EFFECTS OF SEASONAL HEAT STRESS ON THE DIAGNOSIS OF *Mycobacterium avium* subsp. *paratuberculosis* IN TEXAS DAIRY CATTLE

A Thesis

by

SUMMER JOY STRICKLAND

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2004

Major Subject: Epidemiology

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Approved as to style and content by:

H. Morgan Scott (Chair of Committee)	Allen Roussel (Member)
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August 2004

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ABSTRACT

Effects of Seasonal Heat Stress on the Diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* in Texas Dairy Cattle. (August 2004)

Summer Joy Strickland, B.S., Texas A&M University

Chair of Advisory Committee: Dr. H. Morgan Scott

The validity of Johne's disease herd status programs and on-farm disease control programs that rely on established 'cutpoints' (e.g., S/P ratios) for ELISA serological tests such as the HerdChek® (IDEXX Laboratories Inc., Westbrook, Maine) may be susceptible to varied seasonal test accuracy. An observed depression in the proportion of a large central Texas dairy herd classified as "positive" during the months of July and August led to our investigation. We hypothesized that there exists a seasonal variability in serological response to *Mycobacterium avium* subsp. *paratuberculosis* that is directly related to heat stress. We further hypothesized that a reciprocal response may occur during periods of heat stress that results in a greater risk of fecal shedding in subclinically-infected animals.

Starting in October 2002, we invoked a testing regime that included multiple testing of 720 individual adult cows over each of four seasons including spring, summer, fall, and winter. We collected serum on a cyclic, monthly basis from three random groupings of cows, and, based on the ELISA results, collected fecal samples from the 20% of cows with the highest S/P ratios. We continued to sample in this manner for the period of one year and at the end of that period, analyzed the serum en masse.

The ELISA outcome values were treated both as categorical and continuous variables (e.g., S/P ratio). The potential lagged effects of heat stress on S/P ratio, as well as the potential for a change in test result (negative to positive or vice versa) due to heat stress were assessed. The results for fecal culture were analyzed on a categorical scale and were compared to the ELISA results to explore the possibility of a reciprocal response.

In the present study, we did not observe any of the significant seasonal effects of heat stress on S/P ratios and proportion seropositive to MAP that were observed in the historical (and less valid) cross-sectional time-series data conducted in 2001. In addition, we found no evidence to support a hypothesis linking seasonal heat stress to the risk of fecal culture positivity for the causative bacterium for Johne's disease.

DEDICATION

I would like to dedicate this thesis to my parents, Jerry and Pam Strickland, who have supported me in everything I have done. They have always inspired me to do my best, and given me the encouragement I needed to accomplish my goals. I would also like to extend this dedication to the rest of my family and friends, especially my fiancée, Guy. It is because of the support of these people that I have been able to accomplish so many of my goals.

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First, I would like to thank my advisor, Dr. H. Morgan Scott, for his help and guidance. He has challenged and encouraged me throughout the duration of this project, and I now have a much better understanding of the concepts of statistics and epidemiology that will help me in achieving my future goals. I would like to thank Dr. Ellen Jordan for her help throughout the course of this project and for her excellent editorial skills. I would also like to thank Dr. Melissa Libal for her instruction in the lab and for her feedback and suggestions that were very helpful. I would also like to thank Dr. Allen Roussel for his help and advice; I now have a much better understanding of the shape and format scientific literature should take.

I also gratefully acknowledge the efforts of the Texas Veterinary Diagnostic Labs, in particular those of Dr. Libal and her staff, as well as Tess Fernando and Ken Peck. Without their efforts, this project would have been impossible; they provided us with results in an efficient and timely manner, which is difficult with the sheer size and numbers involved in this project. They were always happy to comply with our any request, even if it required more time and effort on their part.

Finally, I would like to extend thanks to my fellow graduate students: Jonathan Hudson, for giving encouragement and advice; Linda Campbell, for her support and friendship; and Ashley Loven, for doing everything first, so I would have an example to follow. I am very thankful that I had the opportunity to share this experience with them.

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CHAPTER I

INTRODUCTION

Johne's disease (JD) is a chronic, enteric disease of ruminants caused by infection with *Mycobacterium avium*. subsp. *paratuberculosis* (MAP), otherwise known as *Mycobacterium paratuberculosis.* This disease is endemic within the United States, with up to 22% of U.S dairy herds classified as Johne's positive (NAHMS, 1997). The economic losses attributable to this disease can be substantial, especially when international trade is taken into consideration in addition to animal productivity issues (Nielsen et al., 2002a).

Johne's disease is usually contracted early in life, most often through the ingestion of fecal material or infected colostrum or milk. The clinical signs of this disease (severe weight loss, diarrhea) will usually develop when an animal has reached 3-5 years of age (Forshell, 2001). The progression of the disease is such that the majority of infected animals in a herd are at the subclinical stage of the infection.

There is no effective, practical treatment for JD, but the disease can be controlled through the identification and culling of infected individuals. On-farm biosecurity measures such as feeding pasteurized milk or milk replacer to neonatal calves and purchasing of animals from known "low-risk" sources are also important in controlling spread of the disease. However, in order to implement these measures, producers must be aware of the extent of the presence of JD in their herd. In an effort to improve awareness and minimize the spread of Johne's disease, the United States Animal Health

This thesis follows the style and format of the Journal of Dairy Science.

Association (USAHA, 1998) in conjunction with federal and state agencies initiated a Voluntary Bovine Johne's Disease Status Program (VBJDSP) for cattle. The program's main purpose is to identify low-risk herds to minimize the inter-herd spread of disease through outside purchase of replacement animals. Programs such as the VBJDSP are based on the use of standardized diagnostic tests, providing increased confidence for freedom from disease at the "herd level" provided that all animals test negative during strategically designed testing schemes.

Detecting these subclinical animals (i.e., infected but not exhibiting clinical signs of JD) is extremely important to controlling the spread of this disease. Unfortunately, the chronic nature of Johne's disease complicates the application and interpretation of these tests (e.g., fecal culture and/or serological testing). Test specificity is usually high, but a low sensitivity makes it very difficult to detect all animals in a herd that are infected (Dargatz et al., 2001; Nielsen et al., 2002b). Since the role of these tests is crucial to on-farm biosecurity programs, potential problems affecting sensitivity and specificity also need to be identified and well characterized.

Background

In early 2001, during the conduct of a herd-health program designed to minimize calfhood exposure to MAP on a dairy farm in Texas, a seasonal pattern of serological response to MAP was observed. Roughly once per month, dairy cows that were in their fourth month of gestation were tested for serum antibodies to MAP using a commercial ELISA test. Upon preliminary examination of those data, a decrease in the proportion of

animals testing positive for Johne's disease during the summer months was noted (particularly during June, July and August). This decrease is illustrated in a categorical scale (i.e., proportion testing positive) in Figure 1.1, and in a continuous scale (presented as sample-to-positive (S/P) ratios) in Figure 1.2. Previous studies have shown that cattle experiencing hyperthermia (i.e., heat stress) can have reduced productivity, food intake, as well as altered endocrine function and energy balance (Wolfenson et al., 2000; De Rensis and Scaramuzzi, 2003), so it is biologically plausible that higher temperatures also will have an effect on cattle immune response to MAP infection.

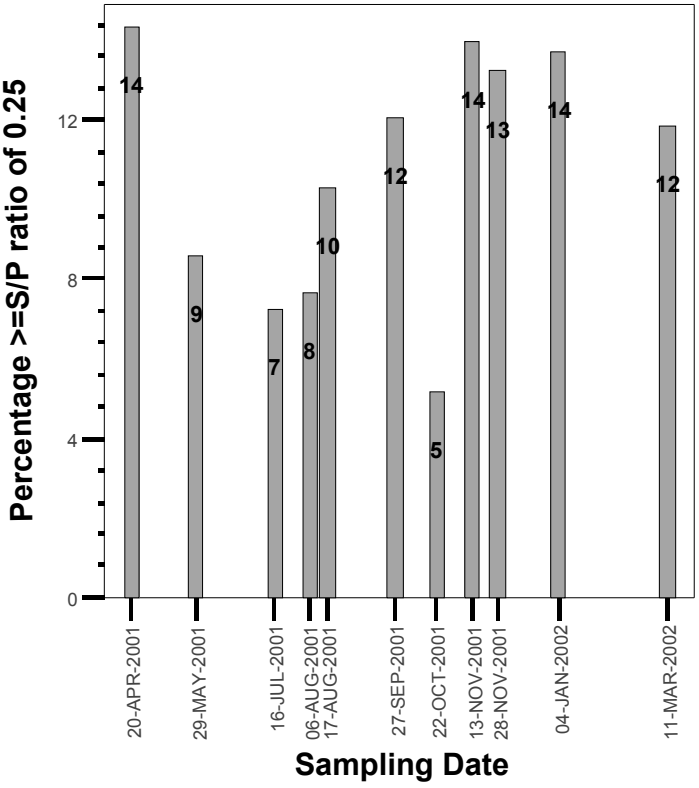

Figure 1.1. Historical cross-sectional time-series preliminary data in this dairy herd showing the percentage of cows testing positive each month for MAP using the commercial ELISA established S/P ratio cutpoint of >=0.25.

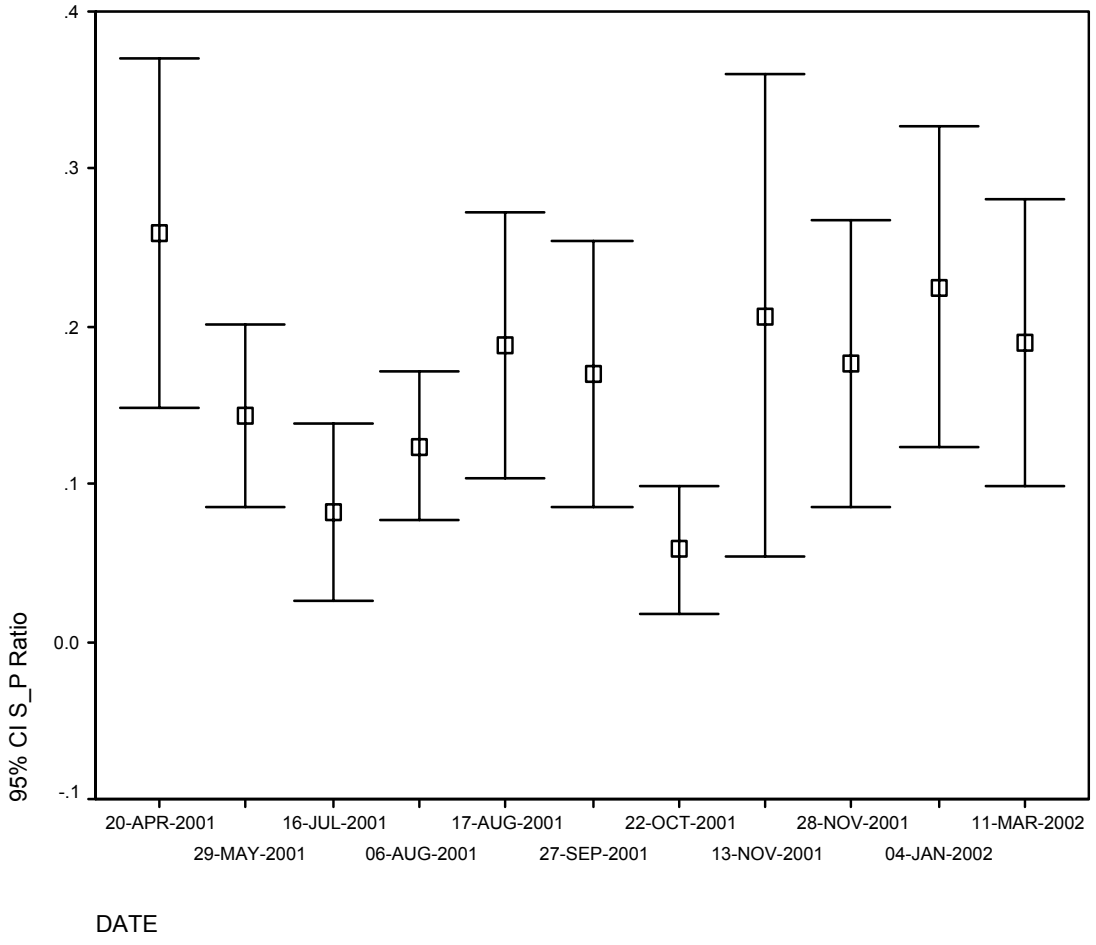

Figure 1.2. Monthly S/P ratio means and 95% confidence intervals from the preliminary cross-sectional data in this dairy herd.

Based on these preliminary cross-sectional data, the following hypothesis was proposed: that seasonal variability in serological response to MAP exists and is directly related to heat (climate) stress, likely mediated through endocrine and immunological mechanisms. A further hypothesis was also proposed: that a reciprocal response may occur (due to immunological suppression) during periods of heat (climate) stress that results in a greater risk of fecal shedding in subclinically-infected animals.

If the first hypothesis is correct, then the validity of Johne's disease herd status programs and on-farm disease control programs that rely on established 'cutpoints' (e.g., S/P ratios) for ELISA serological tests such as the HerdChek® (IDEXX Laboratories Inc., Westbrook, Maine) may be susceptible to varied seasonal test accuracy (as reflected in test sensitivity). In other words, the heat stress may be causing serious problems in the determination of a herd's disease status. The project described herein is designed to explore an observed phenomenon with the potential to undermine efforts to control the spread of MAP at both the farm-level and on a statewide and national level.

CHAPTER II

LITERATURE REVIEW

Natural History of the Disease

Organism

Johne's disease was first described by H.A. Johne and J. Frothingham in 1895 as an enteric disease of cattle caused by acid-fast bacilli. The organism found in the tissues of infected cattle was thought to be a form of *Mycobacterium tuberculosis,* but was later found to be more similar to *Mycobacterium avium*, and has subsequently been identified as *Mycobacterium avium* subsp. *paratuberculosis* (MAP -- Valentin-Weigand and Goethe, 1999). *Mycobacterium avium* and MAP are very closely related, but MAP is slower growing on appropriate *in vitro* culture media (taking up to 16 weeks for visible colonies to appear), and is mycobactin dependent (Thorel et al., 1990). MAP is also identified by the highly specific genomic insertion sequence IS900 (Green et al., 1989), and primarily infects ruminants.

