DEVELOPMENT OF GUIDELINES FOR IMMUNOLOGICAL MONITORING OF GASTROINTESTINAL PARASITES IN DAIRY CATTLE

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ABSTRACT

Gastrointestinal parasites, such as *Ostertagia ostertagi*, adversely affect milk production in dairy cattle. These gastrointestinal nematodes are ubiquitous in temperate climates, but clinical signs of infection are rarely seen in adult cattle. An enzyme-linked immunosorbent assay (ELISA) based on a crude *Ostertagia* antigen has been used to quantify a cow's immunological response to intestinal parasites, and therefore act as a surrogate measurement for parasite load.

Svanova (Uppsala, Sweden) developed a commercial ELISA test (Svanovir®) available in Europe. This test is designed to be used on milk samples, making the collection process very simple, and bulk-tank (BT) or individual cow milk samples can be used. Results from ELISA tests are normalized, using the controls, and reported as optical density ratios (ODRs), thus permitting the ELISA results to be compared between plates, kits and, to a certain extent, studies and regions.

A large clinical trial, involving over 3,000 cows from nearly 40 herds in 9 provinces, was undertaken to predict the amount of milk loss (kg/cow/day) associated with gastrointestinal parasites and to evaluate the ability of the ELISA to predict the benefit from anthelmintic treatment. Milk samples were collected from cows (>200 days in milk) to measure individual ODRs. Producers applied a treatment (randomly allocated as anthelmintic or placebo) to cows as they calved. Milk production records were acquired from Dairy Herd Improvement (DHI) programmes for individual cows on a monthly basis during the study.

The treatment effect (anthelmintic) on milk loss was expected to depend on the level of parasitism in the cow, where low ODR values from the ELISA test indicated low levels of parasitism. As such, the estimates from the interaction between ODR and treatment on milk production would be able to determine

how treatment effect depended on ELISA test results. A fractional polynomial (2-degree) was applied to this interaction since the relationship was non-linear. To increase statistical power, datasets from two previous smaller, yet similar, Canadian studies were incorporated into the analysis. The large combined dataset was able to predict the amount of individual milk loss (kg/cow/day), based on ELISA test results from individual cows in a herd.

The final objective of the thesis was to utilize data from both individual milk and herd BT samples, from several studies in North America (including this clinical trial) and Europe, to develop guidelines for the use of a commercial ELISA test (Svanovir®) to predict production losses (for the herd) associated with gastrointestinal parasites in dairy cattle.

The guidelines required a series of small analyses in order to transform BT ELISA results into averaged individual milk losses (kg/cow/day). Each step along the series used different datasets from various studies. One of the final products is a nomogram (diagram that solves calculations by drawing simple lines) which was designed to interpret ELISA test results to quantify the estimated economic losses associated with intestinal parasites from two BT milk samples taken within one season. While the nomogram reports values using deterministic methods, stochastic processes were applied to estimate uncertainty around the coefficients and identify influential parameters within the guidelines.

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LIST OF ABBREVIATIONS

Note: If the abbreviation is followed by a minuscule 's' in the text, it indicates plurality for the noun in the abbreviation. Example: ODRs are Optical Density Ratios, while ODR is simply Optical Density Ratio.

Ab	Antibody
AB	Alberta (Province)
ABTS	ELISA substrate solution (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]- diammonium salt)
ANTE	Ante-dependent
AR	Auto-regressive
ARMA	Auto-regressive Moving Average
BC	British Columbia (Province)
BLUP	Best Linear Unbiased Predictions
BT	Bulk Tank
CBMRN	Canadian Bovine Mastitis Research Network
CCC	Concordance Correlation Coefficient
CEC	Commission for Environmental Cooperation
CFN	Confined
CI	Confidence Interval
CRT	Concrete
CV	Coefficient of Variance
DC	Days to Conception
DEM	Digital Elevation Model
DFS	Days to First-Service
DHI	Dairy Herd Improvement
DIM	Days in Milk
e-ELISA	Endpoint ELISA
ELISA	Enzyme-link Immunosorbant Assay
eODR	Endpoint ODR
FEC	Fecal Egg Count
FLD	Field
FP	Fractional Polynomial

FSCR	First-Service Conception Risk
HFR	Heifers
ICC	Intraclass Correlation Coefficient
IQR	Interquartile Range
K	Kelvin (Temperature)
k-ELISA	Kinetic ELISA
L_3	Third larval stage
L_4	Fourth larval stage
ln	Natural Logarithm - can be referred to as 'log'
lnSCC	Log transformed SCC
lowess	Locally Weighted Smoothed Scatterplot
LPDAAC	Land Process Distributed Active Archive Center
LST	Land Surface Temperature
MA	Moving Average
MB	Manitoba (Province)
ML	Maximum Likelihood
MLK	Milking Cows
MODIS	Moderate Resolution Imaging Spectroradiometer
NASA	National Aeronautics and Space Administration
NB	New Brunswick (Province)
NCDF	National Cohort of Dairy Farmers
NDVI	Normalized Difference Vegetation Index
NRC	Natural Resource of Canada
NS	Nova Scotia (Province)
NSC	Number of Services per Conception
O. ostertagi	*Ostertagia ostertagi*
OD	Optical Density
ODR	Optical Density Ratio
ON	Ontario (Province)
PCCF	Postal Code Conversion File

PE (PEI)	Prince Edward Island (Province)
PET	Potential Evapotranspiration
PST	Pasture
p-value (*P*)	Predictive value
QC	Québec (Province)
R^2	Correlation squared
SCC	Somatic Cell Count
SD (Std Dev)	Standard Deviation
SE (Std Err)	Standard Error
SK	Saskatchewan (Province)
SPOT	Satellite pour l'Observation de la Terre
SR	Slope Ratio
Std Dev (*SD*)	Standard Deviation
Std Err (*SE*)	Standard Error
UN	Unstructured
VGT	VEGETATION instruments aboard satellites
β	Coefficient (regression parameter estimate)

Chapter 1
General Introduction*

* Manuscript based on this chapter: L. DesCôteaux, R. Vanderstichel, I. Dohoo, & J. Charlier. 2007. Test ELISA pour la détection d'Ostertagia ostertagi chez la vache laitière. Bulletin GTV – Hors série parasitisme des bovins. pp 125-130.

1.1. Introduction

Gastrointestinal 'roundworms' are parasitic worms that commonly infect cattle and are economically detrimental to producers; they belong to the Phylum Nematoda and will be henceforth referred to as nematodes. Of particular distinction is the abomasal nematode *Ostertagia ostertagi*, belonging to the Order Strongylida, Superfamily Trichostrongyloidea, Family Trichostrongylidae (Bowman, 2009), which is considered the most economically important nematode in cattle (Gibbs and Herd, 1986). Other significant nematodes of domestic cattle in North America can be found in the following genera: *Cooperia*, *Haemonchus*, *Trichostrongylus*, *Nematodirus* and *Oesophagostomum* (Yazwinski and Tucker, 2006).

