Reinhold et al., 2008) who show that at enrollment in their study *Chlamydia*-infected calves had lower body weight than *Chlamydia*-non-infected calves. However, over the ensuing five study months the difference in body weight did not change in their study. Hematological parameters of *Chlamydia*-infected calves actually tended to relatively improve over the study period from lower values than in *Chlamydia*-non-infected calves. Thus in the study by Reinhold et al. (Reinhold et al., 2008), low-level chlamydial infection significantly associated with lower health at enrollment, but did not have a measurable negative effect on calf health over the study period.

We ascribe this lack of a negative influence to a protective immune response to the chlamydial infection.

What is the mechanism of growth suppression by subclinical chlamydial infection? One explanation may be malabsorption of nutrients due to the local inflammatory response to intestinal mucosal chlamydial infection. While it is clear that *C. pecorum* resides in the intestinal tract of calves (Storz et al., 1971; Shewen, 1980; Jee et al., 20049), clinical symptoms would have been evident in our study if *C. pecorum* had caused enteritis and/or malabsorption syndrome. In the absence of such symptoms, a more likely explanation is a systemic effect of the sum total of inflammatory mediators released in response to the inapparent *C. pecorum* infection of virtually all mucosal membranes. This would be similar to the exacerbation of insulin resistance in obese mice experimentally infected with *C. pneumoniae* that was mediated by circulating TNF-α released from the lung, the primary infection site (Wang et al., 2009).

To evaluate the possible role of chronic low-level systemic inflammation in the *C. pecorum*-associated growth depression, we analyzed albumin and globulin as global plasma markers of inflammation (Gabay et al., 1999). Albumin is exclusively produced by hepatocytes and inflammation reduces its synthesis, thus albumin is an inverse marker of the acute-phase response (Fleck, 1989). Globulin encompasses all remaining plasma proteins that include hepatocyte-produced proteins such as haptoglobin, but also immunoglobulins, and is a direct marker of the acute-phase response due to its increase during inflammation (Fleck, 1989). Because of their extended plasma half-life, these proteins are better markers for chronic inflammatory conditions than typical acute-phase proteins with short half-lives such as C-reactive protein (Gabay et al., 1999; Cray et al., 2009). Both albumin and globulin highly significantly track *C. pecorum* load and anti-*C. pecorum* IgM levels in the anticipated patterns

(Tables 2, 3; Fig. 4G, H), strongly suggesting that in fact it is the systemic inflammatory response, and in particular its detrimental effect on the liver, that mediate the growth depression at high, but clinically inapparent *C. pecorum* infection.

To further confirm the central role of the liver in chronic inflammation-mediated growth depression, and to address the collinearity of age, chlamydial infection and weight gains, we analyzed plasma insulin-like growth factor-1. IGF-1 is the actual mediator of somatic growth (Collet-Solberg et al., 2000). It is largely produced by hepatocytes, and inflammatory stimuli such as TNF-α, IL-1, or IL-6 reduce its synthesis and plasma levels (Lang et al., 2002). Low plasma IGF-1 invariably results in reduced growth by uncoupling the somatotropic axis via induction of cellular resistance to growth hormone (Shuster et al., 1995; Elsasser et al., 2008). Again, plasma IGF-1 was highly significantly decreased at high chlamydial infection and closely tracked growth rates, confirming that *C. pecorum* infection reduced growth rates of calves via an infection intensity-dependent liver response to *Chlamydia*-induced systemic inflammation (Tables 2, 3; Fig. 4C).

As any biological outcome, growth depression by chlamydial infection is not only influenced by environment and pathogen load, but also by the host genetics-driven response to the inflammatory insult. To account for both chlamydial and host factors, we analyzed the data by hypothesis-free principal component analysis considering both inputs, and separated the data into clusters of related cases (Fig.2, Table 3). The weighted combination of input variables into principal components provided actual biological significance to the interaction of these variables by explaining that anti-chlamydial IgM immunity is both a marker for inflammation driven by chlamydiae (Chlamydial Inflammation Index, Fig. 2) as well as for the protective function of the immune response to chlamydiae (Metabolic Immune Protection Index, Fig. 2). Comparison of

the clusters unambiguously showed that high chlamydial infection intensity highly significantly co-segregated with high plasma globulin and low albumin, IGF-1, and body weight gain. In fact, the difference of 48% in body weight gain was the highest of all contrast analyses in this study, and confirmed that consideration of both chlamydial infection and host response optimally models the outcome.

In a second PCA, grouping of individual animals into high and low responders based on the immune response in week 7 combined with the metabolic response in week 15 allowed us to evaluate the potential for protection by the immune response to an earlier chlamydial infection. In this analysis, high immunity in week 7 highly significantly co-segregated with high metabolic fitness (high albumin, low globulin) in week 15 confirming that chlamydial infection mediates later immunity that protects from disease (reduced growth) but is not able to completely eliminate subsequent chlamydial infections (Fig. 3, 4). This immune protection resulted in week 15 in 66% increased growth rate (14.4 vs. 8.7% bi-weekly weight gain, P=0.027) and 13% increased body weight (89.7 vs 79.4 kg, P=0.059) in protected high-responder calves versus unprotected low-responder calves (Fig. 4A, B).

In summary, this investigation further establishes the negative health effects of endemic subclinical *C. pecorum* infections in cattle. These infections impact liver health and decrease plasma IGF-1 in calves resulting in reduction of somatic growth by up to 48%, while causing no signs of clinical disease other than subtle reddening of the conjunctiva. The worldwide detection of the omnipresence of these chlamydial infections may be still obscured by misleading serological assays (Perez-Martinez et al., 1986; Kaltenboeck et al., 1997, 2005) and the laborious and stochastic nature of PCR detection of low chlamydial burdens (Smieja et al., 2001; Sachse et al., 2009). However, highly sensitive chlamydial genus- and species-specific peptide ELISAs

based on immunodominant proteins identified from all chlamydial genomes may soon be available and correct this shortcoming.

The findings reported here strongly suggest that anti-*C. pecorum* vaccination of calves within the first 4 weeks of life may be an effective way to increase growth similar to anti-chlamydial vaccination of cows that increases udder health (Biesenkamp-Uhe et al., 2007). In the context of the chlamydial depression of growth rates it is also of interest to contemplate a few facts: 1) antibiotics used as feed additives at sub-therapeutic concentrations consistently promote growth, and explanations for this effect center around poorly understood perturbations of the intestinal microbial flora (Feighner et al., 1987; Butaye et al., 2003; Dibner et al., 2005); 2) antibiotic feed additives are extensively used in animal agriculture and are still effective as growth promoters (Gallo et al., 1995; Silbergeld et al., 2008), while many common bacteria have developed resistance against these antibiotics (Silbergeld et al., 2008); 3) chlamydiae can be found in any livestock operation; and 4) due to their sequestered intracellular habitat, chlamydiae are very inefficient at acquisition of antibiotic resistance, and the only antibiotic-resistant strains of chlamydiae with resistance acquired by horizontal gene transfer are strains of *C. suis* isolated from swine (Sandoz et al., 2008). Could it be that in fact it is largely a suppression of chlamydial infections that is responsible for the growth promoting effect of antibiotics? In a scenario of growth depression by chlamydial infection, antibiotic growth promoters would be more effective in large herds and at high population density, poor hygiene and nutrition, and open herd management practices, essentially all situations that match actual observations on the effectiveness of antibiotic growth promoters (Silbergeld et al., 2008). Future vaccination against chlamydiae will test this hypothesis, and if successful, anti-chlamydial vaccines may partially or completely replace antibiotic feed additives as growth promoters.