Infection and Host Response

Infection with MAP occurs most often at a young age (under 6 months), usually via the fecal-oral route or ingestion of colostrum containing the mycobacterium. Some evidence exists that infection can occur through the placenta *in utero* (Sweeney et al., 1992), but ingestion of the mycobacterium seems to be the more common route of infection. The initial immune response (Stage 1) to MAP is cell-mediated and is

detectable by gamma-interferon or delayed-type hypersensitivity (skin) tests. Most of the mechanics of the infection are unknown at this stage, but it is thought that γδ T cells play a significant role in the first line of defense against MAP (Baldwin et al., 2000). Some studies have shown that these cells are highly sensitive to stress, making the animal more susceptible to infection during periods of high stress such as relocation and/or parturition (Kimura et al., 1999; Baldwin et al., 2000).

Stage II of the infection -- the sub-clinical stage -- is marked by the beginning of the humoral response. A low and often undetectable serum antibody titer of IgG is present -- particularly the IgG_1 isotope -- making diagnosis at this time difficult but possible by enzyme-linked immunosorbant assay or ELISA (Collins, 1996). These tests are based on measuring the level of antibody response to an antigen specific to MAP, such as liparabinomannan (LAM), which is a lipopolysaccharide component of the cell wall (Valentin-Weigand and Goethe, 1999). Other antigens specific to MAP have been identified, but very little is known about their role and function in the pathogenesis of the mycobacterium. The infected animal will also shed the bacteria intermittently in the feces, making it possible to make a diagnosis using fecal culture or direct PCR. Despite these difficulties, it is at this stage that diagnosis is most important for herd management of the disease. The infrequent shedding of MAP stimulates a low-level humoral response, with circulating antigen-specific immunoglobulins including IgA, IgM and IgG (Coussens, 2001). Serum IgG levels appear to be the most useful and accurate gauge of infection, especially since IgA cannot be detected in an ELISA (Abbas and Reimann, 1988). However, dramatic fluctuations in IgG titer have been observed to

occur in animals that have been followed throughout a single lactation, particularly at calving (Jakobsen et al., 2000). Another study showed antibody levels to be much higher at the beginning and end of lactation (Nielsen et al., 2002a). It is because of this variability in humoral response that an accurate identification of infected animals at this stage -- and especially at Stage 1 -- is very difficult.

Signs of the early clinical stage (Stage III) of infection will appear after an incubation period of 2 to10 years. These signs include a loss in body weight despite dry matter intake remaining normal, feces eventually becoming more liquid in consistency, and vital signs otherwise remaining normal (Whitlock and Buergelt, 1996). It is at this point that the infected animal is typically shedding larger amounts of the bacteria in the feces and diagnosis of paratuberculosis is much easier to confirm. It has been estimated (Whitlock and Buergelt, 1996) that for every animal in a herd showing clinical signs of Johne's disease, 15 to 20 other cattle are infected within that herd.

In a short period of time (usually 3 to 4 months), the animal will progress to stage IV of the infection -- advanced clinical disease. Stage IV is marked by a severe diarrhea and progressive emaciation of the infected individual. At this time the animal is more likely to be culled due to subsequent losses in milk production, as well as to the severe weight loss (Goodell et al., 2000). If the animal is not sent to slaughter, death will eventually occur as a result of dehydration and malnutrition. There is no effective and economical cure for Johne's disease.

Pathology

Once an animal has been infected with MAP, the bacterium moves from the host's intestinal lumen and enters the subepithelial macrophages (reviewed by Valentin-Weigand and Goethe, 1999; Stabel, 2000). There are many gaps in the current knowledge base, but it is generally accepted that the pathogen eventually begins to interfere with the macrophage's ability to organize the host's bacterial defense mechanisms, resulting in the host's inability to rid itself of the pathogen. Granulomatous lesions in experimentally infected animals have been found in the Peyer's patches and mesenteric lymph nodes very early after ingestion of the pathogen. The most common site of lesions characteristic of chronic enteritis in cattle are found in the ileum. In cattle, the most common clinical manifestations of the disease are severe diarrhea and weight loss; however, sheep and goats do not always exhibit diarrhea.

Detection of Diseased Animals

Tests for the detection of Johne's disease have been reviewed extensively, and it is generally agreed that each has its problems (Collins, 1996; Whitlock et al., 2000; Nielsen et al., 2001). The natural history of the disease makes it very difficult to develop an accurate test for all stages of the disease. Many diagnostic tests have been developed for the purpose of identifying MAP infection in cattle and other ruminants and can be divided into roughly three categories (Kalis, 2001). The first category is based on identification of the organism directly, either in the feces or in tissue. These tests include examining feces and tissue directly using a Ziehl-Neelsen stain to identify acid-

fast bacteria, growth of the organism on selective media, and polymerase-chain reaction (PCR). The second category consists of tests designed to detect antibodies in serum. These include complement fixation (CFT) and absorbed enzyme-linked immunosorbant assay (ELISA). The third category of diagnostic tests for MAP measures the level of cell-mediated response in order to detect an infected animal. These tests include skin tests and gamma interferon assays, and have the advantage of being able to detect infection in young animals, but with very poor specificity. Detailed methodological descriptions for each of these tests are available elsewhere (Collins, 1996; Kennedy, 2001; Kalis, 2001). However, these are beyond the scope of this review, and so instead focus will be on describing the application of two commonly utilized diagnostic tests in individual animals and herds: fecal culture and ELISA.

Fecal Culture

Fecal culture is generally accepted as the 'gold standard', with a specificity approaching 100% but a low sensitivity, ranging from 33-50% (Sockett et al., 1992; Whitlock et al., 2000). In the study by Whitlock, cattle were sampled every six months over a four-year period. At the first testing, sensitivity of fecal culture was estimated to be 38% -- but only 25% when culled cattle were included and assumed to have the same rate of fecal culture positives. At the end of the repeated testing, fecal culture sensitivity was 33%, indicating that repeat testing is necessary for the most accurate picture of infection in the herd. It is possible that the higher test sensitivity was due in part to older cattle being more likely to shed the bacteria, as the study lasted four years. Even with its

low sensitivity, fecal culture is still considered to be the most accurate gauge of a herd's infection status. However, due to the costly process and slow growth of the mycobacterium -- it can take up to 16 weeks to obtain results – it is expensive and inconvenient to implement on a herd level.

ELISA

Several enzyme-linked immunosorbant assays or ELISAs have been developed for diagnosis of Johne's disease and have been studied extensively, particularly in comparison to fecal culture and other immunological assays. These tests are more cost effective than the fecal culture and results are available very quickly. However, the same problems often arise — reasonably high specificity but a low sensitivity, usually between 35-45% (Dargatz et al., 2001; Nielsen et al., 2002b). The variability in ELISA sensitivity has been documented extensively. Whitlock et al. (2000) reported that ELISA has a higher sensitivity in animals that are shedding large amounts of bacteria (approaching 75%) than in those animals that are shedding small amounts (as low as 15%). Overall, when compared to fecal culture with a sensitivity of 33% and specificity approaching 100%, absorbed ELISA had a sensitivity of 40-55% and a specificity of 98.9% (Whitlock et al., 2000). In contrast, a single case study involving an experimentally infected heifer (www.johnes.org) suggested that when serum antibody titer decreased, there was a significant reciprocal increase in fecal shedding. Stabel et al. (2002) found ELISA sensitivity to be lower (around 25%) -- and specificity to be much

lower (only 44%) -- when compared to fecal culture than values reported in previous studies.

Individual ELISA results are subject to substantial variation as well. In 2002, Hirst et al. published the results of a study examining changes in cow ELISA test status using repeated samples. Cows with an S/P ratio equal to or above 0.25 were considered positive (per manufacturer's guidelines) and cows were considered "suspect" if they had an S/P ratio between 0.10 and 0.24. In low prevalence herds (<5% apparent prevalence), 71% of cows with suspect- and 35% with positive-results were negative for the repeated or second sample collected. Cows with negative results were not as likely to change status, and test-suspect cows were more likely to change to negative (71% in low prevalence herds, 35% in high prevalence herds). Barrington et al. (2003) also identified substantial variation in S/P ratio from ELISA utilizing serum, but in this case variation occurred on a day-to-day basis.

Herd Testing Programs

Herd testing programs around the world are typically based on the use of ELISA tests and fecal culture. These include programs implemented by national agencies as well as those used by individual operators at the herd level. Many farms use these tests to control Johne's in their herd as part of best management practices. In the United States, the Voluntary Bovine Johne's Disease Control Program or VBJDCP utilizes these tests to implement a 4-level national herd certification program to identify herds achieving "test negative"status (USDA-APHIS, 2002). The program is also designed to

help herds identified as being "test positive" to reduce the overall prevalence of infection in the herd. This program uses the ELISA test as an initial screening tool and fecal culture -- considered an "official Johne's test" -- is utilized as the "gold standard" to confirm positive status. In both cases these tests are conducted only in laboratories that have met requirements stated by the National Veterinary Services Laboratories. Sensitivity for ELISA and fecal culture were estimated to be 25% and 40% respectively (per agreement by the Herd Status Committee of the National Johne's Working Group), and specificity for the sequential testing was stated as approaching 100% (given follow up of all ELISA positives with fecal culture; USDA-APHIS, 2002). The US and other countries around the world have adopted control programs such as these in an attempt to recognize and control this disease. As public awareness of Johne's disease increases, more and more herds will presumably want to become involved in these voluntary control programs. Potential problems with these diagnostic tests, including some that have not been previously identified, must be addressed.

Factors Impacting Diagnostic Test Accuracy

There are many factors that have the potential to influence the accuracy of diagnostic tests for Johne's disease. In the present study, focus will be specifically on stress and how it can affect the immune functions of the animal. As the effects of climate are to be evaluated, most of this discussion will refer to stress caused by high environmental temperatures and humidity.

Stress and Infection and Immunity

Immune function can be influenced by the response of an individual to stress or stressors in the environment. This has been partially explained by the release of corticosteroids during an acute stress response; corticosteroids being well documented as being immunosuppressive (Sapolsky and Donnelly, 1985; reviewed by Dantzer and Kelley, 1989). Other mediators that have been tentatively identified include pituitary hormones such as growth hormone and prolactin, endogenous opiates, and catecholamines. There are still many unknown reasons for why stress causes immunosuppression, but its effects have been well documented in both human and animal studies (Dantzer and Kelley, 1989; reviewed by Rabin, 1999). The effects of stress have also been demonstrated as a decrease in systemic circulation of γδ T cells (Baldwin et al., 2000), lowering of serum and colostral antibodies (Kelley et al., 1982; Nardone et al., 1997), as well as interference with neutrophil phagocytic activity (Shurin et al., 1994). Though the effects of stress are not always bad, any of the problems mentioned above can influence the interpretation of diagnostic tests (e.g., ELISA) that depend on cutpoints of antibody titer and/or optical density to determine infection status. In the next section, the effect of an important environmental stressor, specifically the effect due to high temperature and humidity, is discussed in more detail.

Heat Stress

It has been shown (Johnson, 1987) that Holstein cattle are at their best production performance at neutral temperatures, specifically at a temperature humidity index

(apparent temperature) of no higher than 72, or in a "thermoneutral zone". Johnson defines the thermoneutral zone as "when the animal is at a state of minimal heat production in which body temperature is within normal range and the thermoregulatory functions of respiration, sweating and vaporization from skin, vasodilation, vasoconstriction and behavioral actions are not 'markedly' altered" (Johnson, 1987). When averages are above 72 (approximately 21^{o}C at moderate humidity), basic physiological functions and performance can be impaired. This is due, in part, to the fact that when cows are stressed, they consume less to avoid increasing the internal heat load (Kelley, 1982), making less energy available for maintenance. In addition, the animal will expend more energy increasing respiratory rates and panting (Correa-Calderon, 2004). Some specific areas that are affected include heat loss mechanisms, energy intake and balance, and endocrine function. The impairment of endocrine function has been well documented in the literature, specifically in regards to reproductive performance (reviewed by Wolfenson et al., 2000; De Rensis and Scaramuzzi, 2003). It has been shown that high temperatures can lead to decreased conception rates and overall poor fertility. Possible explanations for this include poor oocyte quality, impairment of embryo development, and increased embryonic mortality due to elevated body temperatures; as well as impaired follicular steroid production during and after periods of heat stress.

Investigators have disagreed on the subject of whether or not general immunological characteristics of cattle are influenced by temperature and/or heat stress. Conflicting results have been demonstrated from different studies. Some studies have

shown a marked decrease in antibody levels after exposure to high temperatures (Kelley et al., 1982; Nardone et al., 1997;). Kelley et al. (1982), after exposing calves to temperatures of 35^{o}C for 14 days, found a reduction of 27% in plasma concentrations of IgG_1 when compared to calves housed at thermoneutral temperatures. A study by Nardone et al. (1997) showed that the colostrum of heifers exposed to high temperatures had lower concentrations of mean IgG and IgA. Other studies, specifically one by Lacetera et al. (2002), have shown no effect of temperature on antibody levels. Another study by Kelley (1982) showed no effect on levels of IgG when there was a cooling off period at night. While several studies have been conducted on the change in levels of antibodies in cattle undergoing heat stress, most have examined dam's colostrum and antibody transfer; while a few others have evaluated serum antibody levels. However, none have evaluated specific levels of serum antibody when applied to a certain disease, such as Johne's disease and how they are affected by heat stress. In an extensive review of the literature, no studies undertaken to evaluate the potential effect that climate might have on the diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* using standard diagnostic testing such as serum ELISA and fecal culture were identified.

Objectives

The primary objective of this project was to examine the seasonal (i.e., period) effect of heat (or climate) stress on the interpreted results of serological testing using a commercial ELISA for MAP in a large Texas dairy cattle herd. An epidemiological field study design was employed to control for the effects of age -- birth cohort -- and

other potential confounders on the interpretation of a seasonal decrease in the proportion (or, risk) of herd adult cows being categorized as “positive” for Johne’s disease under a contemporary cohort sampling scheme. In addition, the potential for a change in test result (negative to positive or vice versa) for the same cow was examined with a repeated seasonal sampling schedule.

The secondary objective of this project was exploration of the possibility of a reciprocal increase in the risk of fecal shedding for those periods during which heat stress had reduced humoral response as measured by the S/P ratio of the commercial ELISA.

CHAPTER III

MATERIALS AND METHODS

Study Population

The cooperator herd consisted of approximately 3400 cows -- lactating and dry -- located on a commercial dairy in northern Texas. The high producing cows were housed in free-stall barns, while lower-producing cows and non-lactating cows were in dry lots with shades. During the prepartum transition phase, cows and heifers were moved into a free stall barn and then into individual maternity pens. Free stalls and maternity pens were bedded with sand. All housing areas were equipped with headlocks, which were used to restrain cows while collecting coccygeal vein and fecal samples. The cows were milked three times daily in a double-50 parallel parlor, with a rolling herd average >11,000 kg of milk. Due to a recent move from central Texas and expansion of the herd, just over half of the cows had been purchased from outside sources. Approximately 69% of the cows were in their first lactation, 16% were in their second lactation, and 7% in their third (see Figure 3.1), with an annual culling rate of 35-40%.