1.1.1. Life-Cycle for Ostertagia ostertagi

O. ostertagi has a direct life cycle involving four larval stages before becoming an adult capable of shedding eggs into the environment (Bowman, 2009), see Fig. 1.1. The prepatent period, which is the minimum time period from when the infective larvae enter the host to the point where eggs are recovered from feces, is three weeks. The third larval stage (L_3, also known as the infective stage), is ingested by grazing cattle, and depending on the climatic region, has the ability to survive the winter months on pasture (Bowman, 2009). *O. ostertagi* also has the ability to undergo hypobiosis, that is, arrest its larval development (L_4) during harsh environmental conditions (i.e. winter and

drought). Maturation of L_4 (residing in the lumen of gastric glands) into adult nematodes may cause severe pathologic changes to the gastric mucosa. Overtime, cattle develop immunity to nematodes, however, much longer exposure to *O. ostertagi* is necessary for cattle in order to develop immunity in comparison to other nematodes. *O. ostertagi*, therefore, remains a persistent infection in older animals (Armour, 1989; Gibbs, 1988). It is common for the first-season grazers to show clinical signs (e.g. diarrhea, weight loss, etc.), while adult cattle may not. Sub-clinical infections are associated with production losses (Charlier *et al.*, 2009), therefore, identifying and quantifying parasite burden becomes important if producers are going to effectively control the level of parasite infections.

1.2. Methods of Diagnosis

1.2.1. Traditional Methods

Diagnosing gastrointestinal nematodes can be difficult. A diagnostic test capable of both identification of the worm species involved, and quantification of worm burdens would be most useful for the management of gastro-intestinal nematode infections in food production. Apart from counting nematodes retrieved during post-mortem examination, quantification of the infection intensity is traditionally made from fecal egg counts (FEC). Unfortunately, FEC are not as reliable in older cows compared to young calves as the egg shedding is low and intermittent (reducing repeatability), thus false negatives are common (Agneessens *et al.*, 2000; Borgsteede *et al.*, 2000;

Eysker and Ploeger, 2000; Gross *et al.*, 1999). Furthermore, if a high proportion of worms are in the hypobiotic stage (encysted L_4, the last stage before the adult form) then the number of eggs shed will underestimate the real number of adults or potential adults infecting the animal. The FEC technique can only identify 'strongyle-type' eggs which are eggs that look very similar, but come from nematodes belonging to various genera, specifically, *Haemonchus, Ostertagia, Trichostrongylus Cooperia*, *Bunostromum*, *Chabertia*, and *Oesophagostomum.*

1.2.2. Alternative Methods

Since the 1960s, researchers have been exploring alternative ways to diagnose and quantify *O. ostertagi* infections. The two main alternatives to fecal examinations that have been most fully developed are measuring serum pepsinogen, given its association with the degree of infection, and measuring anti-parasite antibodies in serum and/or milk – the most widely used for testing adult dairy cattle.

1.2.2.1. Pepsinogen

As summarized by Berghen *et al.* (1993), *O. ostertagi* larvae cause mucosal damage to the cells responsible for the production of acid that transforms pepsinogen into pepsin. Large amounts of pepsinogen thus remain in the gastric environment. This, together with leakage into the circulation due to the larval damage of the abomasal wall, results in an increased pepsinogen level in serum of infected cattle. An increased serum pepsinogen is thought

to reflect emerging *O. ostertagi* larval stages.

Measuring serum pepsinogen level has been useful in some studies, particularly when investigating first grazing season calves, when there is mucosal damage before the emergence of adult nematodes (Agneessens *et al.*, 2000; Borgsteede *et al.*, 2000; Gross *et al.*, 1999). Unfortunately, other non-parasitic diseases can also be responsible for increased serum pepsinogen level (Gross *et al.*, 1999). Furthermore, in individual adult cows, the level of pepsinogen is not a reliable indicator of the total worm burden present as most of the parasites are in the relatively stable adult or hypobiotic L_4 stages, which are not the main cause of mucosal damage and pepsinogen level increase (Berghen *et al.*, 1993). Nonetheless, high serum pepsinogen level should arouse suspicion of *O. ostertagi* infection (Gross *et al.*, 1999).

1.2.2.2. Enzyme-Linked Immunosorbant Assay

In 1981, Keus *et al.* created an indirect enzyme-linked immunosorbent assay (ELISA) able to measure the level of antibodies against *O. ostertagi* in serum of infected cattle. Briefly, as illustrated in Fig. 1.2, a polystyrene microwell is coated with antigen (in this case, a crude whole worm antigen). The serum sample is then added to the microwell so that antibodies targeting *O. ostertagi* will bind to the coated antigen. Bound antigen-antibody complexes will be detected by means of an enzyme-labeled antiglobulin (anti-antibody), usually described as the conjugate solution. A substrate is then added to the microwell to react with the enzyme and produce a colour change proportional

to the amount of bound antibodies (Tizard, 2004). The colour intensity is measured by a spectrophotometer, quantifying the light absorption ability of the solution at a specific wavelength. Readings from a spectrophotometer are reported as optical densities (ODs), and are measured on a continuous scale (results can take on any value that is measurable by a spectrophotometer). An ELISA plate consists of 96 microwells, including at least two positive controls, two negative controls, and two blank wells (only washing solution is added to the blank well). The final sample OD is adjusted by subtracting the average OD from blank wells, thus removing background 'noise' caused by conjugate and substrate solutions.

1.3. Immunological Response to Nematodes

The immune response against *O. ostertagi* in cattle is very complex and involves both cellular and humoral pathways. Some investigators focused on the cellular response in cattle, particularily the role of leukocytes and their cytokines against gastrointestinal nematodes; their findings are summarized by McClure and Emery (1994). However, measuring cellular response usually requires specialized equipment better suited for research than field conditions.

Vercruysse and Clarebout (1997) described the humoral portion of the immune response, the one that can be measured using an ELISA test. The humoral response involves production of various antibody isotypes, namely IgG1, IgG2, IgA, and IgE, and their interaction with mucosal mast cells. In

short, abundant amounts of anti-parasite IgG (both 1 and 2) antibodies are present during nematode infections. Serum IgA antibodies are limited and may be attributable to a spill-over effect from IgA antibodies present in the gastrointestinal lumen from large antigenic stimulation – it is most notable with the presence of adult nematode. The antibodies, IgE, are found in highest levels in calves when *O. ostertagi* larvae are imbedded in the gastric mucosa (Thatcher *et al.*, 1989). The nematode antigens responsible for eliciting antibody production arise from three sources: excretory-secretory products, cuticular (surface) and somatic (internal) antigens (Vercruysse and Claerebout, 1997).

1.4. investigation of ELISA Tests

1.4.1. ELISA Detection Using Crude Worm as Antigen

Keus *et al.* (1981) prepared their serum ELISA from ground (crushed) third (L_3) and forth (L_4) stage, as well as adults. The use of ground whole worms as an antigen source, however, results in cross reactivity with antibodies against other nematodes (e.g. *Cooperia spp.* and *Dictyocaulus spp.*) and even some trematodes, such as *Fasciola hepatica* (Dohoo *et al.*, 1997; Eysker and Ploeger, 2000; Kloosterman *et al.*, 1984), decreasing the specificity of the test. Therefore, *O. ostertagi* ELISA tests derived from whole worm antigens quantify the overall parasite burden present in the animal, rather than identifying a single parasite species. The low analytical specificity does not necessarily hinder the use of this particular ELISA test, as parasite infections

are nearly always mixed and high levels of *O. ostertagi* antibodies likely reflect a mixed infection (e.g. *O. ostertagi* and *Cooperia spp.*). The analytical sensitivity, on the other hand, is very high for ELISA tests in general, detecting as little as 0.5 nanograms of antibody protein per milliliter of sample fluid (Tizard, 2004). Diagnostic sensitivity and specificity are not real issues because *O. ostertagi* is ubiquitous and the interest lies in quantifying the parasite burden on a continuous scale rather than defining the status of an animal as infected or non-infected.