Chapter 3: Impact of endemic *C. pecorum* infection on performance of dairy cattle

INTRODUCTION

Early post-partum cows are at high risk of uterine infection, and up to 50% of cows experience clinical and subclinical endometritis 40-60 days after calving (Kasimanickam et al. 2004; Gilbert et al., 2005; Sheldon et al., 2008). In addition to predisposing factors such as the retention of the placenta, the endocrine condition of a cow influences uterine infection susceptibility, resulting in highest infection risk during the luteal phase of the oestrous cycle (Lewis, 2003; Sheldon et al., 2008). Uterine infections associate with extended intervals from calving to first insemination or conception, and with increased culling rates for failure to conceive in a timely manner (LeBlanc et al., 2002; Gilbert et al., 2005), thus increasing the cost of production in dairy herds (Bartlett et al., 1986a). Uterine health during the early post-partum period is also a major determinant of total lactation milk performance (Fourichon et al., 1999; Bell et al., 2007; Bertoni et al., 2008), linking essentially low fertility that is the immediate outcome of poor peripartal health to overall dairy cow milk production.

Chlamydia spp. have been sporadically, but consistently linked in cattle to abortion and fertility disorders (McKercher et al., 1966; Nabeya et al., 1991: Griffiths et al., 1995; Cox et al., 1998), and a high prevalence of *Chlamydia* spp.in the genital tract of asymptomatic dairy cows can be found worldwide (Wittenbrink et al., 1993a; Wittenbrink et al., 1993b; DeGraves et al., 2003a; Wehrend et al., 2005; Petit et al., 2008; Kemmerling et al., 2009). After the introduction

of highly sensitive detection methods, it has become evident that obvious disease is only present in a tiny fraction of chlamydial infections of the bovine genital tract (Kaltenboeck et al., 2005; Sachse et al., 2009; Reinhold et al., 2011). Experimental studies have established a causal relationship of *Chlamydia* spp. genital infection in ruminants with infertility and reproductive disorders such as endometritis, late-term abortion and still births (Papp et al., 1993; Papp et al., 1996; Jones et al., 1998).

It is now becoming apparent that chlamydial infections result in multifactorial diseases in which host factors are major determinants of disease outcome (Wehrend et al., 2005; Kemmerling et al., 2009). This leads to the question if the ubiquitous low-level chlamydial infections have a measurable health impact in cattle, and if so, what the pathogenic mechanisms are. We have previously reported a significant influence of *Chlamydia* spp. infection, identified in vaginal swab specimens, on milk quality and quantity of dairy cows (Biesenkamp-Uhe, et al, 2007; Ahluwalia et al., 2010). In the present study, we examined in a large dairy herd the influence of cervical chlamydial infection at first service on fertility and milk production of primiparous dairy cattle. We report 100% seroprevalence and a high detection rate of multiple strains of *C. pecorum* indicating steady-state endemic infection, a highly significant negative correlation between conception rates and cervical chlamydial burdens, and a highly significant increase of milk yield associated with high anti-chlamydial immunity and liver health.

MATERIALS AND METHODS

Dairy herd. The study was performed at Barrington Dairy, a 3,700-Holstein cow commercial dairy in Montezuma, GA, USA. Cows are maintained in 14 free stall housing units of approximately 260 cows each with sand mattress and vertical feeding gates, fed a 53% dry matter, 9.2% protein total mixed ration based on corn silage, brewer's grain and grinded corn grain, and are continuously monitored by two veterinarians and a team of experienced veterinary technicians. Cows are milked three times daily in parallel BouMatic Double 42 and Germania Double 20 milking parlors using standard hygiene and teat-dipping practices. Milk production is measured in 4-week intervals by weighing the collected four-quarter milk from each cow. The average daily milk yield of lactating cows was 32.7 kg containing 3.1% protein and 3.6% fat. Late in gestation, dry cows are kept in pastures, close to calving they are moved to close up barns, isolated in fresh pens to ensure safe calving, and are moved to lactating pens after calving. Approximately 35 days later, they are moved to breeding pens, and after confirmed conception to production pens until they are dried off. Male calves are removed at 1 week of age, and female calves are raised for replacement heifers and dehorned at 4 weeks. They are housed in individual hutches until 8 weeks of age, and subsequently kept in herds of 80-100 calves.

Female calves and cows are routinely vaccinated in appropriate schedules against the following bacterial and viral pathogens: *Escherichia coli*, *Salmonella typhimurium*, *Pasteurella multocida*, *Pasteurella haemolytica* (Endovac-Bovi, Immvac; Scourguard 3(K)/c, J-5 *E .coli* bacterin, Pfizer); *Mannheimia haemolytica* and *Pasteurella multocida* (Once PMH, Merck Health); *Moraxella bovis* (Piliguard Pinkeye-1, Intervet); *Clostridium chauvoei, C. septicum, C.*

novyi, *C. sordellii*, *C. perfringens* types C and D, and *C. haemolyticum* (Vision-8 with Spur, Merck Health; Ultrabac-8, Scourguard 3(K)/c, Pfizer); *C. perfringens* type A toxoid (Novartis); *Campylobacter fetus* and *Leptospira* spp. (Bovi-Shield Gold FP5 VL5, CattleMaster 4+VL5, Pfizer); *Brucella abortus* (RB-51, Professional Biological); bovine respiratory syncytial virus, infectious bovine rhinotracheatis virus, and parainfluenza-3 virus (Inforce 3, Pfizer); bovine viral diarrhea virus (BVD) types I and II (Bovi-Shield Gold FP5 VL5, Pfizer); bovine rotavirus and corona virus (Scourguard 3(K)/c, Pfizer). Ectoparasite control is performed by external application of permethrin and piperonyl butoxide (Synergized De-Lice, Merck Health).

Heifers are first bred at 12 months of age, and cows in week 7 post-partum. For estrus synchronization and timed artificial insemination (TAI), all heifers and cows receive an initial intramuscular (i.m.) dose of 100 µg gonadorelin diacetate tetrahydrate (Merial), a synthetic gonadotropin releasing hormone (GnRH). One week later, cows receive 25 mg of i.m. synthetic prostaglandin F2 alpha (dinoprost tromethamine; Pfizer). Cows receive GnRH again on days 10 and 17, and a second dose of prostaglandin F2 alpha on day 24 followed by a final GnRH administration on day 26 and TAI one day later. Estrous synchronization and TAI is performed by certified in-house veterinary technicians. Semen used for TAI is produced using in-house semen donor bulls.

Experimental design. The investigation was designed as prospective cohort study that examined the effect of natural infection with *Chlamydia* spp. on reproductive performance and milk production in first lactation cows. A total of 451 first-lactation first-service Holstein cows were enrolled in the study over a period of 19 weeks. Sixteen to 31 clinically healthy cows were randomly selected each week from a pool of cows synchronized for the first TAI nine-ten weeks

post-partum, with average insemination at 65 days in milk (DIM). Semen from a total of five bulls with proven high fertility was used throughout the study.