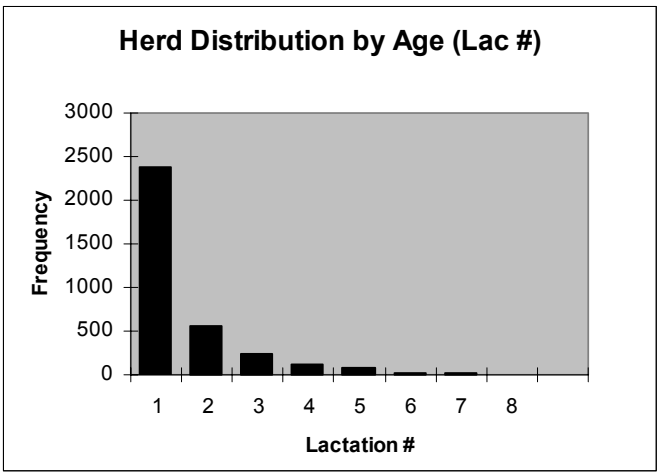

Figure 3.1. Distribution of the herd by age (lactation).

Herd production and health events were recorded and tracked using a commercial on-farm computer software program (DairyComp 305, Valley Agricultural Software, Tulare, CA). Previous serologic results for Johne's disease were recorded in this computer system with negative S/P ratio values recorded as being 0.0 (the computer software did not accept negative numbers) and test interpretation as positive or negative. Basic information regarding cow identification and productivity was extracted from the software program for use in selecting cows for inclusion. Once cows were selected for the research study, information regarding which cycle they were in and whether or not they were to have fecal samples collected was entered into the software program. Lists for subsequent sample collection were then generated using DairyComp305 commands.

Sampling Scheme

Eligible animals were identified through the farm's computerized records, excluding only those animals that were both not pregnant and late in their lactation, as this made them likely to be culled early on in the study (i.e., the ability to follow each cow through four seasons was considered imperative). Cows to be randomly sampled (using a random number generator in Excel -- Microsoft Corp., Redmond, WA) were first stratified on the basis of days in milk (DIM) and lactation number and the selection was weighted towards detecting those animals at greatest risk for identification of JD (i.e., presumably older cows). This was the reverse of herd demographics by age -- with 50% of those selected in their 3rd or greater lactation, 35% in their 2nd lactation, and 15% in their first. These cows were randomly allocated into 3 separate monthly sampling groups, and were then resampled in staggered periods separated by 3 month intervals. Selected cows were distinguished from others by a colored "ear clip" that was attached to both ear tags. Each group was assigned either a red, orange or white clip which would be clearly visible when cows were locked in head gates for feeding. At least 200 cows were randomly sampled per monthly sampling group -- a total of 600 cows over the first three months. These cows were then re-sampled, one sampling group of 200 per month, every three months for a period of one year (i.e., 4 times for each cow). Additional cows (≈20%) were also sampled at the first visit in expectation of some attrition owing to normal herd culling procedures. No interference with the day-to-day operation of the dairy herd was imposed upon the cooperating producer in this regard, nor was the

producer aware of any study test results that could affect his culling practices (i.e., this was a single-blinded study).

Sampling Procedure

At the start of the study (Month 1) blood was collected from first group of 200 cows. Approximately 10 ml of blood was collected from the coccygeal vein of each cow using a red top vacutainer tube and needle. These blood samples (spun down to sera) were evaluated by the Texas Veterinary Medical Diagnostic Laboratory (TVMDL) for Johne's disease using a commercial ELISA test (description below). For this first sampling period only, the 20% of cows with the highest S/P ratio as reported by the TVMDL (40 cows) were then resampled within a short period of the initial blood collection and 25 g of feces was collected from each cow and submitted to TVMDL for fecal culture. Fecal samples were collected directly from the rectum in sterile palpation sleeves using water as the only lubricant. The very same cows identified as the top 20% by S/P ratio at the first testing period were then re-sampled for fecal culture at each of the subsequent 3 seasonal sampling periods. This process was repeated 2 more times (200 blood per month, 40 feces per month) for the first three months, then the cycle was repeated for the next season beginning with the first group of 200 cows. A total of 2,400 ELISA tests (4 periods * 600 cows) and 480 fecal cultures (4 periods * 120 cows) were performed during the study period. See Figure 3.2.

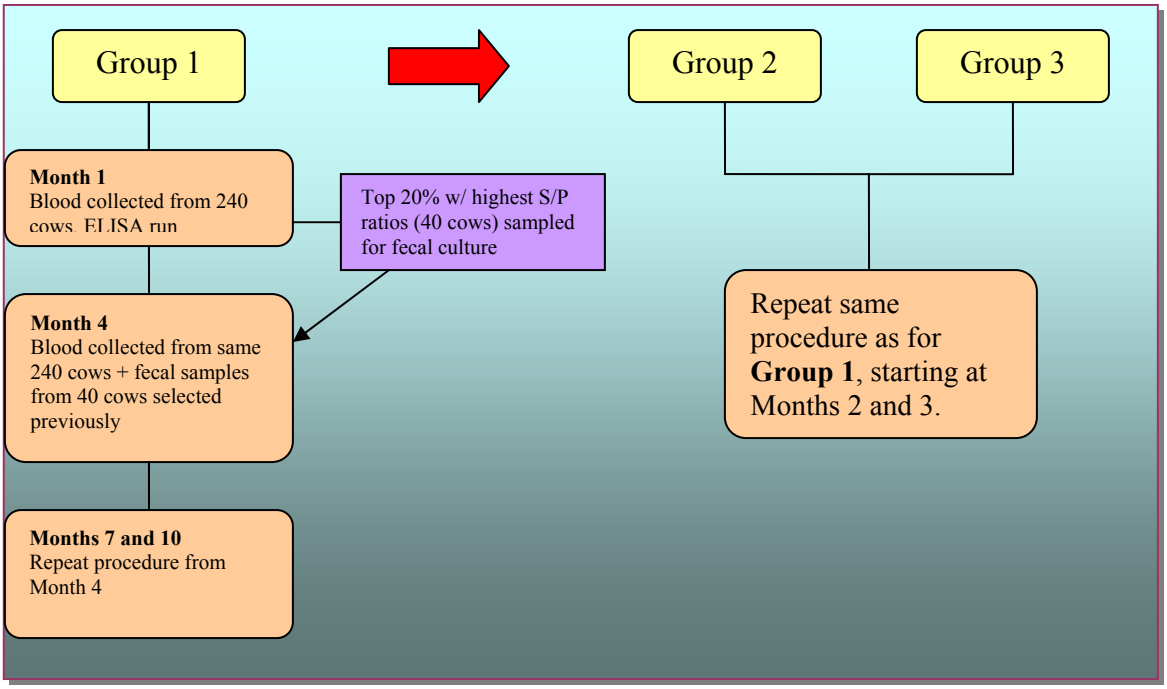

Figure 3.2. Diagram of sampling procedure.

Laboratory Procedure

Serum Antibody

After collection, all blood samples were taken to a nearby laboratory and centrifuged. The serum was decanted into serum tubes and frozen at -70°C. These tubes were sent on to TVMDL in College Station. A commercial solid-phase ELISA kit (HerdChek®, IDEXX Laboratories Inc.,Westbrook, Maine) was used to analyze the serum from the blood samples collected.

All experimental samples were analyzed in duplicate wells per TVMDL protocol following the manufacturer's instructions as follows. The IDEXX test utilized a microtitration format to coat MAP antigens on 96-well plates (IDEXX Laboratories Inc.,

2002). In order to remove cross-reacting antibodies, samples were supplemented with a diluent containing *M. phlei* which was then incubated in the coated wells. Antibodies specific to MAP formed a complex with the coated antigens. Samples were then washed to remove unbound materials and a horseradish peroxidase was added to bind to immunoglobulins bound to the antigen. All unbound conjugate was washed away and an enzyme substrate was added to the wells. The amount of bound immunoglobulin was determined by the rate of conversion of substrate and subsequent color formation was measured spectrophotometrically as optical density values.

As all sera were tested in duplicate, the mean OD value was used as the result for a single serum sample. These results were then converted to S/P ratios using the formula provided by the manufacturer, (mean OD of sample – OD of negative control) / (OD of positive control – OD of negative control) (IDEXX Laboratories Inc., 2002). After the initial evaluation of the first three groups to determine the 20% with highest S/P ratios, all samples (including the first) were subsequently maintained frozen at -70^{o}C upon arrival at TVMDL. At the completion of the collection period of the study, all serum samples were thawed and evaluated in duplicate per TVMDL protocol. In order to limit plate-to-plate and inter-operator variation, each set of samples collected per cow (up to 4) was analyzed on the same plate using a Biomek FX robotic device (Beckman Coulter, Fullerton, CA). The samples were randomly allocated on the plate to minimize within-plate variation.

Fecal Culture

Upon arrival at TVMDL, fecal samples were maintained at -70^{o}C. Throughout the study period, as time and resources permitted, 2-3 grams of each sample were thawed and placed in a 50 ml centrifuge tube and filled to 35 ml volume with distilled water. Samples were mixed on a rotating mixer for 30 minutes. Samples were left standing for 30 minutes and the supernatant fraction was decanted into a new 50 ml tube and centrifuged at 1700 g for 20 minutes. Supernatant was discarded and the pellet resuspended in 30 ml of HPC-BHI (0.9 % cetylpyridinum chloride/1.9% brain heart infusion). Samples were then incubated overnight at 37^{o}C as a decontamination step and then centrifuged at 1700 g for 20 minutes. Supernatant was discarded and the pellet resuspended in 1ml of "antibiotic brew" consisting of sterile water with 50 µg/ml amphotericin B, 100µg/ml vancomycin and 100µg/ml nalidixic acid. Samples were again incubated overnight at 37^{o}C and then inoculated onto Herrold's Egg Yolk Medium (0.2 ml per tube) with four tubes containing Mycobactin J and 1 tube without. Tubes were placed in a slanted position with caps loosened and incubated at 37^{o}C for 1-2 weeks. Tubes (samples) were checked for contamination, caps were tightened and tubes placed in an upright position after one week. Tubes were then incubated for about 8 weeks and checked weekly for appearance of MAP, up to a period of 15 weeks. If no growth was visible after 15 weeks, samples were determined to be negative. If colonies typical for MAP were observed at 15 weeks (i.e., those growing on Mycobactin J only) they were stained using cold acid-fast stain -- acid-fast colonies were confirmed positive for MAP by PCR. If at 8 weeks no significant growth had been observed, the samples

were reincubated for up to 15 weeks and re-examined for growth, and the above procedure was repeated for suspect colonies (TVMDL, 2003).

Climate Data

Climatic conditions were assessed using data from a local north Texas weather station, including monthly mean, monthly mean minimum and monthly mean maximum temperatures in degrees Celsius (mean minimum and mean maximum refer to daily measurements). However, since conditions in the barn and dry-lots were expected to differ from the general ambient regional conditions, an integrated HOBO® weather station data logger system (Onset Computer Corp., Pocasset, MA) was installed to record temperature and relative humidity near the barns, as well as precipitation and wind speed outside. This information was logged on a half-hourly basis and was used as a comparison to the local weather station data. Ultimately, the data from the local weather station was elected for use in this analysis because: 1) temperatures from two months prior to the start of this study (September 2002) needed to be assessed, whereas the HOBO® weather station was only installed on-farm during November 2002, and, 2) each of the mean monthly temperatures from the Hobo and local stations were found to be highly correlated ($R^2 = 0.997$; see Figure 3.3) for the months in which both observations existed.

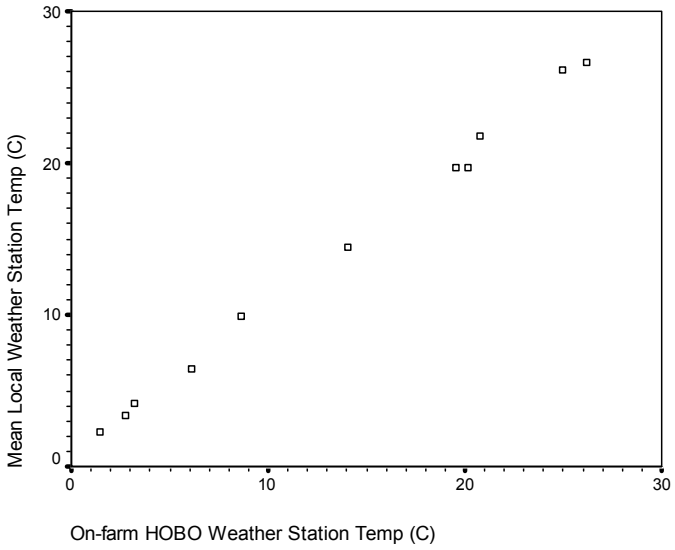

Figure 3.3. Graphical representation of linear correlation ($R^2 = 0.997$) between the on-farm Hobo® data-logger and local weather station.

Statistical Analysis

Variables assessed in the analyses included the following: proportion of cows seropositive to MAP antigen (via ELISA), S/P ratio results from ELISA, fecal culture result, age (in lactation #), stage of lactation (days in milk -- DIM), and mean, mean daily minimum, and mean daily maximum monthly temperatures.

S/P ratio was assessed as a continuous variable. However, since the distribution of S/P ratios in herds is not typically Gaussian normal, a natural log (ln) transformation of the S/P ratio was sometimes performed to better approximate the normal distribution (please refer to Figures 4.2 – 4.5 in the results section). S/P ratio was also assessed categorically on an ordinal scale using quintiles of S/P ratios for cows included in the analyses.

Lactation was analyzed as a categorical variable with four categories. This was due to insufficient numbers in the highest lactations (5-8) and in order to avoid statistical model instability. Categories 1-3 corresponded with lactations 1-3, category 4 included lactations 4-8 in order to more evenly disperse the cattle between groups. (See Tables 3.1 and 3.2).

Table 3.1. Original categories for lactation.

Category	Frequency	Percent
1	107	14.8
2	257	35.5
3	190	26.3
4	91	12.6
5	46	6.4
6	19	2.6
7	11	1.5
8	2	.3
Total	723	100.0

Table 3.2. New categories for lactation compiling groups 4-8 into one (Group 4) in order to avoid statistical model instability.