1.4.2. ELISA Detection Using Excretory-Secretory and Somatic Antigens

Canals and Gasbarre (1990) explain that earlier research found the use of attenuated larvae (radiated whole worm), as a source for vaccination, did not induce a sufficient immune response. Therefore, the use of specific worm antigens (e.g. excretory-secretory or somatic antigen), instead of attenuated larvae, were explored by some investigators to manufacture recombinant vaccines. Canals and Gasbarre (1990) found that excretory-secretory antigens from *O. ostertagi* generated stronger immunological responses and reduced cross-reactivity compared with somatic antigens. Therefore, ELISA tests using either whole worm or excretory-secretory antigens were created to measure the immune response from challenged cattle. Sithole *et al.* (2005b) compared the agreement between ELISA tests, using either whole worm antigens or adult and L_4 secretory-excretory antigens, and found low

agreement between tests (concordance correlations coefficients ranged from 0.31 to 0.56).

Overall, the source of the antigen used for ELISA tests, in a diagnostic setting, may not matter as long as the same source of antigen is consistently used for both the evaluation of the test and the application of the test in a routine diagnostic setting. Acquiring and purifying secretory-excretory and somatic antigens is expensive and consumes more resources than ground whole worms. Therefore, economics and the inherent properties of the diagnostic sensitivity and specificity associated with ground whole worms (as described in 1.4.1) are likely reasons for the favoured use of whole worms as a source of antigens in diagnostic ELISA tests. Svanovir® (Svanova Veterinary Diagnostics, Uppsala, Sweden), an anti-*Ostertagia ostertagi* antibody ELISA test, is available commercially in Europe, and its source for antigens is derived from ground adult *O. ostertagi* worms.

1.4.3. ELISA Repeatability

The initial concern with the ELISA test, for its use diagnostically, was its repeatability. Results varied between plates and also between batches of crude worm antigen (Eysker and Ploeger, 2000). Amongst the different methods of computing and presenting ELISA test results published, namely dilution counts, raw OD, OD percent positivity, and OD ratio (Agneessens *et al.*, 2000; Borgsteede *et al.*, 2000; Guitián *et al.*, 2000; Ploeger *et al.*, 1994; Poot

et al., 1997), it seems that the optical density ratio (ODR) is the most reliable in reducing variability between plates (Charlier *et al.*, 2005b; Sanchez *et al.*, 2001; Sanchez *et al.*, 2002b) and has become the standard format for reporting ELISA antibody results since 2001. After adjusting for the blank controls, the calculation is as follows:

$$ODR = \frac{(OD_{sample} - OD_{negative})}{(OD_{positive} - OD_{negative})}$$

1.5. Validation and Implementation of ELISA Tests for Field Use

The ELISA test for *O. ostertagi* can be a useful diagnostic tool in a production setting as it does not require specialized training or costly equipment. A gold standard is usually required to validate diagnostic tests, however, as has been discussed earlier, there is no gold standard for the antemortem diagnosis of nematode infections in cattle. In an attempt to validate an ELISA test for dairy cattle, four major requirements must be fulfilled: 1) demonstrate that herds with management factors favoring parasitic burdens will have increased ODR levels in bulk tank milk samples; 2) identify a correlation between FEC and ELISA ODR results; 3) associate production losses with elevated levels of *O. ostertagi* antibodies; 4) predict production response to anthelmintic treatment based on measured ELISA ODR either at the individual or herd level. Of these requirements, the last provides the most direct evidence that the ELISA is reliably quantifying the parasite burden.

1.5.1. Bulk Tank ODR and Associated Management and Surrounding Environmental Factors

Several investigators found that bulk tank ELISA ODRs are associated with on-farm management factors which influence parasite burden (Almería *et al.*, 2009; Caldwell *et al.*, 2002; Charlier *et al.*, 2005b; Forbes *et al.*, 2008; Guitián *et al.*, 2000; Sanchez and Dohoo, 2002; Sithole *et al.*, 2005a). Data previously collected in 2005 from farms in Canada, related to farm practices and bulk tank ODRs, are presented in Chapter 2 along with a review of specific factors in previous published studies. The goals of this study were to investigate the effect of farm management practices and surrounding environmental factors on BT ODRs in herds from provinces and ecoregions across Canada.

Overall, studies have found that as exposure to pasture increases, so too does the level of bulk tank ODR.

1.5.2. Correlation Between FEC and Bulk Tank ODR

Caldwell *et al.* (2002) demonstrated low to moderate correlations (0.28 and 0.36 for *O. ostertagi* and *Cooperia spp.*, respectively) between FEC and bulk tank ELISA ODRs. A low to moderate correlation does not necessarily indicate that bulk tank ODRs are a poor indicator of infection since FECs themselves are a poor indicator of parasite burden. Dohoo *et al.* (1997) found higher correlations (0.40, 0.59, and 0.57 for *Dictyocaulus*, *Cooperia* and *Ostertagia*, respectively) between FECs and milk ELISA ODRs when considering other

factors, such as age and stage of lactation, are taken into account and values are averaged for the entire herd. Later, Sanchez *et al.* (2002a) reported a moderately high correlation (0.73) when multiple measurements from individual cows were averaged on a farm over the full year.

1.5.3. Production and ODR

In 1991, Kloosterman *et al.* found negative associations between milk antibody titres and milk yields, and between serum antibody titres and milk yields – i.e. cows with increased antibody levels, reflecting a high parasite burden or exposure, had lower milk yields. Guitian *et al.* (2000) performed a large cross-sectional study in 1998 involving 415 dairy herds in Nova Scotia, and found a significant negative relationship between herd bulk tank OD and milk production in herds exposed to varying levels of pasture during the summer months. Several studies in Prince Edward Island (Sanchez and Dohoo, 2002) and Belgium (Charlier *et al.*, 2005a) followed, and these also found a significant negative relationship between bulk tank ODR and milk yield. These last two studies reported consistent milk yield increases of about 1 kg/cow/day (1.2kg/cow/day and 0.9kg/cow/day, respectively) comparing the lower 25th percentile and the upper 75th percentile of bulk tank ODR taken during the autumn months. Another study, in France, found similar results between the 25th and 75th percentiles of BT ODR from 940 farms – they reported a difference of 1.2 kg milk/cow/day (Guiot *et al.*, 2007). More recently, research on two Spanish islands, Minorca and Girona,

found negative significant correlations between averaged individual ODR values per farm and mean herd milk yields (R^2=0.23, p<0.001), and similarly between bulk tank ODR values and mean herd milk yields (R^2=0.18, p<0.001) (Almería *et al.*, 2009). These negative correlations are consistent with other studies, despite having a study design which introduced selection bias from the multistage sampling, and the questionable use of a random variable to account for the two islands.

Sanchez *et al.* (2004) investigated the effect of milk production (as a predictor), amongst other variables, on individual cow's ODR values; they concluded that ODR values are not greatly influenced by milk yields.