Immediately after TAI, a swab specimen from the posterior portion of the cervix was collected from each cow by use of double-sheathed modified French insemination guns. Both the barrel and the plunger of these guns were shortened to accommodate a trimmed cytobrush swab (Puritan, Hardwood Products) pushed onto the plunger instead of the semen straw. A plastic sheath with the tip removed was pulled over the assembled swab collection gun, and the entire assembly was inserted into the protective plastic cover of the sheaths. For swab collection, this double-sheathed swab collection gun was inserted into the vagina, and the gun pushed through the plastic cover into the cervix. The plunger was pushed to externalize the cytobrush swab into the cervical tunnel and rotated 5 times to collect cells from cervical lining. Then the plunger was pulled to retract the cytobrush into the sheath, and the swab collection gun removed from the vagina. 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) in microcentrifuge tubes. Swab samples were centrifuged at 250×g for 1 min and stored at -80°C without the brush. This method ensured contamination-free collection of cytobrush specimens and preservation of single copies of chlamydial genomes.

Pregnancy was determined 5 weeks after first TAI by ultrasonography-aided rectal palpation. Heparin plasma was collected from each cow in the study after TAI on 65 DIM and 5 weeks after pregnancy check on 100 DIM. Milk production data of each cow were recorded in 4-week intervals and interpolated to calculate milk yield at 30, 65, 100, 137, 170 and 205 DIM. Cows in the study were observed until 205 DIM. Clinical disease and treatment records were

kept for each cow in the study. All animal procedures were approved by the Auburn University Institutional Animal Care and Use Committee.

Blood collection and plasma analyses. Blood was collected by venipuncture of the tail vein in heparin-treated blood collection tubes (BD Vacutainer, Becton Dickinson and Company). Plasma was obtained from heparin blood by centrifugation at 1300×g for 15 min. Plasma colorimetric chemistry analyses (albumin, cholesterol and total protein) were performed by use of an automated cobas c 311 systems analyzer following manufacturer's instruction (Roche/Hitachi). Globulin was calculated as total plasma protein minus albumin. Plasma concentration of bovine luteinizing hormone on day 65 at the first TAI was measured by double-antibody radioimmunoassay using materials provided by the National Hormone and Pituitary Program of NIDDK as previously described (Coleman et al., 1993; Whitlock et al., 2008). Anti-*C. pecorum* IgM were measured by chemiluminescent ELISA in the paired plasma samples for each cow using lysed *C. pecorum* elementary bodies as antigen as described by Poudel et al. (Poudel et al., 2012).

***Chlamydia* spp. detection and ompA genotyping.** *Chlamydia* spp. genomic DNA was detected, quantified, and typed in cervical cytobrush specimens by *Chlamydia* spp. 23S rRNA gene real-time PCR as described (Poudel et al., 2012). In samples with more than 1,000 genomes per PCR the chlamydial species were confirmed and strains typed by *Chlamydia* spp. *ompA* real-time PCR followed by DNA sequencing of both strands of the amplification product as described earlier (Poudel et al., 2012). Eight novel *C. pecorum* partial *ompA* coding sequences were submitted to Genbank (accession # JX311945 - JX311952).

Statistical analyses. All statistical analyses were performed with the Statistica 7.0 software package (StatSoft). Chlamydial load data were logarithmically transformed after addition of 1 to the linear data. Negative results were treated as 0 in the log-transformed data. Differences in PCR positivity of pregnant and open cows, and of chlamydial detection frequency by sampling month were evaluated by Fisher exact test. Temporal differences in chlamydial detection frequency were evaluated by Kruskal-Wallis ANOVA Rank test. The probability of pregnancy in dependence of chlamydial load and anti-chlamydial immune status was modeled by logistic regression.

Principal component analysis (PCA) based on the correlation matrix was used to reduce the dimensionality of the data set and to identify principal components as linear combinations of optimally weighted original predictor variables. Disjoint cluster analysis of principal components based on least-squares estimation of Euclidean distances categorized the cows into natural clusters. Effects between clusters were measured using Student's t-test for normally distributed data as determined by Shapiro-Wilk's W test.

RESULTS

All 451 first-lactation first-service post-partum cows enrolled remained clinically healthy throughout the study period and produced on average 28.94 kg milk per day. Plasma parameters determined at first TAI (cholesterol, albumin, globulin, luteinizing hormone) were in all animals

within the physiological range (Lumsden et al., 1979; Dubreuil et al., 1997; Whitlock et al., 2008). Five weeks after the first timed artificial insemination, 198 cows (43.9%) conceived as determined by ultrasound-assisted rectal palpation, and 253 cows (56.1%) remained open. Of the 198 pregnancies detected, 37 cows (18.7%) experienced embryonic mortality and were bred again later, resulting in full-term pregnancy after single or multiple inseminations in 21 of these cows. Pregnancy rates by bull used did not vary ($P = 0.65$; Kruskal-Wallis ANOVA). A total of 216 cows (47.9%) were positive in the *Chlamydia* spp. 23S rRNA gene PCR of the cervical cytobrush swab collected at first TAI (Table 1). *Chlamydia*-positive cows had lower conception rates after first TAI, and experienced significantly higher embryonic deaths resulting in significantly less full-term pregnancies (29.6%) from first TAI than *Chlamydia*-negative cows (41.3%). However, after multiple inseminations the final pregnancy rates did not differ (Table 1). Thus, chlamydial infection of the upper genital tract resulted in significantly reduced fertility, but not in permanent reproductive disorders.

Chlamydial infection. The average chlamydial load detected in animals positive in the *Chlamydia* spp. 23S rRNA gene PCR was 15.40 genome copies / cervical swab (antilog; range 1 - 673,800 copies). Detection frequency by sampling month from December through April varied from 36.9% to 57.7%, with a significantly lower frequency in February of 38 positive out of 103 cows than in December or March ($P \leq 0.036$; two-tailed Fisher exact test). Tentative species identification by high-resolution melting curve analysis of FRET probe hybridization indicated *C. pecorum* for all positive specimens.

Eighteen highly positive specimens (> 1,000 genome copies/swab) randomly distributed over the 5-month sampling period were re-analyzed by *C. pecorum ompA* FRET PCR amplification and

DNA sequencing of the amplification product. These PCRs confirmed in all cases the *C. pecorum* species identification in the *Chlamydia* spp. 23S RNA gene detection and quantification PCR. However, sequencing of the 18 PCR amplification products of the partial *C. pecorum ompA* gene including variable domain III identified 11 widely divergent *C. pecorum* strains spanning the complete spectrum of *C. pecorum ompA* genotypes (Kaltenboeck et al., 2009; Mohamad et al., 2010; Mohamad et al., unpublished data). Three strains identified in 3 positive specimens had partial *ompA* sequences identical to bovine *C. pecorum* strains 2047, 748/4, and 3257 (Genbank # GQ228191, GQ228188, GQ228190; Mohamad et al., unpublished data). In addition, 8 more novel *C. pecorum ompA* genotypes were identified among the remaining 15 analyzed specimens. These strains were termed Barr58148, Barr41334, Barr57275, Barr10111, Barr41456, Barr57636, Barr58166, and Barr41624 (Genbank # JX311945-JX311952). At any given sampling time point, more than one strain was identified and identical *ompA* genotypes were found at different sampling time points. In two of the 18 swabs, a mixed infection was identified by polymorphic sequencing peaks. Thus, at least eleven *ompA* genotypes of *C. pecorum* circulated simultaneously in the study herd.