Category	Frequency	Percent
1	107	14.8
2	257	35.5
3	190	26.3
4	169	23.4
Total	723	100.0

DIM were analyzed were analyzed as categorical variables. Category 1 included: DIM 0-30, Category 2: DIM 31-60, Category 3: DIM 61-150, Category 4: DIM 151-305,

and Category 5: DIM 305+ (see Table 3.3.). These categories were chosen to represent physiologically important stages of lactation.

Table 3.3. Categories for Days in Milk (DIM).

Category	Frequency	Percent
1	73	10.1
2	56	7.7
3	193	26.7
4	257	35.5
5	144	19.9
Total	723	100.0

Cattle were divided into three sub-groups: cows that were initially randomly selected for inclusion, cows that were excluded from analysis because of insufficient data and, cows that were lost to follow up. These three groups were analyzed separately to determine if any biases were possible based on the final subset of cows eligible for repeated analysis with climate data.

The potential for selection bias owing to cows that were randomly selected but not included in the study (i.e., because they were missed completely during the collection periods) was evaluated using pre-study cow records and existing S/P ratios derived from the DairyComp 305 records provided by the cooperator. Cows missed were assessed as to lactation #, DIM, S/P ratio and proportion seropositive. These cows were then compared to the group of cows from which we were able to obtain at least one sample. Independent sample t-tests (i.e., for S/P ratio), or contingency tables (chi-square) and binary logistic regression (proportion positive) in SPSS 11.5 (SPSS Inc.,

Chicago, IL) were used to compare cows that were missed from those that were found. Due to the nature of the DairyComp program, most of the ELISA results (S/P ratios) obtained from the preliminary data that had been negative numbers were entered as zeros. Because of this, S/P ratios could not be appropriately divided into quintiles to be analyzed as ordinal categorical variables. Therefore these data were analyzed on a continuous scale only. The natural log transformation on S/P ratio was performed to more closely approximate the normal distribution. Basic descriptives of S/P ratio, lactation, DIM and proportion seropositive were calculated for both groups of cows.

Cows with only one sample submitted from the entire study period were excluded from the final analyses -- repeated samples were required to assess seasonality. These cows were likewise evaluated for potential selection bias on the basis of lactation, DIM, S/P ratio and proportion seropositive in comparison to the cows included in the final analysis using independent samples t-tests, chi-square and binary logistic regression in SPSS. Basic descriptives of S/P ratio, lactation, DIM and proportion seropositive were calculated for both groups of cows.

Cows actually lost to follow-up (i.e. physically not present at the end of the study as opposed to being missed at several sampling times) were stratified on the basis of culled/not culled (included deaths) and evaluated by both risk of culling (using logistic regression) and the number of days they were present in the herd (time-to-event of culling) during the study period using non-parametric Kaplan-Meier and semi-parametric Cox regression (Kahn and Sempos, 1989) in SPSS. Categories for

comparison to cows remaining in the study included lactation #, DIM, S/P ratio, quintiles of S/P ratio and proportion seropositive.

Basic descriptive statistics describing cows with repeated samples were tabulated or graphed for S/P ratio and fecal result on the basis of sampling cohort and season of sampling.

Effects of various current and lagged heat-related seasonal climatic factors on the proportion of cows exhibiting seropositivity were tested in a generalized linear modeling (McCullagh and Nelder, 1989) framework (SAS v 8.2 -- PROC GENMOD; SAS Institute, Cary, NC)) using a binomial distribution and a logit link function. Using the 'repeated' statement (by cow) and an auto-regressive (AR(1)) correlation structure, a generalized estimating equation was utilized to adjust for the within-cow dependence of the outcome variable over repeated sampling. Potential explanatory variables that were analyzed as categorical variables included sampling cohort, season of sampling, sampling month, fecal culture result, lactation #, mean, mean daily minimum and mean daily maximum temperatures the month of, month prior to, and two months prior to sampling. Lactation was divided into the same four categories as described above. The effect of climatic factors on actual continuous S/P ratio was assessed using a mixed modeling framework (SAS PROC MIXED) with random effects for cow (with an AR(1) correlation structure) and fixed effects for climate and cow-level factors such as lactation #. The S/P ratio was not ln- transformed since negative S/P values were possible. This model utilized the same variables as in the general linear modeling framework.

The effect of climatic factors on S/P ratio (categorized into quintiles) was assessed in a generalized linear modeling framework using a multinomial distribution and a cumulative logit link function (McCullagh and Nelder, 1989; Hardin and Hilbe, 2003). Using the 'repeat' statement (by cow) and an independent correlation structure, a generalized estimating equation (GEE) was utilized to adjust for the within-cow dependence of the outcome variable. An auto-regressive correlation structure would have been ideal, but the SAS program will not allow a correlation structure other than independent (Hardin and Hilbe, 2003) for the cumulative logit model. However, the GEE paramater estimates remain robust even if correlation structure is mis-stated (SAS Institute, 1996). The GEE parameter estimates are based on robust estimates of the standard errors derived from the empirical covariance matrix.

A transitional model based on a first-order Markov-chain for binary data was used to model the dependence of each change in positive/negative status and was based on the same explanatory variables described above (Diggle et al., 2002). A logistic regression was performed conditioned on the previous response (i.e., positive or negative). This provided consideration for the probability that each subsequent positive or negative test result was conditional (i.e., not independent) on the previous result, while continuing to consider the effects of other explanatory variables.

Fecal culture results were described on the basis of both the proportion of cows exhibiting seropositivity and previous results of a positive or negative test (ELISA or fecal culture). The probability of a cow testing positive for MAP via fecal culture was also evaluated for the effects of mean daily maximum temperature in months present and

1- and 2-months prior to sampling along with lactation # in a general linear modeling framework (SAS PROC GENMOD) using a binomial distribution and a logit link function. Using the 'repeated' statement (by cow) and an auto-regressive (AR(1)) correlation structure -- a generalized estimating equation was utilized to adjust for the within-cow dependence of the outcome variable.

CHAPTER IV

RESULTS

Weather Data

Monthly mean, monthly mean daily maximum and monthly mean daily minimum temperatures (°C) were obtained from a local north Texas weather station and are charted in Figure 4.1 including two months prior to and the duration of the study period.

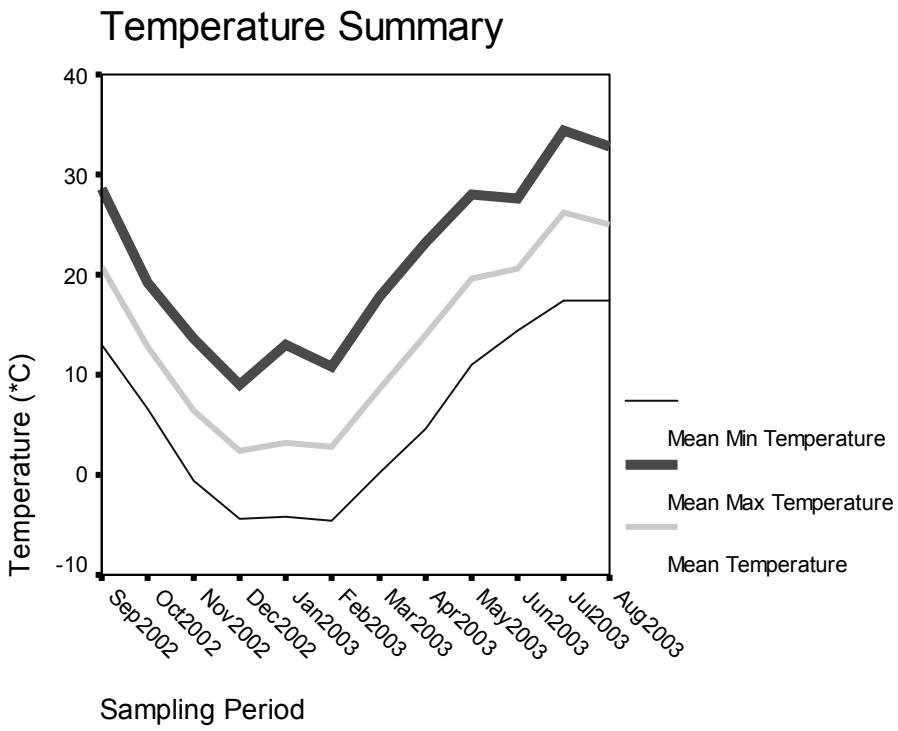

Figure 4.1. Mean monthly temperatures (^{o}C) for study period.

Cows Selected for Inclusion

Of the 723 cows randomly selected from the cooperator herd of approximately 3500 lactating dairy cows, 48 cows were not located in order to obtain a blood and/or fecal sample. Of these 48 cows, 5 were excluded from this analysis because it was determined that they were actually no longer remaining in the herd at the date of collection (e.g., they were culled in the days post-selection but prior to herd visit). An additional 14 cows were unaccounted for -- possibly due to a change in cow ID tag numbering system that occurred halfway through the study. Therefore, this analysis focuses on the differences that may exist between the 29 cows that were truly 'not found' and the 675 that were 'found'.

Basic descriptive statistics for pre-study S/P ratios, lactation number (surrogate for age) and days in milk (DIM) are summarized in Tables 4.1, 4.2, and 4.3 for cows 'found' and 'not found'. The distribution of S/P ratio results (based on previous sampling) for all cows selected for the study is shown in Figure 4.2, and a normal Q-Q plot of those same S/P ratios is shown in Figure 4.3. A natural log transformation was performed on the S/P ratio in order to better approximate a normal distribution and is shown in Figure 4.4, with a normal Q-Q plot of the log transformation of pre-study S/P ratios shown in Figure 4.5.

A summary of the number of cows testing positive for Johne's disease in the two different groups -- 'found' and 'not found' -- is shown in Table 4.4. The group of cows 'not found' during the course of the study differed significantly ($P < 0.05$) from cows 'found' on the basis of positive/negative test status for Johne's disease (based on pre-

study S/P ratios) with a Fisher's exact test p-value of 0.009 and an odds ratio of 0.245. The difference in Log S/P ratio between 'found' and 'not found' was also significant with a t-test p-value of 0.021 and a mean difference -0.7635 (equivalent to 0.4660 S/P difference). When analyzed categorically using a binomial logistic regression, lactation (p-value = 0.323) and DIM (p-value = 0.248) did not appear to differ significantly between the two groups (P > 0.05).

Table 4.1. Descriptive statistics for pre-study S/P ratios: overall and stratified by found/not found.

Cows						
	Mean	**Median**	**Min**	**Max**	**N**	**Std. Deviation**
Found	.067	.003	-.215	2.10	575	.239
Not Found	.134	.030	.000	.860	28	.245
Overall	.071	.008	-.215	2.10	603	.241

Table 4.2. Descriptive statistics for lactation number: overall and stratified by found/not found.

Cows						
	Mean	**Median**	**Min**	**Max**	**N**	**Std. Deviation**
Found	2.73	3.00	1	8	694	1.25
Not Found	2.88	2.00	1	7	29	1.45
Overall	2.76	2	1	8	723	1.35

Table 4.3. Descriptive statistics for Days in Milk (DIM): overall and stratified by found/not found.

Cows						
	Mean	**Median**	**Min**	**Max**	**N**	**Std. Deviation**
Found	197.46	181.5	3	756	694	134.813
Not Found	207.52	221.0	3	709	29	177.133
Overall	197.86	182.00	3	756	723	136.621

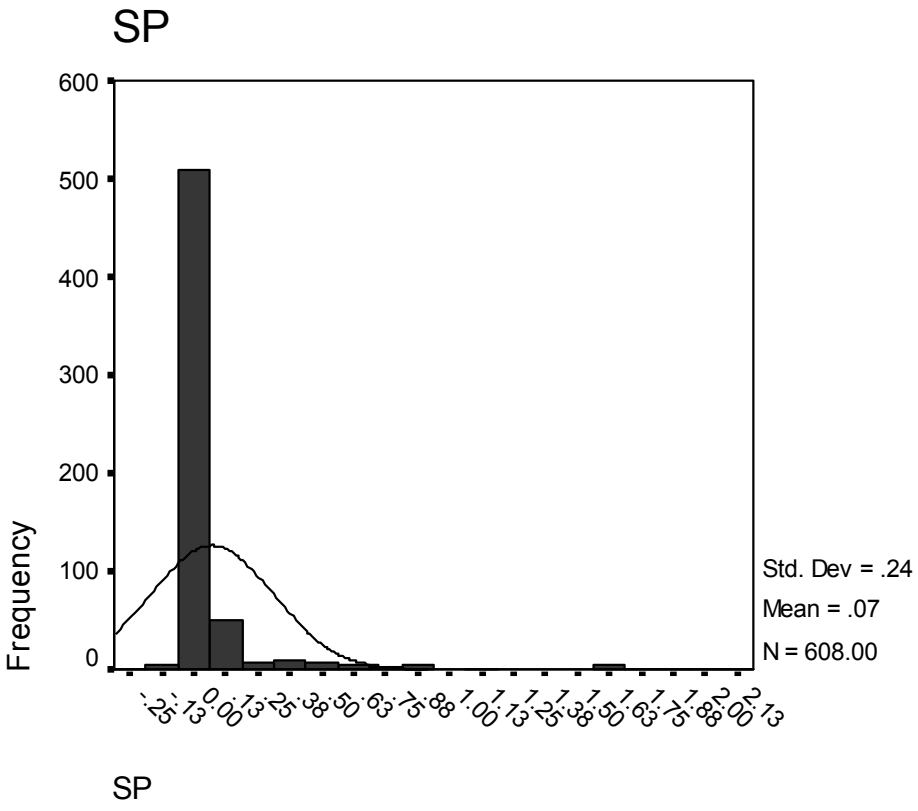

Figure 4.2. Distribution of pre-study S/P ratios of all cows chosen for study.

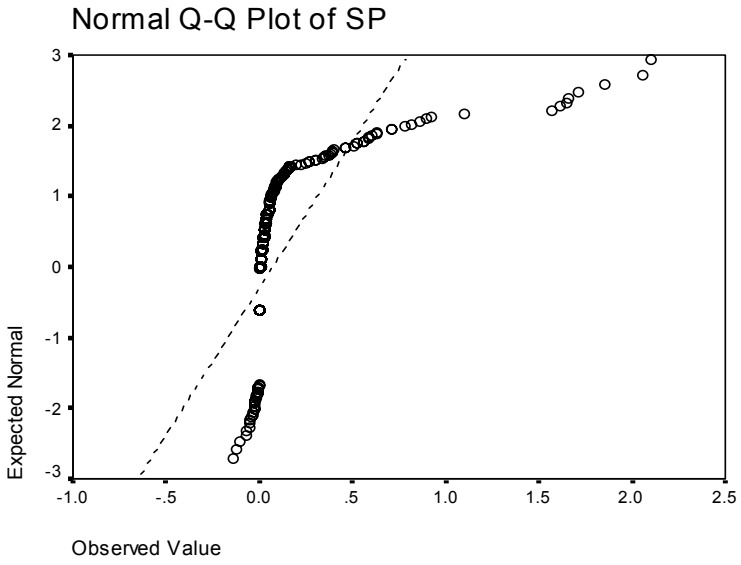

Figure 4.3. Normal Q-Q plot of S/P ratio. Deviation from straight line indicates departure from normality.