1.5.4. Prediction of Production Responses

1.5.4.1. Prediction of Milk Production Response to Anthelmintics

Prediction of response to anthelmintic treatment is the best indicator of validity for the ELISA test. Three pieces of information are necessary for this prediction: 1) an outcome, in this case milk production, 2) a randomized clinical trial between an anthelmintic and a placebo treatment, and 3) ELISA results, quantifying the parasite burden. To predict the milk production response to treatment, the interaction between treatment and ELISA antibody levels needs to be modelled– more specifically, predictions of milk production for both anthelmintic and placebo cows as ODR values increase.

Ploeger *et al.* (1989) were the first to try to predict production response after

treatment, based on ELISA results. In the literature, there are eight studies which have attempted to predict the response of milk production to anthelmintics using ELISA results. These are summarized in Table 5.1. The sample medium (serum vs. milk), the method of procurement (individual cows vs. bulk tank), the treatment options, and the assumed relationship between milk yield and ODR values varied from study to study, however, the direction and values of the estimates remained relatively constant. Statistical significance for the prediction was, unfortunately, a problem for these studies – investigating the interaction between treatment and the continuous ELISA values demands very large sample size to achieve sufficient statistical power.

1.5.4.2. Prediction of Reproductive Responses to Anthelmintics

In dairy cattle, researching the benefits of anthelmintic treatment on production has been mostly focused on milk yields, however, reproductive parameters have also been investigated. Typically, when evaluating treatment effects on reproduction, beef cattle are more popular since fertility determines the profitability of the cow-calf system. More specifically, anthelmintic treatment has been reported to improve body condition scores (ensuring proper body condition for re-breeding), increase conception rates, increase calving rates, reduce calf mortality, and reduce calving to breeding intervals – though these findings are not consistent (Hawkins, 1993).

Walsh *et al.* (1995) found that treated dairy cows had a significantly reduced calving-to-conception intervals, though there were no significant differences

between the calving-to-first service intervals. Only two studies have attempted to predict (using ELISA values) fertility response to an anthelmintic treatment (Sanchez *et al.*, 2002c; Sithole *et al.*, 2005a). Sanchez *et al.* (2002c) found that treated cows with high levels of ODR had a significant reduction in the number of breeding-to-conception rates when compared to the placebo group with elevated ODR values. Sithole *et al.* (2005a), however, failed to show any beneficial treatment effect – it is worth noting that their study cows came from semi-confined or totally confined herds so they likely had low parasite burdens. Additionally, Sithole *et al.* (2005a) did not have enough statistical power in their study, therefore, the results regarding reproductive performance are not conclusive.

1.6. Guidelines for Sampling to Determine Anthelmintic Response

While the ELISA seems to be the preferred diagnostic test to quantify the parasite burden in dairy cattle, producers and veterinarians are still missing some critical pieces of information for its application in the field. Though there is sufficient information supporting the use of milk rather than serum as the sample medium for the test, there are no clear guidelines for sampling methods, either individual milking cows or bulk tank samples taken from the herd. Also, once the samples are taken and the results given, there is little information about the interpretation of the ELISA values.

Forbes *et al.* (2008) created a chart to aid with the interpretation of bulk tank samples taken from European herds. This chart should only be used as a

general guide, especially since it is very crude. The authors arbitrarily chose the value of 0.5 as a cutpoint for bulk tank ODRs, which was derived from the 25th percentile of measurements from bulk tank samples of many studies, including theirs. They suggest that if bulk tank ODR values are less than or equal to 0.5, gastrointestinal parasites had no effect on milk production, and if bulk tank ODR values are greater than 0.5, milk yields will decline according to an amount determined by an estimated coefficient from another study (Charlier *et al.*, 2005a). It is not clear from the manuscript how the error bars were derived, and the relationship between milk yield and ODR was assumed to be linear. Given there were no other current options to predict milk losses from ODR values, this chart has its uses, but the interpreter should use great caution.

1.7. Objectives and Direction of Thesis

Measuring *O. ostertagi* milk-antibodies can improve the production of dairy cattle by providing information to make rational decisions about parasite control. Modern anthelmintic medications are effective at removing gastrointestinal nematode infections, however, unnecessary use can have detrimental effects on the environment (Floate, 2006), and may promote anthelmintic resistance (Coles, 2005), not to mention the expense of the medications to the producers. It is likely that, in the near future, ELISA tests measuring anti-parasite antibodies will be incorporated into herds' routine herd-health monitoring to give producers the right tools to make appropriate

decisions regarding parasites on their dairy farms. The theme to this thesis was, therefore, to investigate how an ELISA test can be used to quantify parasite levels and production losses in dairy cattle

The overall specific goal of this research was to develop guidelines for the use of an ELISA test to monitor and predict the effect that herd parasite burdens have on dairy production in Canada. Producers want to have an economical test with easy sample collection methods and applicable interpretations. Bulk tank samples are therefore preferred over individual cow samples. However, predicting milk loss from bulk tank samples would have required a study that involved hundreds of herds with a treatment that was applied to the entire herd (placebo or anthelmintic); a study of such magnitude was, unfortunately, not feasible.

Predicting milk loss from individual samples, however, is achievable with a clinical trial including large numbers of individual cows from relatively few herds (e.g. 40) with known access to pasture or grassed paddocks within Canada (as explained in Chapter 5). Additionally, data collected from the clinical trial permitted the investigation of the effect of anthelmintic treatment on reproductive parameters in dairy cattle using an ELISA test from those individual milk samples (described in Chapter 6).

It is possible, through a series of analyses, to convert individual milk loss predictions into estimates of average milk loss for the herd from BT ODR

samples. Chapter 7 explains the series of analyses necessary, using data from many studies, to estimate the average individual milk loss for the herd. The processes undertaken in Chapter 7 are used to develop sampling guidelines and interpretation on the use of an *Ostertagia ostertagi* ELISA to predict production losses from gastrointestinal parasites in dairy cattle.

Additionally, other objectives arose from the project, more specifically dealing with logistical issues such as the effect of storage and transportation on milk ELISA results from samples collected on farms (Chapter 3), and investigating the use of a kinetic ELISA method instead of the currently recommended endpoint ELISA method; there are potential benefits from kinetic methods, as described in Chapter 4.