Anti-*C. pecorum* IgM antibodies. All cows in the study had highly positive plasma anti-*C. pecorum* IgM levels with a mean value of 127,262 rlu/sec (13,509 - 611,236; range) at first TAI and 83,822 rlu/sec (1,064 - 660,139; range) at pregnancy check five weeks later. At pregnancy check, a higher number of the cows (n = 320) showed decreased anti-*C. pecorum* IgM levels compared to 131 cows that had increased anti-*C. pecorum* IgM.

Table 1. Chlamydial detection and reproductive performance.

	Cervical *Chlamydia* spp. 23S rRNA gene PCR					
	negative			**positive**		
Animals, N	235	(52.1%)		216	(47.9%)	
Conception after 1st TAI	113[a]	(48.1%)		85	(39.4%)	
Embryonic deaths[1]	16[a]	(14.2%)		21	(24.7%)	
Full-term pregnant after 1st TAI	97[A]	(41.3%)		64	(29.6%)	
Pregnant after final TAI	200	(85.1%)		178	(82.4%)	
Open period all cows, days[2]	106.14	±	7.55	112.50	±	7.81
Open period pregnant cows, days[3]	95.19	±	6.07	103.19	±	6.22

Open periods are shown ± 95% confidence interval

[1] cows conceived at 1st TAI but did not maintain pregnancy

[2] days between partum and conception for pregnancy maintained for ≥ 180 days or final TAI for cows that did not conceive

[3] days between partum and conception for pregnancy maintained for ≥ 180 days

[a.A] = significantly different from "positive"; one-tailed Fisher exact test

[a] = $P < 0.05$; [A] = $P < 0.01$

Physiological effects of *C. pecorum* infection. Previous studies have shown that chlamydial infection results in multifactorial diseases in which an environmentally and genetically restricted host response to the infection is a critical contributor to the physiological outcome (Wehrend et al., 2005; Ahluwalia et al., submitted; Poudel et al., 2012). To evaluate the influence of both *C. pecorum* infection and host response we combined parameters for infection (*C. pecorum* load and day 65 and 100 plasma anti-*C. pecorum* IgM and their ratio [day 100/65 trend]) and surrogate parameters of host metabolic status and acute phase response (plasma cholesterol, albumin, and globulin; Bertoni et al., 2008) in hypothesis-free multivariate modeling by principal component analysis. Of all combinations of these parameters, the combination of cervical chlamydial load, cholesterol, and albumin best explained data variance. Only two principal components (PC) composed of these original variables explained 80% of the variance (Fig 1). Based on the dominant contribution of original variables we termed PC 1 "Metabolic health index" (cholesterol + albumin = 99%; Fig 1) and PC 2 "Cervical *C. pecorum* index" (Log *C. pecorum* / swab = 97%; Fig 1). Cluster analysis by factor scores of these two principal components grouped all cows into three distinct populations (Fig 1). Cows in cluster "low fertility/low milk" (n = 52) were characterized by poor fertility and milk production. They were all *C. pecorum* positive, with significantly higher *C. pecorum* genomes/cervical swab at first TAI ($P < 10^{-4}$) and lower pregnancy rates 5 weeks later ($P < 0.01$) than cows of the other two clusters (Table 2). Cows in both "high fertility/low milk" (n = 206) and "high fertility/high milk" (n = 193) clusters had similar low chlamydial prevalence and loads and high pregnancy rates, but differed highly significantly ($P = 0.004$) in total milk production from days 1-205 in milk (DIM). The increased milk production of "high fertility/high milk" cows was highly significantly accompanied by higher plasma cholesterol, albumin, and globulin, as well as a significant

increase of anti-*C. pecorum* IgM between 65 DIM and 100 DIM (P = 0.018), most clearly expressed as highly significantly elevated day 100/65 anti-*C. pecorum* IgM trend (P = 0.008). Thus, in summary cows with low fertility had significantly increased cervical *C. pecorum* loads while animals with low milk production had significantly decreased metabolic health and a decreased day 100/65 anti-*C. pecorum* IgM response trajectory, but not increased cervical *C. pecorum* loads.

Influence of *C. pecorum* infection on fertility. Based on the PCA observations we hypothesized that the local response to genital tract chlamydial infection is the major driver of chlamydial influence on fertility while the systemic response to chlamydial infection of all mucosal membranes interacts with metabolic health to influence milk production. Thus, fertility would be influenced by cervical chlamydial burden but not by anti-chlamydial IgM levels. To ascertain this hypothesis, we modeled fertility by logistic regression against either the cervical *C. pecorum* burden or the progression of anti-*C. pecorum* IgM levels from 65 DIM to 100 DIM (anti-*C. pecorum* IgM trend). Figure 2 shows the linear relationship of cervical *C. pecorum* burden (Fig. 2A) or anti-*C. pecorum* IgM trend (Fig. 2B) with the logit-transformed probability of pregnancy. Cows with high cervical *C. pecorum* had highly significantly reduced probability of pregnancy (Fig 2A; P = 0.0002). For each log increase in cervical chlamydial load, cows were 46.2% more likely to remain open (OR = 1.462). Thus, the probability of pregnancy for a cow without detectable *C. pecorum* genomes per cervical swab at first TAI was 49.3%, but dropped to 23.8% for cows with 1,000 *C. pecorum* genomes per swab, and to 9.6% for the highest detected cervical *C. pecorum* load of 673,800 genomes per swab. In contrast, the

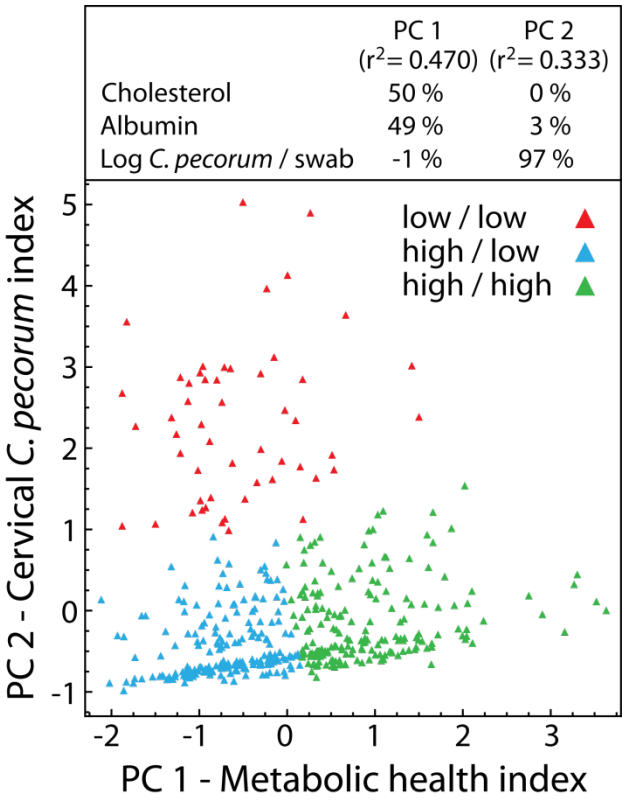

Figure 1. Cluster analysis based on PCA-1 modeling of data using cervical *C. pecorum* burden and surrogate markers for metabolic health**.** Data of all 451 first service cows included in the study were analyzed by principal component analysis using the cervical chlamydial burden (*C. pecorum*, Log $_{10}$) at timed artificial insemination on day 65 in milk and plasma concentrations of cholesterol and albumin. Two principal components (PC) explained 80% of the variance and were termed according to their biological significance. The percentages indicate the variable contributions to the partial r^2, and inverse orientation is indicated by "-". Cluster analysis based on these principal components separated all animals into three distinct clusters that were termed on the basis of reproductive and milk performance as shown in Table 2 (low/low = low fertility and low milk; high/low = high fertility and low milk; high/high = high fertility and high milk).