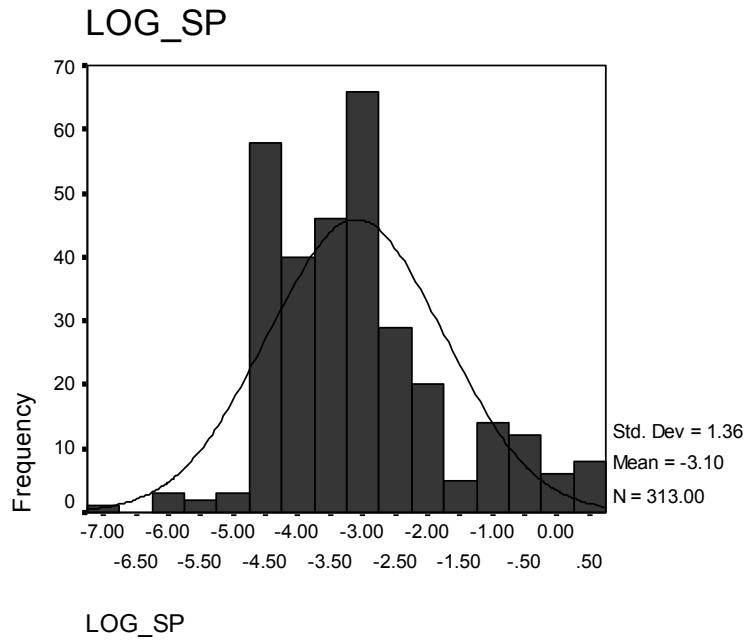

Figure 4.4. Distribution of natural log transformed pre-study S/P ratios of cows chosen for study.

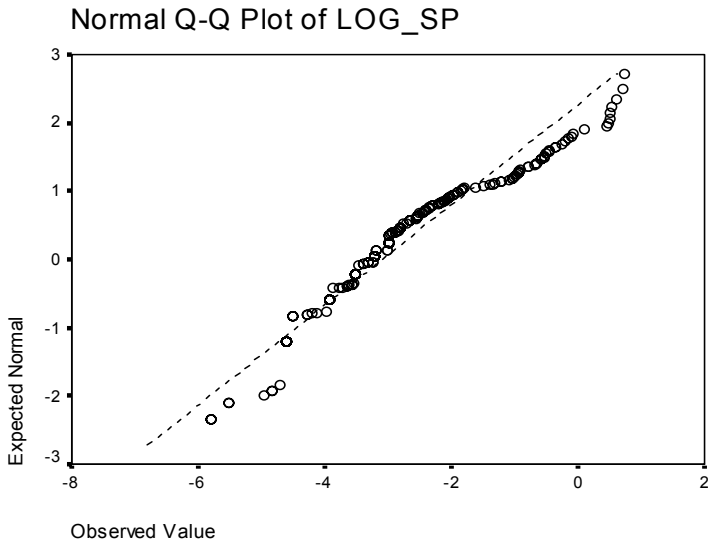

Figure 4.5. Normal Q-Q plot of S/P ratio after log transformation showing a closer approximation of the normal distribution.

Table 4.4. Cows testing positive or negative (result) to Johne's disease cross-tabulated by found/not found status.

		FOUND		Total
		0 (no)	1 (yes)	
RESULT	**0 (-)**	22	539	561
	1 (+)	6	36	42
Overall		28	575	603

Cows Included/Excluded from Analysis

At least one serum sample was obtained from 675 of the 723 cows randomly selected for this study. Analysis on repeated samples (i.e., at least 2 seasonal samples) was performed on 539 of those cows -- 136 were excluded due to the fact that only one serum sample had been obtained for those animals throughout the course of the study. For the purposes of this analysis, only those S/P ratio results obtained at the first sampling date were utilized. Of the 136 animals with only a single sample, 30 had a

single sample collected at sampling dates other than the first, so no results were obtained that could be used in this analysis. The remaining results compare the 106 cows excluded from the seasonality study from the 539 that were included (though only 448 had S/P ratio results from first collection period).

Descriptive statistics for S/P ratio, lactation, and DIM are summarized in Tables 4.5 to 4.7 for cows included and excluded from repeated sample analysis.

A summary of the number of cows testing positive for Johne's disease in the two different groups -- included and excluded -- is shown in Table 4.8. The group of cows not included in analysis during the course of the study differed significantly ($P < 0.05$) from cows included based on positive/negative status for Johne's disease with a Pearson chi-square value of 14.269, p-value of <0.0001 and an odds ratio of 0.296. S/P ratio was found to differ significantly between the two groups ($P < 0.05$) when analyzed using an independent samples t test with p-value = 0.002 and mean difference of -0.086. When analyzed categorically (by stratifying S/P into quintiles), S/P ratio and lactation were both found to be significantly associated ($P < 0.05$) with study inclusion, with results shown in Tables 4.9 and 4.10. In particular, ordered S/P quintiles exhibited a strong 'dose reponse' indicating higher probabilities of not being included as S/P categories rose. DIM were not found to differ significantly between cows included/excluded ($P > 0.05$).

Table 4.5. Descriptive statistics for S/P ratios: overall and stratified by included/excluded (lower overall numbers due to missing values).

Cows						
	Mean	**Median**	**Min**	**Max**	**N**	**Std. Deviation**
Included	.0761	.0235	-.1821	1.913	448	.2308
Excluded	.1633	.0735	-.0270	1.593	106	.2607
Overall	.0925	.0297	-.1821	1.913	554	.2380

Table 4.6. Descriptive statistics for lactation number: overall and stratified by included/excluded.

Cows						
	Mean	**Median**	**Min**	**Max**	**N**	**Std. Deviation**
Included	2.72	2.00	1	8	535	1.398
Excluded	3.10	3.00	1	7	106	1.390
Overall	2.76	3.00	1	8	641	1.353

Table 4.7. Descriptive statistics for days in milk (DIM): overall and stratified by included/excluded (lower overall numbers due to missing values).

Cows						
	Mean	**Median**	**Min**	**Max**	**N**	**Std. Deviation**
Included	191.8	172.0	3	650	526	132.3
Excluded	216.5	211.5	3	756	107	139.6
Overall	194.9	177.0	3	756	633	132.5

Table 4.8. Cows testing positive or negative to Johne's disease cross-tabulated by included (analyzed) / excluded (not analyzed) status.

		ANALYZE		**Total**
		0 (no)	**1 (yes)**	
POS_SP	**0 (-)**	89	424	513
	1 (+)	17	24	41
Overall		106	448	554

Table 4.9. S/P ratio analyzed by quintiles using a binomial logistic regression showing a significant difference ($P < 0.05$) in the log odds of being included versus excluded. For each incremental increase in S/P value the odds of being included in the analysis decreased.

Category	B	S.E.	df	Sig.	Exp(B)
SPQUINT			4	.000	
SPQUINT(1)	-.108	.506	1	.831	.898
SPQUINT(2)	-.610	.465	1	.190	.543
SPQUINT(3)	-1.459	.427	1	.001	.233
SPQUINT(4)	-2.252	.415	1	.000	.105
Constant	2.545	.367	1	.000	12.748

Table 4.10. Lactation analyzed categorically using a binomial logistic regression showing a significant difference ($P < 0.05$) in ages between included and excluded cows.

Category	B	S.E.	df	Sig.	Exp(B)
LACT4			3	.037	
LACT4(1)	-.396	.384	1	.302	.673
LACT4(2)	-.535	.391	1	.171	.585
LACT4(3)	-.989	.386	1	.010	.372
Constant	2.152	.334	1	.000	8.600

Cows Lost to Follow-up

Six hundred fifty-four cows were evaluated as to their herd status at the end of the study. This subset included all cows with at least one sample collected during the study period. Cows were determined to be either lost to follow up, or present at the end of the study (defined as the last scheduled sampling date). Cows lost to follow up were categorized on the basis of culled/not culled and the relation of time to this event and analyzed with S/P ratio quintiles, positive/negative status to Johne's disease, lactation and DIM. Twenty-one cows were excluded from this analysis because they were sold

for dairy purposes (i.e not culled). Cows that died were considered censored observations.

Positive/negative status to Johne's disease and S/P quintiles were found to be significantly ($P<0.05$) related to status of culled/not culled; results of the binary logistic regression are shown in Tables 4.11 and 4.12. Positive/negative status to Johne's disease was found to be significantly associated with a decreased time-to-culling ($P < 0.05$) with a p-value < 0.0001 and hazard ratio (HR) of 3.018 utilizing Cox proportional hazards regression. Increasing S/P quintiles were likewise found to be significantly associated with a decreased time-to-culling in a dose-response gradient ($P < 0.05$) -- results of the Cox regression are shown in Table 4.13. Survival curves for positive/negative status and S/P quintiles are shown in Figures 4.6 and 4.7. No similar association with either risk of culling or time-to-culling was demonstrated for either lactation number or DIM.

Table 4.11. Results of binary logistic regression examining association of culled/not culled status with positive/negative status to Johne's disease.

	B	S.E.	df	Sig.	Exp(B)
POS_NEG(1)	1.565	.344	1	.000	4.782
Constant	-.908	.098	1	.000	.403

Table 4.12. Results of binary logistic regression examining association of culled/not culled status with S/P ratio quintiles.

	B	S.E.	df	Sig.	Exp(B)
SP_QUINT			4	.000	
SP_QUINT(1)	.371	.316	1	.241	1.449
SP_QUINT(2)	.196	.323	1	.544	1.216
SP_QUINT(3)	.680	.309	1	.028	1.973
SP_QUINT(4)	1.300	.303	1	.000	3.671
Constant	-1.319	.235	1	.000	.267

Table 4.13. Results of Cox regression comparing S/P quintiles and culled status (culled/not culled).

	B	SE	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
						Lower	Upper
SP_QUINT			4	.000			
SP_QUINT(1)	.319	.275	1	.247	1.376	.802	2.359
SP_QUINT(2)	.207	.284	1	.466	1.230	.705	2.145
SP_QUINT(3)	.621	.266	1	.019	1.861	1.106	3.131
SP_QUINT(4)	1.024	.252	1	.000	2.785	1.699	4.565

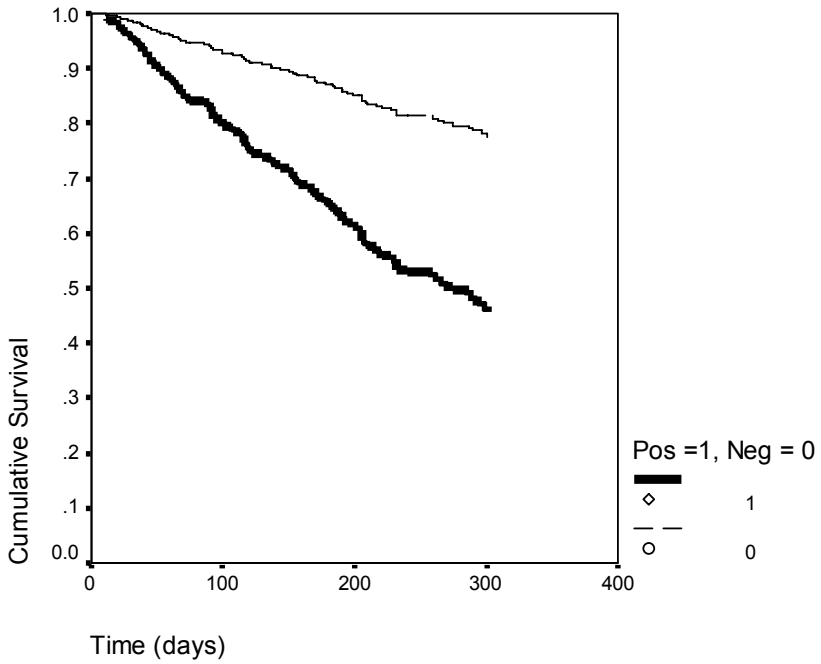

Figure 4.6. Survival plot for time-to-culling of Johne's serum ELISA positive/negative cows.

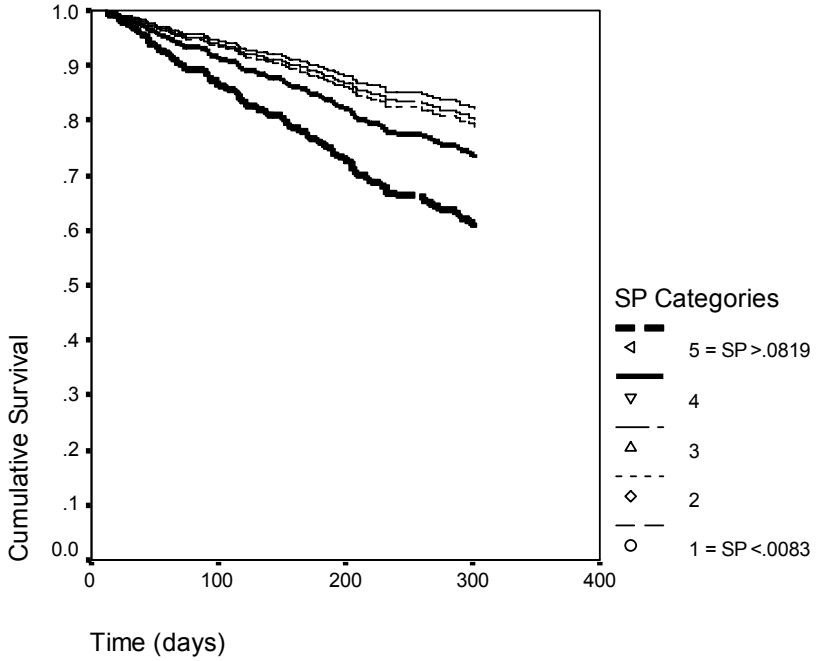

Figure 4.7. Survival plot of time to culling by ELISA SP ratio quintiles consisting of the following: 1 < .0083; 2 ≥ .0083, < .0215; 3 ≥ .0215, < .0396; 4 ≥ .0396, <.0819; 5 ≥ .0819.