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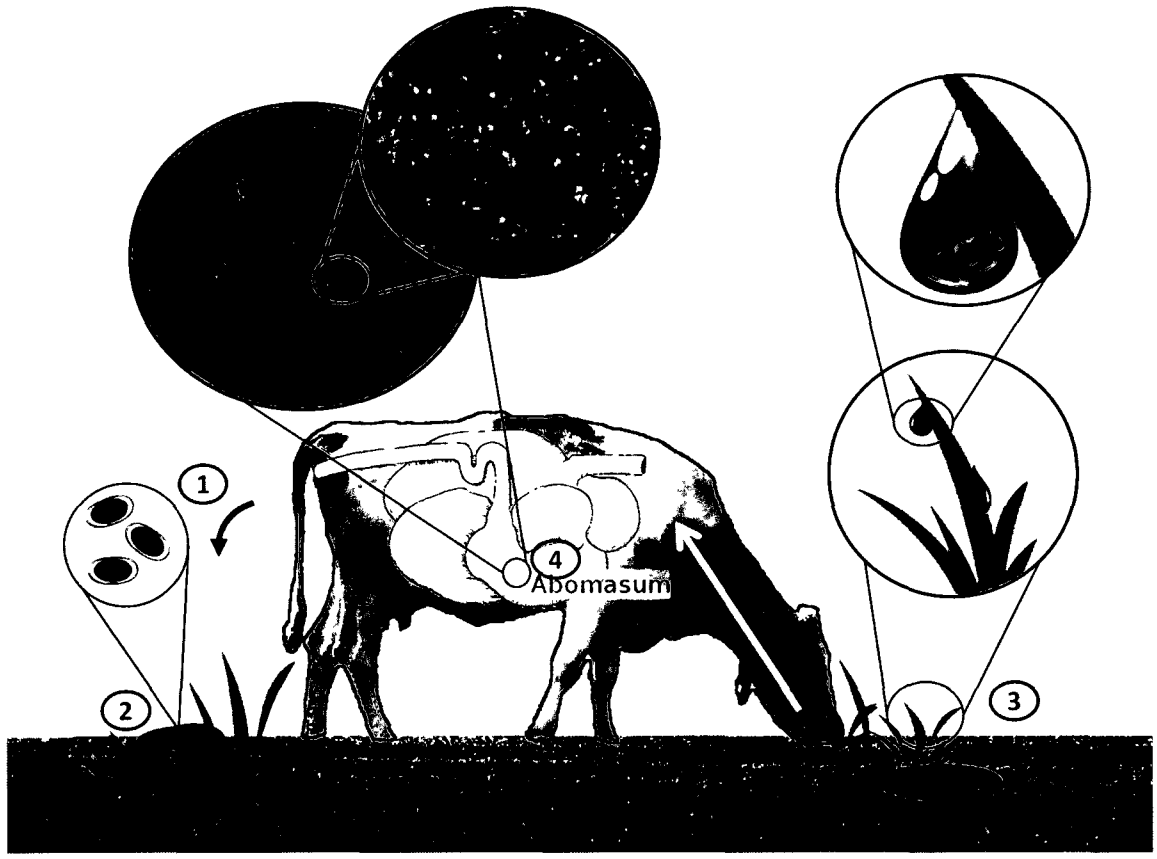

Figure 1.1. The life cycle of *Ostertagia ostertagi*. (1) Viable *O. ostertagi* eggs are shed through feces. (2) Eggs hatch and the organism matures through three larval stages (L_1, L_2, and L_3) in the manure and on pasture. (3) L_3 larvae are able to overwinter, and are easily ingested by cattle when they are present in water droplets and on grass blades. (4) L_3 larvae can then mature through the fourth larval stage in the gastric glands (shown in photographs), and then matures into adulthood where it remains permanently in the abomasum. Adult nematodes will shed eggs into the gut lumen, where the eggs get incorporated in the feces. *O. ostertagi* has the ability to undergo hypobiosis during the last larval stage (L_4), arresting the maturation process until favorable environmental conditions return. The prepatent period, which is the minimum time from ingestion of larvae to egg shedding, is 3 weeks.

Pictures of abomasal lesions are courtesy of the Department of Pathology and Microbiology, AVC.

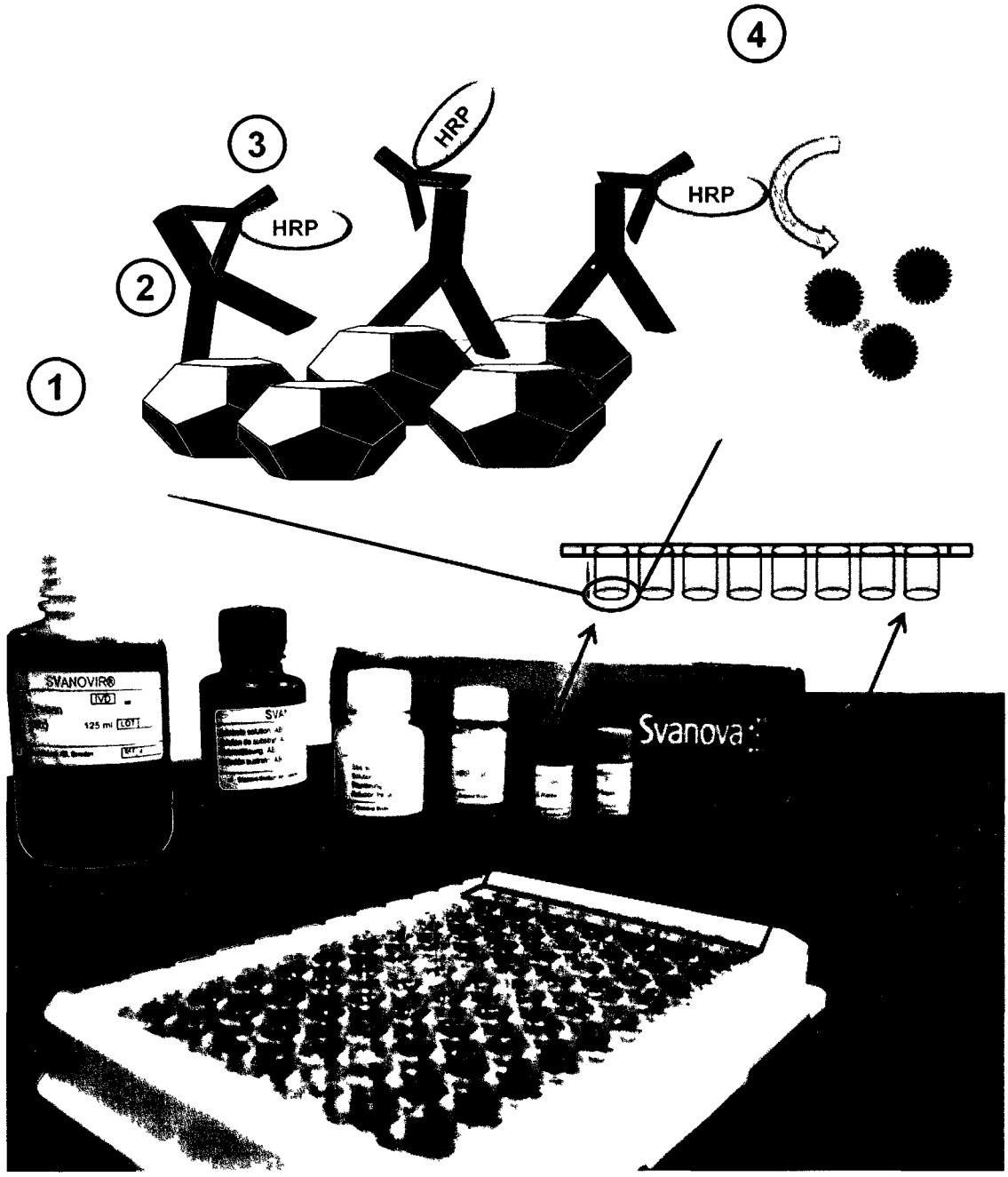

Figure 1.2. Schematic illustrating the procedures for an enzyme-linked immunosorbent assay. (1) Polystyrene microwells are coated with crushed whole *Ostertagia ostertagi*, formulating the antigen (2) Anti-parasite antibodies, found in the sample (milk), adhere to the antigen. (3) Enzyme-labeled antiglobulin, called the conjugate solution, binds to the anti-parasite antibodies. (4) Chromogens, found in the substrate solution, react with the enzymes present on the antiglobulins; the reaction transforms the clear solution into colour, proportional to the amount of antibodies present. The larger the number of anti-parasite antibodies in the sample, the greater the amount of colour intensity the solution inside the well will acquire.