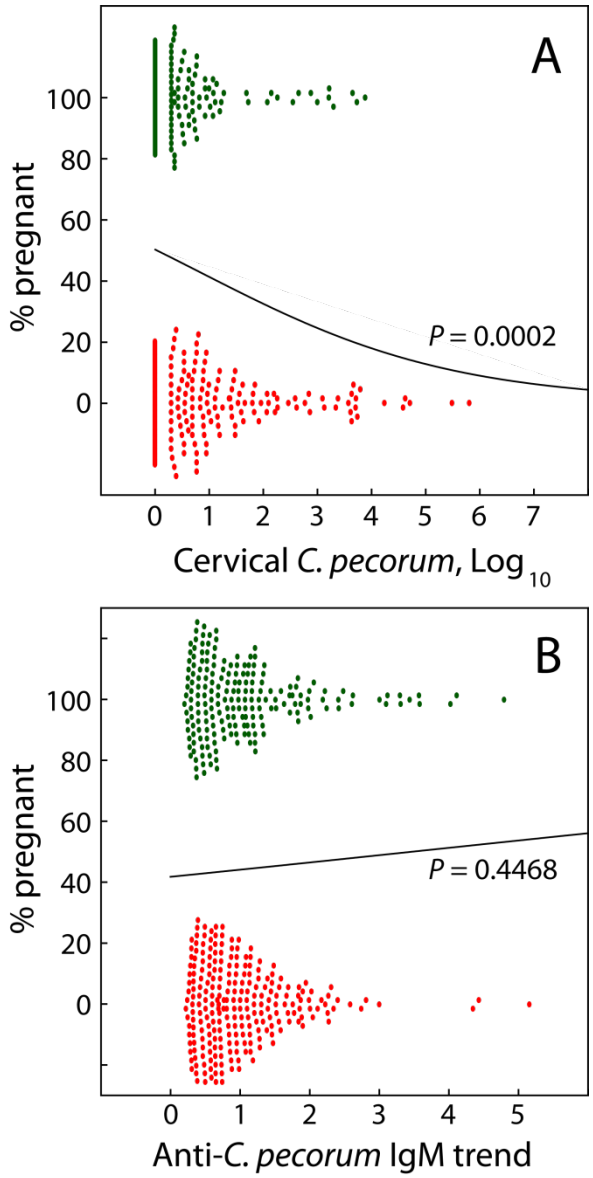

Figure 2. Logistic regression modeling of probability of pregnancy in relation to cervical *C. pecorum* burden and time-trajectory of anti-*C. pecorum* IgM concentrations. Linear relationships between cervical chlamydial load (A) or anti-chlamydial immune trajectory (B) and pregnancy outcome (1 or 0) five weeks after artificial insemination were modeled as logit-transformed probability of pregnancy. Data points in red correspond to 0 % pregnancy, and in green to 100% pregnancy (open vs. pregnant). (A) The cervical *C. pecorum* load had a highly

significant effect on the probability of pregnancy. Cows with higher log cervical *C. pecorum* had lower probability of being pregnant (odds ratio (OR) per log cervical chlamydial burden = 0.684, $P = 0.0002$; Chi-square test). For each unit increase in log chlamydial burden, animals were 46.2 % more likely to remain open (OR = 1/0.684 = 1.462). The zero in the abscissa indicates *C. pecorum*-negative animals. (B) The anti-*C. pecorum* immune trend had no significant effect on probability of pregnancy, with animals with higher day 100/65 anti-*C. pecorum* IgM trend showing only marginally higher pregnancy rates (OR = 1.099, $P = 0.4468$; Chi-square test).

trend of the *C. pecorum* immune response had no significant influence on reproductive performance (Fig 2B). The probability of pregnancy increased only marginally in animals with higher day 100/65 anti-*C. pecorum* IgM trend (OR = 1.099).

We observed after multiple inseminations a final pregnancy status of approximately 81% in low fertility cows with high cervical *C. pecorum* infection at first TAI (Table 2), not different from cows with high fertility, and therefore excluded major, irreversible inflammatory damage of the uterine endothelium as the cause of infertility. Similarly, embryonic death rates did not differ between low and high fertility cows. To further understand the possible mechanisms of transient suppression of fertility by natural *C. pecorum* infection of the upper genital tract, we analyzed plasma luteinizing hormone (LH) concentrations at first TAI. LH is considered the best peripheral marker of endocrine regulation of reproduction in dairy cows because of its elevated plasma concentration in response to pulsed stimulation by gonadotropin-releasing hormone (Vizcarra et al., 1997), and early post-partum uterine inflammation is known to down-regulate reproductive hormone levels (Herath et al., 2009; Sheldon et al., 2009). Plasma levels of circulating LH in *C. pecorum* PCR-positive cows were 109.22 ± 12.16 ng/L (n = 216; 95% CI), highly significantly lower than the 137.23 ± 15.45 ng/L of cows with no detectable *C. pecorum* genomes (n = 235; $P = 0.006$). Among *C. pecorum*-positive animals, LH levels were not significantly correlated with chlamydial load. Thus, plasma LH is highly significantly reduced in animals with cervical chlamydial infection, but this reduction is not dependent on chlamydial burden such as pregnancy rates are.

Table 2. Influence of cervical *C. pecorum* infection and host response to infection on performance