Cows with Repeated Samples

Basic descriptive statistics of S/P ratios for the 539 cows with repeated samples (i.e., included in all subsequent analyses) are shown in Tables 4.14 and 4.15 stratified by sampling cohort and sampling season. Summary counts of positive/negative status to Johne's disease by S/P ratio for cows with repeated samples are shown below in Tables 4.16 and 4.17 by sampling cohort and sampling season. Summary counts of fecal results for cows with repeated samples are shown in Tables 4.18 and 4.19 by sampling cohort and sampling season. Summary counts for positive/negative status to Johne's disease by fecal culture result and S/P ratio are shown in Table 4.20; evaluation of the level of agreement between the two tests resulted in a Kappa statistic = 0.465.

Table 4.14. Descriptive statistics for S/P ratio by sampling cohort.

Cohort					
Start Date	**Mean**	**Median**	**Min**	**Max**	**Std. Deviation**
October	.0747	.0259	-.1821	2.855	.2630
November	.1001	.0219	-.0282	2.054	.2868
December	.0923	.0254	-.0912	1.769	.2609

Table 4.15. Descriptive statistics for S/P ratio by sampling season.

Season					
	Mean	**Median**	**Min**	**Max**	**Std. Deviation**
Fall	.0760	.0235	-.1821	1.913	.2299
Winter	.0968	.0230	-.1715	2.357	.2986
Spring	.0850	.0229	-.1715	2.631	.2556
Summer	.1002	.0270	-.1351	2.855	.2948

Table 4.16. Summary counts of positive/negative S/P ratio stratified by sampling cohort.

		POS_SP		**Total**
		0 (-)	1 (+)	
COHORT	**DEC**	519	39	558
	NOV	545	46	591
	OCT	520	31	551
Overall		1584	116	1700

Table 4.17. Summary counts of positive/negative S/P ratio stratified by sampling season.

		SP ratio		**Total**
		0 (-)	1(+)	
SEASON	**Fall**	425	24	449
	Winter	418	34	452
	Spring	374	28	402
	Summer	367	30	397
Overall		1584	116	1700

Table 4.18. Summary counts of positive/negative status (fecal culture result conducted on samples from cows with the top 20% of initial S/P ratios) and stratified by sampling cohort.

		fecal result		Total
		0 (-)	1(+)	
COHORT	**DEC**	93	14	107
	NOV	91	14	105
	OCT	90	4	94
Total		274	32	306

Table 4.19. Summary counts of negative/positive status (fecal culture result conducted on samples from cows with the top 20% of initial S/P ratios) and stratified by sampling season.

		fecal result		Total
		0 (-)	1(+)	
SEASON	**Fall**	82	8	90
	Winter	67	6	73
	Spring	59	13	72
	Summer	66	5	71
Overall		274	32	306

Table 4.20. Summary counts of negative/positive status to Johne's disease by fecal culture and S/P ratio.

		S/P Ratio		Total
		0 (-)	**1 (+)**	
Fecal result	**0 (-)**	234	30	264
	1 (+)	7	21	28
Overall		241	51	292

When S/P ratio was analyzed as a continuous variable in a mixed model analysis for the repeated seasonal samples, S/P ratios for season ($F = 2.74$, p-value = 0.0422) were shown to be significantly different ($P<0.05$) from the beginning to end of the study period (a full factorial model indicated that cohort by season interaction was non-

significant (P=0.733)). Results of the post-hoc t-tests comparing each season relative to summer (baseline) are shown in Table 4.21. Fecal result was also highly significantly associated with S/P ratio (F = 34.07, p-value = <0.0001; n=292); results are shown for the t-test in Table 4.22. Lactation number; sampling cohort; sampling month; and mean, mean daily minimum and mean daily maximum monthly temperatures in the month of, month prior to and 2 months prior to sampling did not demonstrate a significant effect on S/P ratio (P > 0.05); results are summarized in Table 4.23, and shown specifically (as an illustrative example) for mean daily maximum monthly temperatures in Table 4.24.

Table 4.21. Results of mixed model analysis for S/P ratio stratified by sampling season.

Season					
	Estimate	**Std. Error**	**DF**	**t value**	**Sign.**
Fall	-0.0336	0.0123	1158	-2.72	0.0066
Winter	-0.0168	0.0108	1158	-1.56	0.1186
Spring	-0.0080	0.0085	1158	-0.94	0.3484
Summer (Baseline)	0.0	—	—	—	—
Intercept	0.1104	0.0129	536	8.55	<.0001

Table 4.22. Results of mixed model analysis for S/P ratio by proportion of cows positive/negative by fecal culture result.

Fecal Result					
	Estimate	**Std. Error**	**DF**	**t value**	**Sign.**
0 (-)	-0.3363	0.0576	12	-5.84	<.0001
1 (+)	0	--	--	--	--
Intercept	0.4967	0.0583	109	8.52	<.0001

Table 4.23. Results of mixed model analysis for S/P ratio cross-tabulated by lactation number and climatic data.

Cow Variables					
		Num DF	**Den DF**	**F-value**	**Pr > F**
	Lactation	3	533	1.27	0.2837
Climate Variables					
	Sampling Cohort	2	534	0.31	0.7305
	Sampling Month	11	1150	1.13	0.3360
	Mean Max Temp	11	1150	1.13	0.3360
	Mean Max Temp (1 mo. previous)	10	1150	1.22	0.2738
	Mean Max Temp (2 mos. previous)	11	1150	1.13	0.3360
	Mean MinTemp	11	1150	1.13	0.3360
	Mean Min Temp (1 mo. previous)	9	1150	1.32	0.2237
	Mean Min Temp (2 mos. previous)	11	1150	1.13	0.3360
	Mean Temp	11	1150	1.13	0.3360
	Mean Temp (1 mo. previous)	9	1061	0.33	0.9651
	Mean Temp (2 mos. previous)	11	1150	1.13	0.3360

Table 4.24. Results of mixed model analysis for S/P ratio stratified by mean daily maximum monthly temperature.

Monthly Mean daily maximum Temperature (^{o}C)					
	Estimate	**Std. Error**	**DF**	**t value**	**Sign.**
8.94	-0.0292	0.0311	1150	-0.94	0.3488
10.88	-0.0199	0.0305	1150	-0.65	0.5133
12.95	-0.0325	0.0305	1150	-1.06	0.2871
13.50	-0.0160	0.0205	1150	-0.78	0.4368
17.88	-0.0013	0.0181	1150	-0.07	0.9420
19.11	-0.0580	0.0306	1150	-1.90	0.0578
23.23	-0.0132	0.0308	1150	-0.43	0.6675
27.72	-0.0035	0.0140	1150	-0.25	0.8053
28.00	-0.0109	0.0307	1150	-0.35	0.7229
28.56 (Baseline)	0.0	--	--	--	--
32.70	-0.0087	0.0310	1150	-0.28	0.7790
34.44	0.0086	0.0319	1150	0.27	0.7872
Intercept	0.1115	0.0217	536	5.14	<.0001

S/P ratio was also assessed with the above variables restricting the dataset to only those cows with S/P values above 0.05. Lactation number, fecal culture result, sampling season, sampling cohort, sampling month and, mean, mean daily minimum and mean daily maximum monthly temperatures in the month of, month prior to and 2 months prior to sampling did not show a significant effect on S/P ratio ($P > 0.05$) -- results are summarized in Table 4.25 and listed specifically for mean daily maximum monthly temperatures in Table 4.26, lactation number in Table 4.27 and fecal culture result in Table 4.28.

Table 4.25. Results of mixed model analysis for only those cows with S/P ratios above 0.05 cross-tabulated by cow and climate variables.

Cow Variables					
		Num DF	**Den DF**	**F-value**	**Pr > F**
	Lactation	3	129	2.58	0.0561
	Fecal Result	2	28	3.19	0.0567
Climate Variables					
	Sampling Season	3	303	0.30	0.8254
	Sampling Cohort	2	130	0.34	0.7138
	Sampling Month	11	295	0.81	0.6279
	Mean Max Temp	11	295	0.81	0.6279
	Mean Max Temp (1 mo. previous)	10	296	0.89	0.5472
	Mean Max Temp (2 mos. previous)	11	295	0.81	0.6279
	Mean MinTemp	11	295	0.81	0.6279
	Mean Min Temp (1 mo. previous)	9	297	0.99	0.4490
	Mean Min Temp (2 mos. previous)	11	295	0.81	0.6279
	Mean Temp	11	295	0.81	0.6279
	Mean Temp (1 mo. previous)	9	272	0.30	0.9745
	Mean Temp (2 mos. previous)	11	295	0.81	0.6279

Table 4.26. Results of mixed model analysis for only those cows with S/P ratios above 0.05 cross-tabulated by mean daily maximum monthly temperature.

Monthly Mean daily maximum Temperature (^{o}C)					
	Estimate	**Std. Error**	**DF**	**t value**	**Sign.**
Intercept	0.3114	0.0760	132	4.10	<.0001
8.94	-0.0131	0.1062	295	-0.12	0.9021
10.88	-0.0539	0.1050	295	-0.51	0.6083
12.95	-0.0642	0.1056	295	-0.61	0.5438
13.50	0.0467	0.0701	295	0.62	0.5341
17.88	0.0652	0.0618	295	1.06	0.2920
19.11	-0.1245	0.1055	295	-1.18	0.2388
23.23	-0.0163	0.1061	295	-0.15	0.8777
27.72	0.0073	0.0489	295	0.15	0.8807
28.00	-0.0116	0.1063	295	-0.11	0.9133
28.56 (Baseline)	0	--	--	--	--
32.70	-0.0116	0.1074	295	-0.11	0.9139
34.44	0.0380	0.1092	295	0.35	0.7284

Table 4.27. Results of mixed model analysis for only those cows with S/P ratios above 0.05 cross-tabulated by lactation number categories.

Lactation Category					
	Estimate	**Std. Error**	**DF**	**t value**	**Sign.**
Intercept	0.2260	0.0834	129	2.71	0.0077
1 (baseline)	0	--	--	--	--
2	0.2118	0.1085	129	1.95	0.0530
3	0.0807	0.1085	129	0.74	0.4584
4	-0.0586	0.1131	129	-0.52	0.6052

Table 4.28. Results of mixed model analysis for only those cows with S/P ratios above 0.05 cross-tabulated by fecal culture result.

Fecal Culture Result					
	Estimate	**Std. Error**	**DF**	**t value**	**Sign.**
Intercept	0.4526	0.0841	132	5.38	<.0001
Missing	-0.1562	0.0860	28	-1.82	0.0799
0 (-)	-0.1892	0.0757	28	-2.50	0.0186
1 (+) (baseline)	0	--	--	--	--

The proportion of cows seropositive to Johne's disease (positive/negative based on S/P ratio cutpoint of 0.25), when tested in a general linear modeling (GLM) framework were found to be significantly associated (P<.005) with fecal culture result (chi-square = 15.81, p-value = 0.0004) and lactation (chi-square = 8.90, p-value = .0307), with results shown in Tables 4.29 and 4.30. Sampling cohort; sampling season; sampling month; and mean, mean daily minimum and mean daily maximum monthly temperatures in the month of, month prior to and 2 months prior to sampling did not show a significant effect on S/P ratio (P > 0.05); results are summarized in Table 4.31, and listed specifically for mean daily maximum monthly temperatures in Table 4.32.

Table 4.29. Results of GLM for positive/negative S/P ratio with fecal result.

Fecal Culture Result					
	Estimate	**Std. Error**	**95 % Confidence Limits**	**Z**	**Sig.**
Intercept	1.0986	0.4856	0.1468 2.0504	2.26	0.0237
Missing (.)	-4.1261	0.5142	-5.1339 -3.1184	-8.02	<.0001
0 (-)	-3.1527	0.4916	-4.1162 -2.1893	-6.41	<.0001
1 (+)	0.0000	0.0000	0.0000 0.0000	--	--

Table 4.30. Results of GLM for positive/negative S/P ratio with categories of lactation number.

Lactation Category					
	Estimate	**Std. Error**	**95 % Confidence Limits**	**Z**	**Sig.**
Intercept	-3.4965	0.4600	-4.3982 -2.5948	-7.60	<.0001
1	0.7008	0.5818	-0.4395 1.8410	1.20	0.2284
2	1.1624	0.5063	0.1701 2.1546	2.30	0.0217
3	1.0590	0.5252	0.0296 2.0884	2.02	0.0438
4	0.0000	0.0000	0.0000 0.0000	--	--

Table 4.31. Summary of GLM results with positive/negative S/P ratio by categories of climate data.

Climate Variables			
	DF	**Chi-square**	**Pr > Chi Sq**
Sampling Season	3	3.72	0.2932
Sampling Cohort	22	1.09	0.5812
Sampling Month	11	14.73	0.1951
Mean Max Temp	11	14.73	0.1951
Mean Max Temp (1 mo. previous)	10	12.63	0.2450
Mean Max Temp (2 mos. previous)	11	14.73	0.1951
Mean MinTemp	11	14.73	0.1951
Mean Min Temp (1 mo. previous)	9	13.60	0.1374
Mean Min Temp (2 mos. previous)	11	14.73	0.1951
Mean Temp	11	14.73	0.1951
Mean Temp (1 mo. previous)	9	4.81	0.8509
Mean Temp (2 mos. previous)	11	14.73	0.1951

Table 4.32. Results of GLM for positive/negative S/P ratio by mean monthly maximum temperature.

Monthly Mean daily maximum Temperature (^{o}C)					
	Estimate	**Std. Error**	**Wald 95% Confidence Limits**	**Chi-Square**	**Sig.**
Intercept	2.1768	0.3181	1.5533 2.8004	46.81	<.0001
8.94	0.5395	0.4842	-0.4095 1.4886	1.24	0.2652
10.88	0.1999	0.4304	-0.6438 1.0435	0.22	0.6424
12.95	0.7136	0.4829	-0.2329 1.6600	2.18	0.1395
13.50	0.3622	0.4372	-0.4947 1.2190	0.69	0.4075
17.88	0.1561	0.4308	-0.6883 1.0005	0.13	0.7171
19.11	1.4608	0.5982	0.2884 2.6331	5.96	0.0146
23.23	0.5879	0.4838	-0.3604 1.5362	1.48	0.2243
27.72	0.4135	0.4697	-0.5072 1.3341	0.77	0.3788
28.00	0.2695	0.4472	-0.6070 1.1460	0.36	0.5468
28.56	0.2728	0.4379	-0.5855 1.1310	0.39	0.5334
32.70	0.7525	0.5017	-0.2309 1.7358	2.25	0.1337
34.44	0.0000	0.0000	0.0000 0.0000	.	
Scale	1.0000	0.0000	1.0000 1.0000		

S/P ratio divided into quintiles, when analyzed using a cumulative multinomial logistic regression model, was found to be significantly associated ($P<0.05$) with lactation number (chi-square = 10.84, p-value = 0.0126), results are shown in Table 4.33. Odds ratios for comparison of the probability of being in a lower S/P quintile for each lactation category relative to lactation 1 are as follows: 2 – 0.9749, 3 – 1.2515 and 4 – 1.6876. Sampling cohort; sampling season; sampling month; and mean daily maximum monthly temperatures in the month of, month prior to and 2 months prior to sampling did not show a significant effect on S/P ratio quintiles ($P > 0.05$); results are summarized in Table 4.34, and listed specifically for mean daily maximum monthly temperatures in the month of sampling in Table 4.35.