Chapter 2

Effects of Farm Management Practices and Environmental Factors on Bulk Tank Milk Antibodies Against Gastrointestinal Parasites In Dairy Farms Across Canada

2.1. Introduction

Gastrointestinal nematode infections in dairy cattle are very common and economically detrimental to producers (Anderson, 2000; Charlier *et al.*, 2009; Gibbs and Herd, 1986). Enzyme-linked immunosorbent assays (ELISA) have been used as a diagnostic tool to quantify levels of gastrointestinal nematodes in dairy cattle by measuring *Ostertagia ostertagi* antibodies circulating in milk (Forbes *et al.*, 2008; Keus *et al.*, 1981; Kloosterman *et al.*, 1993). Higher levels of *O. ostertagi* antibodies measured by ELISA methods, and normalized as optical density ratios (ODRs) (Sanchez *et al.*, 2002b; Vanderstichel *et al.*, 2010) are associated with decreased production in dairy cattle (Charlier *et al.*, 2007; Sanchez *et al.*, 2002a; Sanchez *et al.*, 2002c; Sithole *et al.*, 2005).

On-farm management practices can influence the exposure of cattle to nematode infections (e.g. pasturing techniques, and anthelmintic usage). In Canada, four studies involving different provinces in eastern Canada investigated the effect management practices had on *O. ostertagi* antibodies in bulk-tank (BT) milk samples (Caldwell *et al.*, 2002; Guitián *et al.*, 2000; Hovingh, 1998; Sanchez and Dohoo, 2002). Overall, exposure to pasture was associated with higher BT ODRs, and some of the anthelmintic treatment practices were significantly associated with lower ODR values.

In addition, two European studies using a commercially available *O. ostertagi*

ELISA milk test, Svanovir® (Svanova Veterinary Diagnostics, Uppsala, Sweden) investigated the relationship between farm management practices and BT ODR values (Almería *et al.*, 2009; Charlier *et al.*, 2005a). Both studies found positive associations with pasture exposure and BT ODRs, but no associations with anthelmintic treatments.

Environmental and climatic data, such as land elevation and precipitation, have the potential to describe conditions which may be favorable to the development of nematodes. These environmental data, collected from remote sensing via satellites and from local weather stations, have never been investigated as potential predictors for bulk-tank anti-parasite antibody levels. The objectives of this study were to investigate the effect of farm management practices and surrounding environmental factors on BT ODRs in herds from provinces across Canada, and also further examine potential effects of various anthelmintic treatment protocols on BT ODRs.

2.2. Materials and Methods

2.2.1. Study Population, Sample Collection and Laboratory Methods

Herds were randomly selected to participate in a mastitis research project (Olde Riekerink *et al.*, 2006); the randomization process involved generating a list of producers, stratified and weighted by provinces. The selected producers were sent an additional questionnaire, pertaining to this study, and were asked if they were willing to participate in this study.

Four bulk tank milk samples were collected between December 2003 and April 2005 from each enrolled farm. The Bulk-tank milk samples were acquired during routine on-farm Dairy Herd Improvement programs and shipped frozen to the Atlantic Veterinary College; freezing does not adversely affect ELISA results (Charlier *et al.*, 2005c; Sanchez *et al.*, 2002b; Vanderstichel *et al.*, 2010). The indirect crude-antigen *O. ostertagi* ELISA was performed on all BT samples as described by Sanchez *et al.* (2002b).

2.2.2. Questionnaire

In 2005, the producers received a two-page questionnaire containing 6 closed-format questions on pasturing methods and anthelmintic treatments during the autumn of 2003 and throughout the seasons in 2004 (Appendix A). To increase the response rates, the same questionnaire was sent a second time, one month later, to those producers who did not reply to the initial questionnaire. The questionnaire was available in both of Canada's official languages (French and English). The questionnaire responses were digitally recorded in duplicate and verified using EpiData Entry 3.1 (2008).

2.2.3. Normalized Difference Vegetation Index and Environmental Data

The French Satellite pour l'Observation de la Terre (SPOT) programme has VEGETATION (VGT) instruments aboard two satellites (SPOT 4 and 5), capable of reading four spectral bands (blue, red, near-infrared, and shortwave-infrared) at 1 kilometer resolution. From these spectral bands, a normalized

difference vegetation index (NDVI) can be calculated to quantify the amount of live green vegetation present. The NDVI data was acquired from Natural Resources Canada, online, via the GeoGratis portal (http://geogratis.cgdi.gc.ca). The dataset (Canada 1-km, 10-day, SPOT/VEGETATION composites for growing season 1998-2004), derived from SPOT VGT, contained 10-day composite NDVI digital rasters from April 11 to October 21 yearly between 1998 and 2005.

The properties of NDVI are such that values range between -1 and 1 (Trishchenko *et al.*, 2002). More specifically, vegetation ranges approximately between 0.1 and 0.7, and soil or rock approximately ranges between 0 and 0.1, though there is some flexibility in these estimates. The greater the vegetation absorbs energy, the greater the NDVI values (Kidwell, 1997). Natural Resources Canada (NRC), however, reported their NDVI values on a transformed scale: (NDVI+1)*10,000. On this scale, vegetation would be expected to have NDVI values that range between 11,000 and 17,000. For the analysis, the NDVI variable from Natural Resources Canada was further transformed to rescale the model constant and coefficients; the final transformation was: $(NDVI_{NRC}/1000)-10$. On this final scale, vegetation would be expected to have NVDI values that range between 1 and 7. The median NDVI value within a 5-km radius, from the 2004 growing season, was taken for each herd in the study – it will henceforth be referred as 'NDVI-04'.

Land surface temperature (LST) data, provided by Land Processes Distributed Active Archive Center (LPDAAC), was measured from a Moderate Resolution Imaging Spectroradiometer (MODIS) aboard the Terra satellite managed by the National Aeronautics and Space Administration (NASA). The dataset contained monthly composited and averaged emissivity values globally, starting in 2000, at 0.05 degree latitude and longitude grids. Land surface temperatures were expressed as scaled Kelvin (K) and were transformed into degrees Celsius (°C).

The elevation data came from the digital elevation model (DEM) file (Canada3D – Digital Elevation Model of the Canadian Landmass) which was also available online from GeoGratis. The DEM expressed elevation as meters, and was kept in its original scale.

2.2.4. Climate Data

Air temperatures and precipitation measurements were taken from Environment Canada's National Climate Data and Information Archives (www.climate.weatheroffice.gc.ca), specifically, within the climate summaries. Monthly values for each weather station across Canada were taken between April and October 2004.

2.2.5. Farm Locations

The farm locations (latitude and longitude) were derived from the postal code

conversion file of April 2007(PCCF), made available by Statistics Canada (http://www.statcan.gc.ca). The methods of acquiring representative points given in latitude/longitude coordinates for the PCCF are explained in Appendix E in the PCCF Guide, and vary depending on the level of information (e.g. block-faces vs. dissemination areas). A 5 kilometer radius was created around each farm coordinates to calculate the surrounding median NDVI, LST, and elevation values, as well as identify the nearest weather stations. A five kilometer radius was arbitrarily chosen to represent the approximate surrounding pasturing areas of a farm, and since the locations given in the PCCF do not accurately locate the physical location of a farm.