	PCA-1 Clusters		
	low fertility / low milk	high fertility / low milk	high fertility / high milk
Animals, N	52	206	193
C. pecorum PCR-positive animals	52[A, B] (100.0%)	81 (39.3%)	83 (43.1%)
C. pecorum / positive cervical swab[1]	1121.00[A,B] ± 749.11	3.36 ± 0.75	4.63 ± 1.20
Anti-*C. pecorum* IgM day 65, rlu	129,921 ± 31,417	127,467 ± 13,186	126,327 ± 14,198
Anti-*C. pecorum* IgM day 100, rlu	77,148 ± 25,073	75,287[b] ± 9399	94,729 ± 13,294
Anti-*C. pecorum* IgM Trend day 100/65	0.74 ± 0.21	0.75[B] ± 0.10	0.96 ± 0.12
Cholesterol, g/L	2.54[A,B] ± 0.19	2.30[B] ± 0.07	3.57 ± 0.10
Albumin, g/dL	4.17[A,B] ± 0.21	3.77[B] ± 0.07	4.88 ± 0.11
Globulin, g/dL	5.88[b] ± 0.27	5.88[B] ± 0.14	6.31 ± 0.17
PC 1 – Metabolic health index	-0.56[B] ± 0.21	-0.71[B] ± 0.07	0.90 ± 0.10
PC 2 – Cervical *C. pecorum* index	2.32[A,B] ± 0.27	-0.45[B] ± 0.05	-0.15 ± 0.07
Conception after 1st TAI[2]	13[A,B] (25.0%)	92 (44.7%)	93 (48.2%)
Pregnant after final TAI[2]	42 (80.8%)	172 (83.9%)	163 (84.9%)
Open period all cows, days[3]	123.02[b] ± 16.35	110.42 ± 8.33	104.15 ± 7.91
Open period pregnant cows, days[4]	111.74[b] ± 13.95	98.81 ± 6.50	95.84 ± 6.46
Luteinizing hormone, ng/L	106.73 ± 32.71	131.48 ± 16.76	120.21 ± 12.36
Milk day 30, kg	26.67 ± 2.17	27.60 ± 1.07	28.75 ± 0.99
Milk day 65, kg	28.53 [B] ± 2.02	29.47[B] ± 0.86	31.10 ± 0.83
Milk day 100, kg	29.74 ± 1.67	28.74[B] ± 0.84	31.25 ± 0.78
Milk day 135, kg	29.77 ± 1.81	28.87[B] ± 0.86	31.15 ± 0.82
Milk day 170, kg	26.29[b] ± 1.87	26.45[B] ± 0.90	28.12 ± 0.76
Milk day 205, kg	26.13 ± 1.94	25.48[b] ± 0.94	27.15 ± 0.85
Milk days 1-65, kg	1766.07[b] ± 125.81	1831.74 ± 58.40	1908.62 ± 55.71
Milk days 66-135, kg	2061.26[b] ± 114.23	2034.71[B] ± 52.22	2182.50 ± 50.28
Milk days 136-205, kg	1898.36 ± 119.37	1891.18[B] ± 55.44	2001.77 ± 50.00
Milk days 1-205, kg	5725.70[b] ± 321.84	5822.11[B] ± 132.29	6099.87 ± 136.25

Data are shown ± 95% confidence interval

[1] antilog of mean logarithm of *C. pecorum* genome copies / swab; significance refers only to PCR-positive animals

[2] significance evaluated by one-tailed Fisher exact test

[3] days between partum and conception for pregnancy maintained for ≥ 180 days or final TAI for cows that did not conceive

[4] days between partum and conception for pregnancy maintained for ≥ 180 days

[a, A] = significantly different from “high fertility/low milk cluster”

[b, B] = significantly different from “high fertility/high milk cluster”

[a,b] = $P < 0.05$; [A,B] = $P < 0.01$

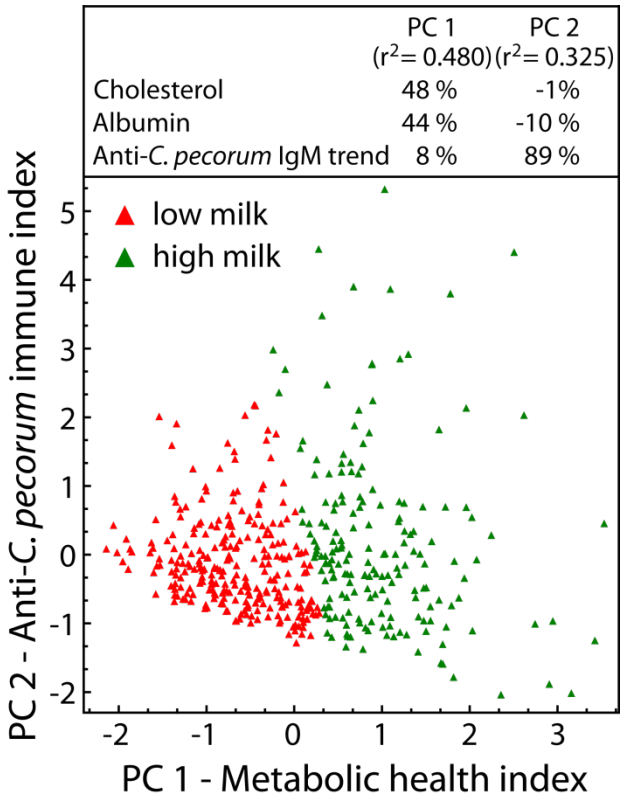

Figure 3. Cluster analysis based on PCA-2 modeling of data using the day 100/65 trend of anti-*C. pecorum* IgM and surrogate markers for liver health. Data of all 451 cows were analyzed by principal component analysis using the trend of anti-*C. pecorum* IgM between days 65 and 100 in milk and plasma concentrations of cholesterol and albumin. Two principal components (PC) explained 81% of the variance and were termed according to their biological significance. The percentages indicate the variable contributions to the partial r^2, and inverse orientation is indicated by "-". Cluster analysis based on these principal components separated all animals into two distinct clusters that were termed on the basis of milk performance as shown in Table 3.

Influence of *C. pecorum* infection on milk production. While the PCA and logistic regression analyses identified the role of localized chlamydial infection in fertility, these analyses also pointed towards an influence of the systemic response to the chlamydial infection on milk production. The trajectory of the anti-*C. pecorum* response (IgM trend) and metabolic health separated in PCA-1 the high fertility cows into low- and high-milk clusters (Table 2). To corroborate this observation in the complete data set, we modeled indicators of metabolic health (cholesterol and albumin) and day 100/65 anti-*C. pecorum* IgM in another PCA (Fig. 3). Metabolic health index and anti-*C. pecorum* immune index explained 81% of the variance observed. Cluster analysis based on these two PCs grouped all cows in the study (n=451) into two distinct populations with different milk production that we termed "low milk" and "high milk" clusters (Fig 3). Cows of the "low milk" cluster produced highly significantly less milk than "high milk" cows at all milk sampling time points subsequent to the first TAI on 65 DIM (Table 3; Fig. 4), evident as 290 kg lower milk yield between 65 and 205 DIM, an approximately 6.9% reduced milk production. This lower milk yield associated highly significantly with lower anti-*C. pecorum* IgM on 100 DIM but higher IgM on 65 DIM, resulting in an average 59% anti-chlamydial immune trend between 65 and 100 DIM in low milk cows as compared to a 120% immune trend in high milk cows ($P < 10^{-4}$). This differential anti-*C. pecorum* immune response is the dominant original variable in the anti-*C. pecorum* immune index that explains 32.5% of the data variance in PCA-2 (Fig. 3). Metabolic health as indicated by dominant original variables plasma cholesterol and albumin is also highly significantly lower in "low milk" cows ($P < 10^{-4}$) and explains 48% of the variance. Interestingly, reproductive performance parameters did not differ between low and high milk clusters (Table 3). Thus, high metabolic health and an

increasing anti-*C. pecorum* immune response are the key drivers that improve milk yield in high preforming cows.