Table 4.33. Analysis of GEE parameter estimates (empirical standard error estimates) for S/P ratio quintiles and categories of lactation number.

	Parameter	**Estimate**	**Std. Error**	**95% Confidence Limits**	**Z**	**Pr > |Z|**
S/P Ratio Quintiles						
	Intercept1	-1.7558	0.1549	-2.0594 1.4522	-11.34	<.0001
	Intercept2	-0.7691	0.1447	-1.0528 0.4855	-5.31	<.0001
	Intercept3	0.0585	0.1432	-0.2221 0.3392	0.41	0.6828
	Intercept4	1.0484	0.1463	0.7617 1.3351	7.17	<.0001
Lactation Category						
	1	0.5233	0.2223	0.0877 0.9589	2.35	0.0185
	2	0.5487	0.1813	0.1934 0.9039	3.03	0.0025
	3	0.2989	0.1959	-0.0850 0.6829	1.53	0.1270
	4	0.0000	0.0000	0.0000 0.0000	--	--

Table 4.34. Summary of GEE analysis parameter estimates for S/P ratio quintiles by categories of climatic data.

Climate Variables			
	DF	**Chi-square**	**Pr > Chi Sq**
Sampling Season	2	3.59	0.1661
Sampling Cohort	3	6.11	0.1066
Sampling Month	11	15.73	0.1515
Mean Max Temp	11	15.73	0.1515
Mean Max Temp (1 mo. previous)	10	13.82	0.1814
Mean Max Temp (2 mos. previous)	11	15.73	0.1515

Table 4.35. Analysis of GEE parameter estimates (empirical standard error estimates) for S/P ratio quintiles and monthly mean daily maximum temperature.

	Parameter	Estimate	Std. Error	95% Confidence Limits	Z	Pr > |Z|
S/P Ratio Quintiles						
	Intercept1	-1.6878	0.1831	-2.0467 -1.3290	-9.22	<.0001
	Intercept2	-0.7059	0.1747	-1.0483 -0.3635	-4.04	<.0001
	Intercept3	0.1153	0.1756	-0.2288 0.4594	0.66	0.5114
	Intercept4	1.1010	0.1801	0.7479 1.4540	6.11	<.0001
Monthly Mean daily maximum Temperature (^{o}C)						
	8.94	0.2558	0.2326	-0.2001 0.7117	1.10	0.2714
	10.88	0.2197	0.2255	-0.2223 0.6617	0.97	0.3300
	12.95	0.1894	0.1520	-0.1085 0.4874	1.25	0.2127
	13.50	0.4083	0.2202	-0.0232 0.8398	1.85	0.0637
	17.88	0.6936	0.2276	0.2474 1.1398	3.05	0.0023
	19.11	0.1922	0.1692	-0.1394 0.5238	1.14	0.2559
	23.23	0.2950	0.1636	-0.0256 0.6157	1.80	0.0714
	27.72	0.4091	0.2305	-0.0428 0.8609	1.77	0.0760
	28.00	0.3226	0.2322	-0.1326 0.7777	1.39	0.1648
	28.56	0.3712	0.2273	-0.0743 0.8168	1.63	0.1025
	32.70	0.0925	0.2252	-0.3488 0.5339	0.41	0.6811
	34.44	0.0000	0.0000	0.0000 0.0000	--	--

Transition Model

Summary counts indicating the frequency of a change in positive/negative Johne's disease status (as measured using serum ELISA) from the previous test result for cows with repeated samples are shown in Tables 4.36 and Table 4.37 for both previous positive/negative test result and lactation number. The previous Johne's test result (positive/negative) was highly associated with transition to a similar result (Fisher's Exact Test p-value of <.0001) and with lactation number (Pearson chi-square value of

14.908 and p-value of 0.002). When evaluated together in the same regression model, only previous test status remained significant (P<0.05) with results shown in Table 4.38. Mean, mean daily minimum and mean daily maximum monthly temperatures in the month of, month prior to and 2 months prior to sampling; sampling cohort; and sampling season did not appear to differ significantly (P>0.05) for change (transition) in positive/negative status to Johne's disease, conditional on previous test result.

Table 4.36. Summary counts for change in positive/negative status relative to previous positive/negative S/P ratio.

		Trans_pos		Total
		0	1	
POS_SP	0	947	31	978
	1	14	45	59
Overall		961	76	1037

Table 4.37. Summary counts for change in positive/negative status and relative to categories of lactation number.

		Trans_pos		Total
		0	1	
Lactation Category	1	186	14	200
	2	401	46	447
	3	306	25	331
	4	266	7	273
Overall		1159	92	1251

Table 4.38. Results of logistic regression for change in positive/negative status (to positive) as associated with current positive/negative S/P ratio and categories of lactation number.

	B	S.E.	df	Sig.	Exp(B)
POS_SP(1)	4.578	.366	1	.000	97.273
Lactation (4)			3	.069	
Lactation (1)	.248	.437	1	.570	1.282
Lactation (2)	-.290	.493	1	.556	.748
Lactation (3)	-1.134	.615	1	.065	.322
Constant	-3.282	.380	1	.000	.038

Of the 539 cows with repeated samples, 111 had at least one fecal sample collected, with summary counts shown in the above Tables 4.18 and 4.19 stratified by sampling cohort and season. Fecal results did not appear to differ significantly between sampling cohorts or sampling season (P>0.05). Summary counts are also shown in Table 4.39 for fecal results with positive/negative S/P ratio and change in positive/negative status to Johne's disease as determined by fecal culture. When analyzed in a general linear modeling framework, fecal result was significantly associated (P<0.05) with positive/negative S/P ratio (chi-square = 14.32, p-value = 0.0002); results are shown in Table 4.40. Sampling cohort was found to be significantly associated (P<0.05) with fecal culture results when analyzed in a general linear modeling framework (chi-square = 8.99, p-value = 0.0112); results are shown in Table 4.41. Sampling season, sampling month, and lactation were not significantly associated

(P>0.05) with fecal culture result. The mean daily maximum monthly temperatures in the month of, month prior to and 2 months prior to sampling were unable to be analyzed in GEE due to insufficient data in all cohorts. However, the initial (non GEE-robust) parameter estimates and standard errors were calculated and all were found not to be significantly associated (P>0.05) with fecal culture result.

Table 4.39. Summary counts for fecal culture result with positive/negative S/P ratio and change in fecal culture result (Trans fecal result 0/1).

S/P Positive (1), Negative (0)			Trans fecal result		Total
			0	1	
0	Fecal culture result	0	130	2	132
		1	3	1	4
			133	3	136
1	Fecal culture result	0	12	6	18
		1	3	5	8
	Total		15	11	26

Table 4.40. Results of GLM for fecal result with positive/negative S/P ratio.

	Estimate	Std. Error	95% CI	z value	Sig.
Intercept	0.3567	0.3024	-0.2360 0.9493	1.18	0.2382
S/P 0 (-)	3.1527	0.4916	2.1893 4.1162	6.41	<.0001
S/P 1 (+)	0.0000	0.0000	0.0000 0.0000	--	--

Table 4.41. Results of GLM for fecal culture result with sampling cohort.

		Estimate	**Std. Error**	**95% CI**	**z value**	**Sig.**
	Intercept	2.8332	0.6243	1.6096 4.0568	4.54	<.0001
Cohort	**OCT**	0.0000	0.0000	0.0000 0.0000	--	--
	NOV	-1.8386	0.7050	-3.2204 -0.4568	-2.61	0.0091
	DEC	-1.3588	0.7188	-2.7676 0.0500	-1.89	0.0587

CHAPTER V

DISCUSSION AND CONCLUSION

In the present study (conducted from October 2002 – September 2003), there were no consistent across-cohort seasonal effects on S/P ratios and/or proportion seropositive to *Mycobacterium avium* subsp. *paratuberculosis* that had been observed in the historical (and less valid) cross-sectional time-series data collected in 2001. However, in the mixed model analysis of S/P ratio, a significant association ($P<0.05$) with season was detected (note that the season*cohort interaction was non-significant at $P=0.733$). This was likely due to a single cohort in which a trend toward increasing S/P ratio over time was shown throughout the course of the study. This is consistent with the literature (Collins, 1996; Holmes, 2004), which states that serological response increases over time with advancement of the disease. However, the other two cohorts did not exhibit this increase in S/P ratios over the study period. It is also possible that this finding was more consistent with the hypothesis of a seasonal effect on variation of S/P ratio results. The single cohort involved with the significant association was the cohort first sampled in October, 2002. It is possible that low S/P values in that month were due to an extended lagged effect of the previous summer temperatures. The subsequent cohort groups began in November and December, respectively. However, since results were inconsistent for the other models, it cannot be concluded that temperature has a significant effect on S/P ratio results. In the evaluation of the transitional model, no significant effects were found associating S/P ratio or seropositivity with temperature.

However, variation in seropositivity within repeated samples per cow was noted on several occasions, consistent with findings of other investigators (e.g., Hirst et al., 2002). In addition, we found no evidence to support a hypothesis linking seasonal heat-stress to the risk of fecal culture positivity for the causative bacterium for Johne's disease. There are two possible reasons that the lack of repeatability (with the historical data) may have occurred. First, the initial observed summer depression in S/P ratio and the proportion of cows seropositive to Johne's disease could simply have been an artifact, and not truly have been associated with temperature or heat stress. Another possibility is that there actually was an historical association with S/P ratio and heat stress, but that shortcomings in the present study proved sufficient to thwart proper assessment of the association. Discussion of each of these possibilities in further detail follows.

Artifact

The original data, for which declines in both S/P ratio and risk of seropositivity were observed during high-temperature months (see Figures 1.1 and 1.2) were evaluated as a cross-sectional study, with a single blood sample collected from specified cows only once per lactation. In other words, an entirely different group of cows was sampled during each subsequent month. Therefore, it is possible that the large decreases in S/P ratio observed in July and October 2001 (Figure 1.2) are related only to the features (e.g., age, infection status) of the specific groups of cows sampled at those time periods, rather than to ambient climatic conditions at the time. They might have been a group of very young cows, or possibly a group of cows at the same stage of MAP infection. For

these reasons the present investigation was designed as a cohort study with repeated sampling in order to more properly evaluate this observed phenomenon.

An additional problem with the historical data may relate to the manner in which the blood samples were analyzed. The historical samples were analyzed on a monthly basis with no measures in place to eliminate or reduce potential plate-to-plate or inter-operator variation. Extremely high values for S/P ratio -- some above 6.0 -- were recorded for certain cows. This alone could have accounted for the decrease in following months when samples were analyzed on different plates and possibly with different operators (no automated devices were used at that time). Again, it is for these reasons that methods suited to reducing inter-plate and inter-operator variability in the evaluation of blood samples were specified.

True Association

The historical cross-sectional data were obtained from the cooperator herd approximately one year prior to the beginning of the present study. During the course of that year, the cooperator herd physically relocated from central Texas to a new establishment in northern Texas. Mean temperatures for the central Texas region are usually somewhat higher than those in north Texas, (Figure 5.1), but the absolute temperature difference is not extreme. Rather, the key difference is in relative humidity; which is much higher in the central Texas location. Because of this humidity difference, there is little or no significant cooling-off period during the nighttime, and the daily temperature humidity index (THI) is usually much higher. Typically northern Texas

does not experience the levels of humidity that central Texas does throughout the summer, and therefore does not experience the high THI levels without nighttime cooling that central Texas does. According to some authors (Kelley, 1982; Johnson, 1987), if the nighttime temperature cools to an acceptable thermoneutral zone (THI = 72, Johnson, 1987), the effects of any heat-stress incurred during the day can be alleviated. As is noted in Figure 5.2, THI levels for the area and time period of the previous study were significantly higher than those for north Texas, reaching almost 80 during certain periods, and especially higher than 72 during the summer months. It was not determined if the cattle in the present study experienced periods of heat stress sufficient to cause any immunological suppression. It does seem likely that a linear relation between heat stress and the outcome variables is unlikely. Rather, a threshold effect at a critical cutpoint would instead be expected. Therefore, statistical analyses that are predicated on treating heat indices as continuous (as opposed to categorical) variables could be problematic. Another aspect that may have exaggerated the results from the previous study was the fact that results were obtained during a particularly hot summer, with temperatures higher than the normal average, even for that area (Figure 5.1 and 5.2).

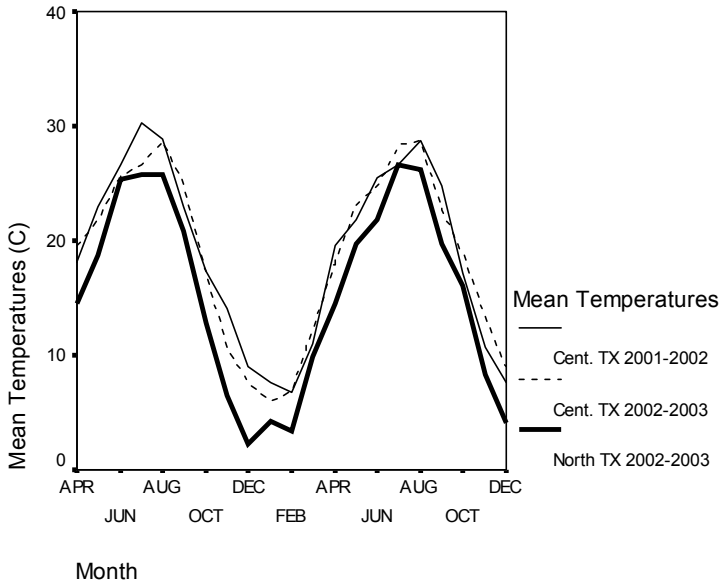

Figure 5.1. Mean monthly temperatures (^{o}C) for central Texas during the previous study (2001-2002) and mean monthly temperatures for central Texas and north Texas during the current study (2002-2003).