All individual digital satellite images available for the 2004 growing season (April 11 to October 21, total of 20 files) were averaged (arithmetic mean) to create one digital image for NDVI, LST, and elevation values for the growing season of 2004. The climate data for each farm was assigned from the nearest weather station. Four variables were used to explain the weather patterns during April to October 2004, two explaining air temperatures and two explaining precipitation. The averaged monthly mean air temperatures, and the sum of the degree days above 18°C were used as air temperature variables. Total precipitation (total millimeters for the growing season) and the number of days with precipitation greater than 1 millimeter were used to explain precipitation.

All manipulations of digital maps, rasters, and DEMs were performed in ArcGIS 9.3 (2009), and all maps are projected as North America Lambert Conformal Conic.

2.2.6. Statistical Analysis

Unless otherwise stated, all statistical analyses, including summary and descriptive statistics, were performed in Stata11 (2009).

2.2.6.1. Multivariable Mixed Analysis

A multilevel mixed-effects linear regression, with BT ODR as the dependent variable, was fit using restricted maximum likelihood estimator. There were two random effect variables (province and herd), with no additional correlation among residuals because the repeated measures sequence was short (maximum of 4 observations) and there were differing time gaps between sampling collections which gave inconsistent monthly intervals.

All fixed effect variables, except for the environmental variables, were derived from the questionnaire. The season variable, based on the BT collection month, was categorized into three seasons (November-February, March-June, and July-October) to represent three stages of the parasite infection during the year. Specifically, the hypobiotic stage during late fall and winter, the re-emergence of adult nematodes from their dormant stage and the survival of overwintering L_3 on pasture in the spring, and lastly, the peaking infection

load in cattle and on pasture during the summer months (Charlier *et al.*, 2007; Yazwinski and Tucker, 2006). A stepwise selection process, including any variables with a p value<0.15, was used to identify possible significant predictors.

Best linear unbiased predictions (BLUPs) were calculated for both random effects (province and herd), and were plotted against the predicted outcome to verify the assumption of homoscedasticity. The residuals from the bulk tank ODRs were evaluated by plotting the standardized residuals against the predicted outcomes. Normality assumptions were verified by plotting the quantiles of the BLUPs, for both random effects and the lowest level residuals (BT ODRs) against quantiles of normal distribution. The residuals at the herd and the ODR level were not normally distributed and a Box-Cox analysis (Box and Cox, 1964) was used to determine the most appropriated transformation. (Dohoo *et al.*, 2009)

2.3. Results

A total of 195 herds from 9 provinces (British Columbia[BC] 26; Alberta[AB] 22; Saskatchewan[SK] 7; Manitoba[MB] 11; Ontario[ON] 45; Québec[QC] 22; New Brunswick[NB] 22; Nova Scotia[NS] 19; Prince Edward Island[PE] 21) responded to the questionnaire. Figure 2.1 shows the 5-km radius around the postal code coordinates from participating herds. All of the Canadian provinces were included in the study, except for Newfoundland. In 2005, Newfoundland represented a small fraction of the Canadian dairy industry,

with 41 farms out of the National total of 15,522. The two provinces with the largest representation of farms by province in the study were New Brunswick (8.5%) and Prince Edward Island (8.5%), while the two provinces with the lowest representation were Québec (0.3%) and Ontario (0.9%).

Figure 2.2 shows the hierarchical structure to the data with total number of units at each level. The bulk-tank milk sampling months were December 2003; March, June, August, September, and December 2004; March, and April 2005. The majority of the samples were taken in December 2003 (126=18.7%), and March, June, and September 2004 (127=18.8%, 169=25.0%, and 164=24.3%, respectively).

The overall average BT ODR value was 0.327 (IQR 0.152 to 0.473), though the average varied by provinces (Table 2.1). The highest averages were found in the Maritime provinces (NB, NS, and PE), and the lowest averages were in the most westerly provinces (BC and AB).

2.3.1. Questionnaire Responses

The type of housing, during the summer of 2004, was defined in the questionnaire as: 'Confined' (CFN), totally confined in the barn 24 hours per day; 'Concrete' (CRT), given access to a concrete or gravel surface exercise yard outdoors some time each day; 'Field' (FLD), given access to a small field for the purpose of exercise but not primarily for grazing; 'Pastured' (PST), spent some time grazing and met some their nutritional requirements from

pasture. Figure 2.3 is a horizontal bar graph summarizing the housing type responses for heifers and milking cows by province. The majority of the herds in the Maritime provinces (NB, NS, and PE) had their milking cows on pasture during the summer months, while this pattern was less evident in the other provinces across Canada.

Two questions pertained to pasture sharing practices, (1) whether milking cows were mixed with heifers, and (2) whether heifers were mixed with dry cows. The interaction between the two variables answered the question of whether producers mixed all three groups on the same pasture, though not necessarily at the same time (summarized in Table 2.2). There were more herds that mixed all three groups (Heifers, Dry Cows, and Milking Cows) on pasture compared to those which did not mix any of their groups (55/122=45% vs. 16/122= 13%). If only two groups were going to be mixed on pasture, it was more likely to be Heifers with Milking Cows (47/122=39%) than Heifers with Dry Cows (4/122=3%).

Anthelmintic treatments were recorded separately for heifers and milking cows between autumn of 2003 and 2004; the findings are summarized in Table 2.3. Generally, heifers received at least one pour-on or an injectable form of an anthelmintic in the spring or summer, and another in the fall. Due to the cyclic nature of milking cows, anthelmintic treatments can be broken into individual (e.g. at calving or dry off) or whole herd. The most popular

answer was to treat all milking cows at once (59/177=33%).

2.3.2. Normalized Difference Vegetation Index and Environmental Data

Figure 2.4 shows the digital raster containing averaged NDVI values for the 2004 growing season with a 5-km radius surrounding farm location. It is possible to see the difference in vegetation index of the Great Plains (outlined by data obtained from the Commission for Environmental Cooperation (CEC) online: www.cec.org). Herds within the Great Plains (an ecoregion also referred to as the prairies) had lower NDVI-04 values ($p<0.001$, Wilcoxon rank-sum test) than herds that were not (5.031 vs. 6.670). The range of NDVI-04 values within provinces varied, where Alberta, Québec, and Ontario had the highest (3.575, 2.085, and 1.983 NDVI units, respectively) and Nova Scotia had the lowest (0.479 NDVI units).

Herds in the study were found within 13 different ecoregions across Canada, as demarcated by the CEC (Fig. 2.5). The Commission for Environmental Cooperation states that ecoregions are "based on the premise that a hierarchy of ecological regions can be identified through the analysis of the patterns and the composition of both living and nonliving phenomena, such as geology, physiography, vegetation, climate, soils, land use, wildlife, and hydrology, that affects or reflect differences in ecosystem quality and integrity." The data was made available with three levels of details within ecoregions, and the most detailed level was chosen for the analysis (Level-3).

Land surface temperatures are shown across Canada (Figure 2.6). The median LST value (°C) within a 5-km radius was recorded for each herd. Again, the outline of the Great Plains are visible from averaged surface temperatures for the 2004 growing season, and herds within the prairies had higher median LSTs ($p<0.001$, Wilcoxon rank-sum test) than herds that were not (24.3 vs. 22.4 °C). Herds in the Maritime provinces had cooler average surface temperatures (21.5 °C) than other herds in the country (23.2 °C, $p<0.001$, Wilcoxon rank-sum test).