DISCUSSION

The present study we analyzed the impact of chlamydial herd infection on fertility in dairy cows, using parameters that allowed mechanistic evaluation of the influence of the local genital tract response to chlamydial infection as well as the metabolic health and systemic host response. Overall, chlamydial infections reduced conception rates and full-term pregnancies after first insemination by up to 12% (Table 1). Our results further identify intensity of the genital tract infection as the dominant regulator of chlamydial effects on fertility, with high level chlamydial infections reducing fertility by up to 40% (Table 2; Fig. 2A), but not resulting in permanent lesions that would prevent conception later (Tables 1, 2). Interestingly, systemic metabolic responses to chlamydial infection had no detectable influence on fertility. The systemic reduction of plasma luteinizing hormone concentrations, associated with chlamydial genital tract infection independent of infection intensity, may have a minor depressive effect on fertility. In contrast, increased milk production over the first 205 days in lactation highly significantly associated with liver health (high plasma cholesterol and albumin) at first TAI and increasing anti-*C. pecorum* immunity from first TAI forward.

Table 3. Influence of anti-*C. pecorum* IgM trajectory and metabolic health and performance

	PCA-2 Clusters					
	low milk			high milk		
Animals, N	267			184		
C. pecorum PCR-positive animals, %	134	(50.2%)		82	(44.6%)	
C. pecorum / positive cervical swab[1]	19.77	±	10.77	10.23	±	5.59
Anti-*C. pecorum* IgM day 65, rlu	139,437[A]	±	12,575	109,596	±	13,014
Anti-*C. pecorum* IgM day 100, rlu	67,610[A]	±	7962	107,346	±	14,280
Anti-*C. pecorum* IgM Trend day 100/65	0.59[A]	±	0.06	1.20	±	0.14
Cholesterol, g/L	2.36[A]	±	0.06	3.61	±	0.11
Albumin, g/dL	3.90[A]	±	0.07	4.85	±	0.13
Globulin, g/dL	5.82[A]	±	0.13	6.41	±	0.16
PC 1 – Metabolic health index	-0.66[A]	±	0.07	0.96	±	0.10
PC 2 – Anti-*C. pecorum* immune index	-0.13[A]	±	0.08	0.18	±	0.19
Conception after 1st TAI[2]	111	(41.57%)		87	(47.28%)	
Pregnant after final TAI[2]	224	(84.21%)		153	(83.60%)	
Open period all cows, days[3]	110.78	±	7.18	106.89	±	8.29
Open period pregnant cows, days[4]	101.18	±	5.95	95.71	±	6.26
Luteinizing hormone, ng/L	124.98	±	13.62	122.10	±	14.59
Milk day 30, kg	27.72	±	0.94	28.36	±	1.02
Milk day 65, kg	29.44[a]	±	0.77	30.96	±	0.87
Milk day 100, kg	28.89[A]	±	0.73	31.46	±	0.81
Milk day 135, kg	29.00[A]	±	0.76	31.33	±	0.84
Milk day 170, kg	26.37[A]	±	0.77	28.27	±	0.70
Milk day 205, kg	25.49[A]	±	0.81	27.42	±	0.89
Milk days 1-65, kg	1835.00	±	51.84	1889.11	±	57.47
Milk days 66-135, kg	2037.28[A]	±	46.40	2194.46	±	50.94
Milk days 136-205, kg	1885.49[A]	±	48.00	2018.00	±	52.34
Milk days 1-205, kg	5804.31[A]	±	121.48	6112.70	±	138.80

Data are shown ± 95% confidence interval

[1] antilog of mean logarithm of *C. pecorum* genome copies / swab; significance refers only to PCR-positive animals

[2] significance evaluated by one-tailed Fisher exact test

[3] days between partum and conception for pregnancy maintained for ≥ 180 days or final TAI for cows that did not conceive

[4] days between partum and conception for pregnancy maintained for ≥ 180 days

[a, A]= significantly different from “high milk cluster”

[a] = $P < 0.05$; [A]= $P < 0.01$

Epidemiology of the *C. pecorum* infection. The study dairy had never been examined for chlamydial infection, but the presence of chlamydiae in the herd and a 100% seroprevalence conform to a number of investigations (Reinhold et al., 2011). The high chlamydial detection rate of 47.9% with single cervical sampling was unexpected, but it is consistent with the observation that chlamydial shedding peaks during estrus (Papp et al., 1994). The extremely high plasma anti-*C. pecorum* IgM antibody concentrations, approximately 10-fold higher than the highest antibody levels that we observed in a simultaneous investigation on another farm (Poudel et al., 2012), strongly imply a high steady state of an endemic *C. pecorum* infection (Nasell, 2002). It has been known for decades from many epidemiological and animal model studies, and is confirmed in this study, that immune resistance to chlamydial infection is not immediately sterilizing; that it is largely based on Th1 cellular immunity; and that early after immunizing infection animals are highly resistant to challenge infection, but within 10-20 weeks become again susceptible to re-challenge with the same chlamydial strain despite manifest antibody immunity (Morrison et al., 2002; Morrison et al., 2005; Miyairi et al., 2010; Rank et al., 2010). Therefore, the appropriate herd infection model is an endemic susceptible-infected-susceptible (SIS) model with variable re-infection rate driven by immune heterogeneity (Greenhalgh et al., 2000; Thieme et al., 2002). Such endemic infections with cyclical host immune response become more stable by the parallel existence of multiple strains of a pathogen, and are characterized by rapidly dampened oscillations of pathogen prevalence (Andreasen et al., 1997; Lin et al., 1999; Thieme et al., 2002; Simpson et al., 2012). With 11 distinct *C. pecorum* strains found in 18 animals, this study herd certainly represents such a multi-strain system of endemic infection, and the significantly reduced detection rate in February, midway through the sampling period, may represent such an oscillation. An additional factor favoring co-existence

of multiple strains may be the repeated sequestration and mixing of cows while moving through close up barns, fresh pens, breeding pens, lactating pens, and dry pastures.

***C. pecorum* infection and fertility.** The results of this study indicate distinct effector mechanisms by which *C. pecorum* impacts the performance of dairy cows. It is clear that the local inflammatory response of the genital tract is the dominant mediator of suppression of fertility, consistent with epidemiological and experimental studies (Wittenbrink et al., 1993, 1994; Bowen et al., 1978). Our data, however, also indicate a systemic effect of the *C. pecorum* infection that manifests itself in a downregulation of luteinizing hormone as well as, importantly, in an acute phase response of the liver that strongly associates with reduced milk production.

Among the dual mechanisms by which *C. pecorum* genital infection affects reproductive performance (Sheldon et al., 2009), the local response dominates. The infection intensity-dependent reduction in conception rates suggests a central role of chlamydial inflammatory stimuli, presumably largely LPS, in endometrial damage by the host immune response (Maxion et al., 2004; Sheldon et al., 2009). As the study of Bowen et al. (1978) suggests, it may be the inflammatory environment of the uterus, but not an infection of the embryo itself, that prevents implantation of a fertilized egg and thus causes infertility. In addition, it is well established that uterine infections also affect the central/neuro-endocrine regulation of fertility (Sheldon et al., 2002; Sheldon et al., 2009). The failure of *C. pecorum* infected animals to conceive may therefore also be attributed to an LPS-mediated suppression of the pulsatile release of GnRH from the hypothalamus and of LH from the pituitary (Peter et al., 1989; Karsch et al., 2002). The inability to ovulate a dominant follicle due to decreased LH concentration may thus explain in part the decrease in conception rates of *C. pecorum* infected cows.