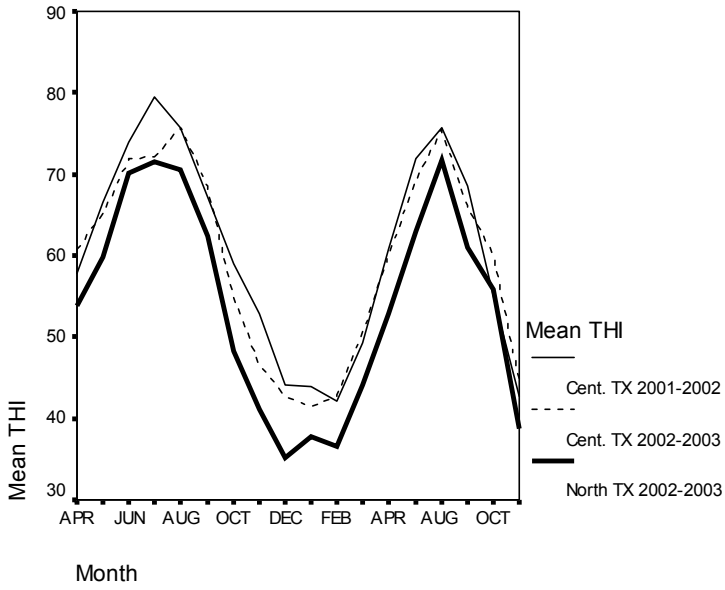

Figure 5.2. Mean monthly temperature humidity index (THI) for central Texas during the previous study (2001-2002), and mean monthly THI for central Texas and north Texas during the current study (2002-2003).

A second issue that might have obscured the relationship of S/P ratio to heat stress in the present study is that entirely different groups of cows were sampled in each of the respective studies. In the present study, one group of cows was followed over time, and this group did not include many, if any of the cows sampled in the previous study. According to the data, mean S/P ratio for cows in the previous study were significantly higher ($P<0.05$) than those for the current study, with mean values of 0.158 for the preliminary study and 0.089 in the present study. These differences over time are shown on a monthly basis in Figure 5.3. It is possible that the mean S/P ratios for the previous study were inflated because of the extremely high observations in S/P ratio mentioned earlier, and so median results (which are less susceptible to outliers) are shown in Figure 5.4. In addition, cows in the previous study had a mean monthly seropositive proportion of 0.099, while the present study had 0.067 seropositive, shown monthly in Figure 5.5. Concurrent with the conduct of the historical cross-sectional study, whose data were collected in response to a management-driven desire to manage seropositive cows differently from seronegative animals, control measures for Johne's disease were implemented. It is also possible that these more recent results indicating lowered S/P values and decreased seropositivity -- while good for the herd manager -- may have obscured observation of a significant variability in S/P ratios during the present study.

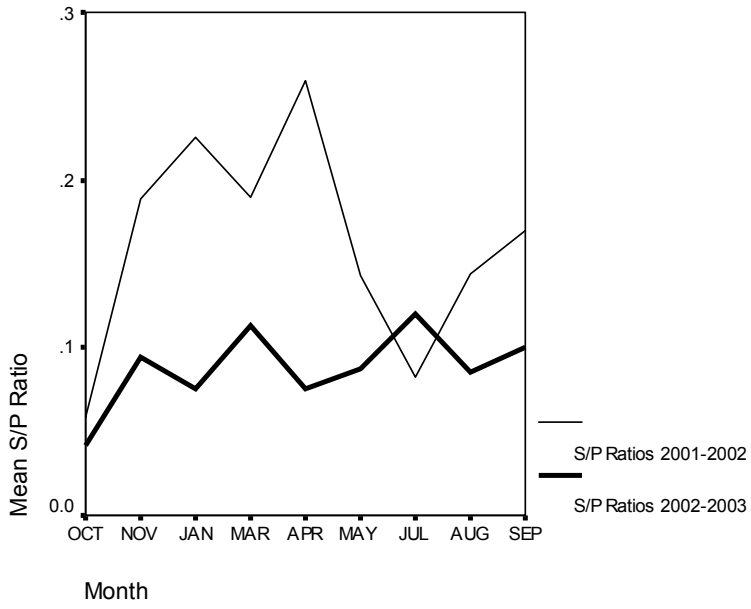

Figure 5.3. Mean S/P ratios for the calendar months during the previous study and the present study (1 year later).

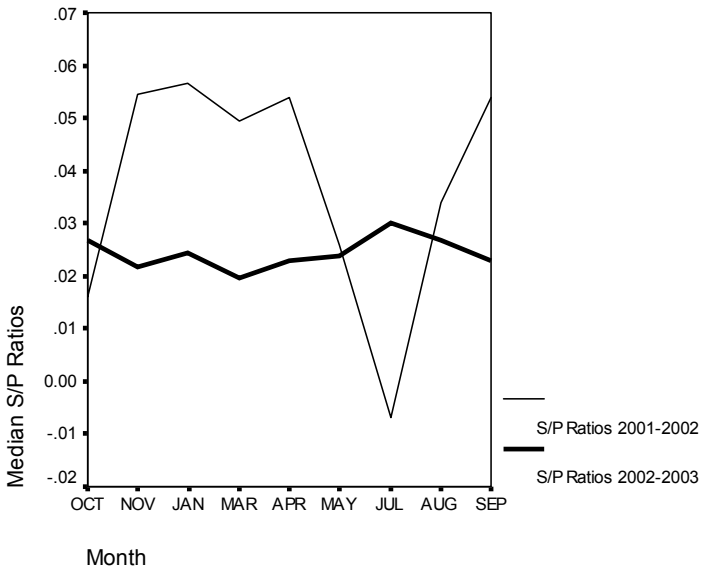

Figure 5.4. Median S/P ratios for the calendar months during the previous study and the present study (1 year later).

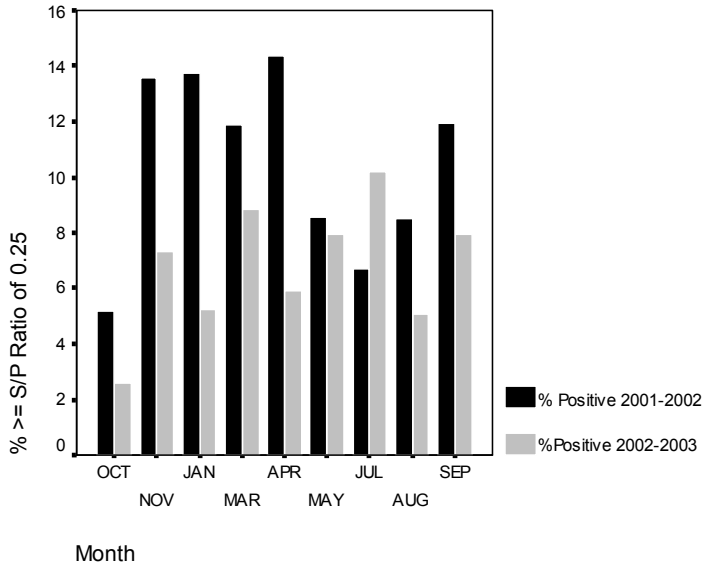

Figure 5.5. Proportion of cows seropositive to Johne's disease during the calendar months of the previous study and the present study (1 year later).

A third issue that might have affected the results is the finding that Johne's positive cows were at a much greater risk for culling. In other words a so-called "healthy worker survivor effect" might have aptly applied in this longitudinal study design. Since seropositive cows were being culled at a higher rate than seronegative cows, they were more likely to be lost to follow-up than seronegative or healthy cows. In the present study, the odds of a cow that was culled to be positive to Johne's disease by S/P ratio were approximately 5 times greater than seronegative cows. This was similar to results of another study (Wilson et al., 1993) in which a six-fold increase in culling rate for seropositive cows was identified. In addition, cows with S/P results in the top quintile (above 0.0819), were culled from the herd at a lactation risk of 32.1%, compared to animals with S/P ratios in the lowest quintile (less than 0.0083), with a

lactation culling risk of 18.3%. These results are somewhat lower than those identified by Goodell et al. (2000) in which cows with S/P ratios above 0.10 were removed from the herd at a rate of 35.8% to 50.0%, compared to cows with an S/P ratio of less than 0.09 with a removal rate of 30.4%. As illustrated in the scatterplot (Figure 5.6), older animals (animals in higher lactations) had uniformly (i.e., low variance) low S/P ratios, suggesting that culling pressures on animals with higher S/P ratios had significantly affected herd structure as it related to Johne's disease status. While others have evaluated total effects of Johne's disease status on culling (Merkal et al., 1975; Benedictus et al., 1987), no studies were found that described the effect on overall herd structure associated with Johne's disease status. In addition, some of the cows chosen for the study were missed at each collection period, due to several possible reasons. In order to obtain a sample from an individual cow, the cow had to be present in the allotted pen and locked in a head gate at feeding time. In a group of cows containing a significantly higher percentage of cows seropositive to Johne's disease, it is likely that sick cows might not go up to eat, and therefore would not be locked in a head gate. This would be a rather unforeseen form of 'selection' bias! Sick cows are also likely to be in other pens (e.g., sick pens) lacking an ordered feeding schedule and therefore not usually locked up. Any cows that repeatedly missed feeding and lock-up were likely to be ill, and would therefore be less likely to have a sample collected. After an extensive search of the literature, no other studies were found that evaluated this potential for selection bias. Since cows with the higher S/P ratios were no longer present in the herd towards the end of the study and some cows with possible clinical signs of Johne's disease (and

therefore high S/P ratios) were not sampled, it was more difficult to accurately describe any associations with heat stress. If high S/P ratio results were missing from already lowered mean S/P ratios for the study population, it reduced the discriminating ability to differentiate any significant fluctuations in S/P ratio or proportion-infected data. It was imperative, in a seasonal study such as this one, to be able to follow the same cows over time (as best as is possible), and when a significant proportion of the cows were lost to follow up, seasonal variation was much more difficult, if not impossible to assess.

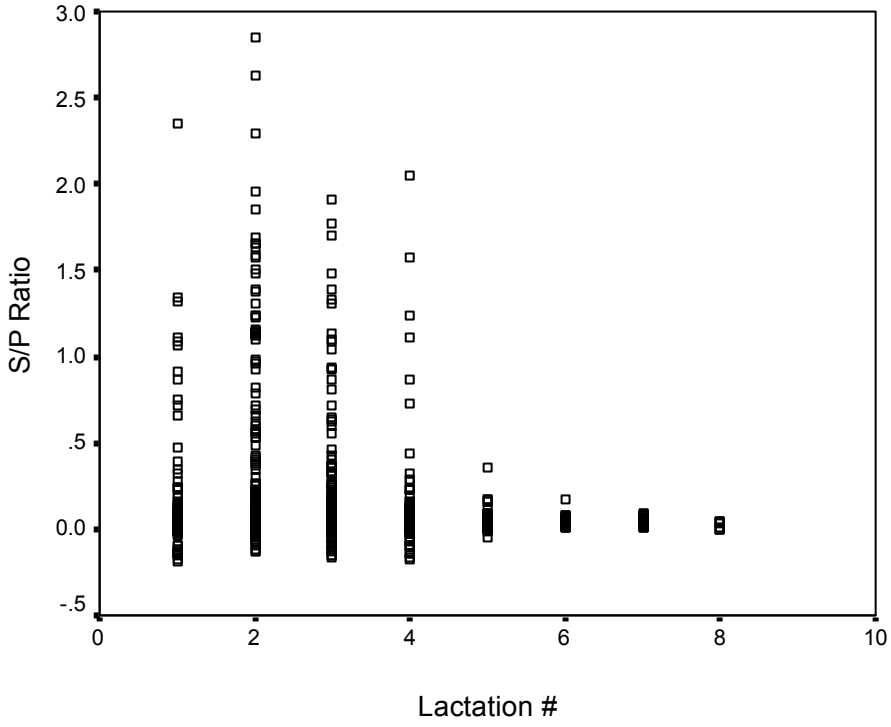

Figure 5.6. Scatterplot of S/P ratios by lactation number (age) of cows in the present study.

A fourth issue affecting the present study was the fact that the duration of this study was for only for one year. Because this study began in the autumn months and ended in the summer months, the possibility of a subsequent increase in S/P ratio or proportion of cows seropositive or fecal negative or positive to Johne's disease when colder months ensued was not evaluated. A spring start, or ideally a study period of at least two years would have been ideal. Unfortunately, the timeframe of funding for this study precluded a study period of more than a single year. In addition, the 'natural' herd pressures of culling on seropositive animals would make such a study difficult to undertake.

Recommendations for Future Studies

In order to properly evaluate the phenomenon of a depression in S/P ratios during the summer months, several aspects need to be addressed. In an ideal situation, an experimental, non-commercial herd would be utilized in order to restrict the culling of infected animals and to allow for full follow-up. As mentioned previously, the ability to follow cows over time is extremely important for the evaluation of seasonal fluctuations in S/P ratio. Since a situation involving such an experimental herd with a large enough population of Johne's positive cows is unlikely and prohibitively expensive to maintain, commercial herds would probably have to be utilized. Since it is only those cows that are borderline positive/negative that are in danger of being misdiagnosed, the study population should consist only of those cows with S/P ratios > 0.10, including those with 'positive' values. In order to reduce the bias associated with early culling, an initial

screening of a larger number of herds, and a restricted sampling frame with cows less than the third lactation in age and with S/P ratio values > 0.01 would be ideal. Cows in lactations >2 would be excluded in order to make full follow-up of animals involved in the study a higher probability.

Once an adequate study population had been determined, in an ideal situation the temperatures and humidity would be applied in experimental conditions that could be controlled and accurately evaluated, such as those attained in climatic chambers. Since this situation is impractical, the study should instead be conducted in areas with THI's high enough to initiate and maintain prolonged heat stress. The study period should consist of more than a single year. Ideally, a study of two years would be very useful in showing any effects of season temperatures. If this is the case, a spring start would be preferable to identify any associated decrease in S/P ratio during the summer months, but would also include the subsequent fall and winter months, necessary for an observed increase in S/P during cooler months. In order to determine if levels of heat stress reached by animals are great enough to cause immunological suppression, daily rectal temperatures and respiratory rates should also be recorded during midday feeding on a subset of study cows. According to Johnson (1987), rectal temperatures can be very appropriate indicators of heat stress.

If the observed depression of S/P ratios during the summer months was indeed determined to be artifactual, no further studies would be necessary to test this hypothesis.

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PUBLICATIONS

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