The significantly increased embryonic death in *C. pecorum* infected animals suggests that the uterine endometrial response to chlamydial infection can have detrimental effects beyond conception and even after implantation of the embryo. A critical influence of maternal immune effector mechanisms on the survival of embryos has been shown in a murine model of early embryonic loss (Haddad et al., 1997). Activated macrophages and natural killer cells present at implantation sites produce nitric oxide (NO) in response to inflammatory stimuli such as LPS, and the cytotoxic NO molecule directly induces embryonic mortality (Haddad et al., 1995). Recently, Hoelker et al. have published similar observations in cows when they demonstrated that subclinical endometritis alters gene expression in the embryo, including genes responsible for membrane stability, cell cycle and apoptosis (Hoelker et al., 2012). Such changes may well be the cause of increased embryonic deaths in cows with *C. pecorum* infection of the genital tract.

***C. pecorum* infection and milk production.** The regulation of milk production in response to chlamydial infection is intriguing. Unlike on fertility, chlamydial infection and organism burden appear to have only marginal, if any, influence on milk production (Tables 2, 3). However, increased anti-*C. pecorum* plasma IgM at 100 DIM, and even more significantly an upward trend of these antibodies after 65 DIM, associated with elevated milk yield for up to 135 days subsequent to the first TAI (Table 3). This is consistent with earlier reports that link elevated anti-chlamydial antibodies to improved milk quality and performance (Biesenkamp-Uhe et al., 2007; Ahluwalia et al., 2010). An abundance of data indicates that immunoprotection against chlamydial infections requires $CD4^+$ T cell (Th1) immunity (Morrison et al., 2002; Morrison et al., 2005; Miyairi et al., 2010; Rank et al., 2010). Thus, we consider the short-lived

anti-chlamydial IgM response (Husband et al., 1972) not the direct effector, but a surrogate quantitative marker for protective Th1 immunity induced by a recent natural chlamydial infection.

The synergistic association between anti-chlamydial immunity and metabolic markers for liver health (cholesterol, albumin) with milk production strongly suggests that increased immunity reduces chlamydial colonization under the continuous infectious pressure in the herd, and thereby also reduces the systemic inflammatory stress resulting from these infections. It has been known for a long time that inflammatory cytokines and immune activation can have severe effects on hepatic metabolism, and that these effects can be tracked as acute phase response (Fleck, 1989; Waldron et al., 2003; Bertoni et al., 2008; Zebeli et al., 2009).

Several studies have demonstrated that inflammatory mediators influence milk production by direct and indirect mechanisms (Waldron et al., 2003; Bertoni et al., 2008; Zebeli et al., 2009). Pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 released by liver macrophages in response to bacterial LPS activate synthesis of acute phase proteins (APP) while liver proteins produced abundantly under normal condition are decreased under the influence of inflammatory cytokines (Fleck, 1989; Waldron et al., 2003; Bertoni et al., 2008; Zebeli et al., 2009). Classical acute phase proteins (APP) such as C-reactive protein, serum amyloid A, ceruloplasmin, or haptoglobin are synthesized in the liver as rapid and short-term response to inflammatory stimuli. In contrast, negative APP that are constitutively produced in the liver such as albumin or cholesterol binding protein (measured as plasma cholesterol concentration), are down-regulated as a slow and long-term response to inflammatory stimuli, and therefore provide for better long-term markers of liver stress than positive APP (Nicholson et al., 2000; Bertoni et al., 2008).

It is thought that inflammatory stress precipitates reduction of milk production by a negative energy balance that is caused by both impaired liver function and central anorexia. Impaired liver function results in metabolic diversion from regular anabolic to catabolic nutrient channeling and ultimately in a negative energy balance (Elsasser et al., 2000; Bertoni et al., 2008). Central anorexia is driven by proinflammatory cytokines TNF-α, IL-1β, and IL-6 that can reduce feed intake directly through their receptors in brain or by inducing hepatocyte synthesis of the acute phase protein, leptin, which in turn reduces feed intake by acting on receptors that are involved in satiety and energy expenditure and are present in hypothalamic nuclei (Shuster et al., 1991; Johnson et al., 2001).

The second PCA (Fig. 3) indicates that liver health as well as anti-chlamydial immunity are highly variable, and high milk-producing cows always show relatively high liver health combined with either low or high anti-chlamydial immunity. This suggests a scenario in which good liver health is an absolute requirement for high milk production, and in which anti-chlamydial immunity contributes to liver health in combination with other determinants of liver health such as nutritional status or host genetics (Bertoni et al., 2008; Zebeli et al., 2009). In other words, chlamydial disease in dairy cows is truly multifactorial in the sense that these contributing factors may exacerbate as well as compensate each other, resulting in either low or high milk-producing phenotypes.

Economic impact of *C. pecorum* infection of dairy cows. Our study showed substantial suppression of fertility and milk production by chlamydial infection in dairy cattle. Full-term pregnancies after first insemination of *Chlamydia*-infected cows was reduced by 28%, and 1-205 DIM milk yield in cows with low anti-chlamydial immune trend by 7%. Therefore, *C. pecorum*

infections of dairy herds, while clinically asymptomatic, have a profound impact on dairy production, potentially erasing the profit margin of dairy operations. These direct numbers for fertility and milk losses clearly underestimate the actual losses, since reproductive failure directly increases operational cost by delayed conception, extra synchronizations and inseminations, medicinal cost and increased culling (Bartlett et al., 1986b), and indirectly by decreasing total productive life of dairy cows. In addition, the losses that we detected represent the difference between relatively immune-protected versus relatively susceptible cows in a situation of continuous and high herd exposure to *C. pecorum*. Thus, we cannot say what the performance difference would be compared to truly *C. pecorum*-free animals Hence, herd-level intervention trials are required to correctly estimate the incurred economic losses in which reproductive performance and milk production of infected mock-vaccinated herds is contrasted to herds protected by an effective vaccine against *C. pecorum*.

Chapter 4: Overall conclusions

Our studies show that the chronic, subclinical *C. pecorum* infections can have a significant impact on health, growth and performance of the livestock. In neonatal calves, we saw reduction in growth rates of up to 48% in *C. pecorum* infected animals. In dairy cattle, there was a 7 % drop in milk production and 28% reduction in fertility. Reduced pregnancies, additional heat synchronizations and inseminations, suppressed milk production, increased culling rates each can substantially reduce return and increase operation costs, thereby erasing the profit margin. Additionally, stunted growth rates in calves may as well have profound economic impact by delayed maturity and puberty resulting in decreased productive life. Similar *Chlamydia*-induced potential losses may very well occur in other livestock farming systems. Our findings of very high plasma anti-*C. pecorum* IgM antibody concentrations in all animals studied, calves and cows, and simultaneous prevalence of multiple strains of *C. pecorum* suggest a high steady-state of an endemic *C. pecorum* infection. *C. pecorum* strain-specific ELISAs are being developed in the laboratory of Bernhard Kaltenboeck. Such methods will help to further elucidate the significance of multi-strain *C. pecorum* endemic infections.

In summary, these studies confirm the enormous economic impact of low-level asymptomatic *C. pecorum* infection and the need for efficacious vaccinations against *C. pecorum* infection. Such vaccines are also required to establish causality of chlamydial infection in the observed effects on health, growth and performance of livestock and thus to assess the true economic impact by allowing the comparison of performance of truly protected versus unprotected herds.

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