The DEM showed that most of the study herds in the Maritimes were located in low lands (median=36m), while herds in Québec (median=111m), Ontario (median=265m), Manitoba (median=280m), Alberta (median=790m), and in eastern British Columbia (median=664m) were more elevated (Figure 2.7). The elevation of herds in the western portion of British Columbia were much lower (median=91.5m).

Many environmental variables were highly correlated (Table 2.4). Between both air temperature variables, and both precipitation variables, the correlations were 0.692 and 0.965, respectively. Elevation was negatively correlated with the other environmental variables, except median LST, indicating that higher elevation was associated with decreased precipitation, air temperature, and vegetation; the largest negative elevation correlation was with NDVI-04, followed by air temperature variables, and lastly, precipitation.

NDVI-04 seemed equally correlated with precipitation and air temperature variables. The highest LST correlation was with NDVI-04, followed with both precipitation variables; LST was not correlated with air temperatures, positively correlated with elevation, and negatively correlated with the vegetation index. Due to inherent collinearity within environmental variables, only one variable at a time was used in the linear mixed models.

2.3.3. Multivariable Mixed Model

The final 'province' model (Table 2.5) included all nine provinces, with 115 herds and 412 BT ODR values. The model diagnostics revealed a non-normal residual distribution at the ODR and herd level. A Box-Cox analysis (theta=0.4243, $p<0.001$) demonstrated that a square root transformation would be more appropriate for this data. Prior to the square root transformation, the ODR values had to be positive (0.10 was added) and to increase the model coefficient values, the ODRs were subsequently multiplied by 100. The final transformation was: ([(ODR+0.10)*100]^0.5). The model assumptions of heteroscedasticity and normality were verified; there were no visual indications of any violations after the transformation of the BT ODR values.

A second model, 'ecoregions' (Table 2.6), had the same parameters as the 'province' model, except that provinces were substituted with ecoregions. There were 412 BT ODR values from 115 herds within 13 ecoregions.

2.3.4. Random Effects

The estimated variances in the 'province' model at the province, herd, and BT ODR levels were 0.2996, 0.8750 and 0.5934, respectively (Table 2.5). The intraclass correlation coefficient (ICC) can be calculated from the variance estimates for each level; it quantifies the similarities between any BT ODR measures in relation to the herd or province. The 'province' model estimated the ICC between any two bulk-tank ODR measures within a herd to be 0.664, and between any two BT ODR measures within a province (but from different herds) to be 0.169.

Similarly, in the 'ecoregions' model (Table 2.6), the ICC between any two BT ODR measures within a herd to be 0.660 and between any two BT ODR measures within an ecoregion to be 0.135.

2.3.5. Fixed Effects

The coefficients of the final model are not on the original scale, therefore, interpretation of each value requires individual calculations to back-transform estimates into their original scale (BT ODRs). The following interpretations of effects from fixed variables assume that all other variables in the model are held constant, and were derived from the final 'province' model.

The BT ODR was estimated to have decreased in the spring (March-June) by 0.016 ODR units, when compared to BT ODR values in the autumn, and then

increased sharply by 0.066 ODR units during the summer months.

For both heifers and milking cows, being exposed to pasture during the summer months significantly increased the BT ODRs (0.095 and 0.071, respectively). Although being exposed to a concrete area or a small field during the summer was not statistically different than being confined, the effects were positive, ranging from 0.011 to 0.038 ODRs for both heifers and milking cows.

Sharing a pasture with two groups of cattle, either heifers & dry cows, or heifers & milking cows, seemed to have beneficial effects (-0.021 [p=0.078], and -0.076 [p<0.001]). However, when all three groups had access to the same pastures (not necessarily during the same time), the benefits of sharing two groups was replaced with a slight overall negative effect of 0.012 ODR units.

Two significant treatment variables were included in the final model: treating cows at calving, and treating the entire herd at least once a year. The beneficial effects of both protocols were very similar. Treating the whole herd decreased the BT ODR by 0.035 units, while treating cows at calving decreased the BT ODR by 0.039 units.

An increase in the median vegetation index by one unit (1,000 on the Natural Resources Canada scale, or 0.1 on the conventional NDVI scale) was estimated to increase the BT ODR by 0.025 units (p=0.025). The effect of

elevation was investigated by substituting NDVI-04 with the elevation variable, while keeping the remaining parameters the same in the model (model not shown). The effect of increasing the farm's median surrounding 5-km of land by 1000m (representing the range in the data) was estimated to decrease the BT ODR by 0.154 units (p=0.005).

Substituting NDVI-04 with the number of rainy days, while keeping the other parameters (model not shown), showed that for each additional 10 rainy days during the growing season (after an initial 40 days of rainfall), the BT ODR would increase by 0.019 units (p=0.027). There was on average 68 rainy days on the farms (median=68, standard deviation=11).

The land surface temperature variable was significant when it was substituted for the NDVI-04 variable (model not shown). Higher median LST was protective, where for every increase of 1°C, averaged over the growing season, the BT ODR would decrease by 0.019 (p=0.043).

Table 2.6 shows the coefficient estimates for the same variables in the 'ecoregion' model. The same number of BT ODRs and herds were kept in the model, and the estimates and respective statistical significance remained very similar, except for NDVI-04 and the variance estimates for the three levels (ecoregions, herds and repeated BT measures). The total variance was slightly smaller in the 'ecoregion' model (1.7462 instead of 1.7680), however, the Akaiki Information Criteria, as explained by Dohoo *et al.* (2009), favors the

'province' model (1208.185 instead of 1210.888). The change in the impact from the vegetation index was minor (ODR effect of 0.022 instead of 0.025 per one NDVI-04 unit) and became non-significant (p=0.08). The intraclass correlation coefficient at the ecoregion level was reduced compared to the provinces in the 'province' model (0.135 vs. 0.169, respectively), while the ICC at the residual level was virtually the same (0.660 vs. 0.664).

The environmental parameters were taken during the growing season in 2004, however, 18.7% of the BT ODRs samples were taken in December of 2003, and 13% after September 2004. A reduced model, including only those milk samples taken in 2004, was used to assess this potential bias. The reduced model (not shown), including 312 BT ODRs from 110 herds within 9 provinces, reported nearly identical finding to the 'province' model, therefore, the bias was deemed negligible.

2.4. Discussion

The purpose of the questionnaire was not to perform a survey of the dairy management practices in Canada, but to gather information to serve as predictors for the bulk tank ODR values. As such, equal or proportional representation of farms within provinces across Canada was not necessary, as long as most provinces had some representation in the study to capture the differences across Canada.

Not surprisingly, there were strong correlations between environmental

predictors. Both low lying coastlines (eastern and western low elevation areas) had higher NDVI values, and the prairies had lower NDVI and precipitation. Only one of those predictors could be chosen for the final model, and NDVI-04 captured many meteorological factors in one variable. Image maps showed recorded vegetation indices every square kilometer, while climate information came from 1,183 weather stations across Canada and each farm's data was derived from its nearest weather station; weather stations' distances ranged from 1.2 to 53.5 kilometers (median=13.5) from the postal code coordinates for farms. The accuracy of the geographical data was limited to the quality of the data source. Other than province and postal code, there was no other specific information about farm location. Perhaps this is less important when regional environmental conditions are relatively similar (e.g. within the Maritimes or the prairies); it is when the different regional conditions are compared overall across a large country, with diverse ecological regions, that environmental and ecological effects are seen.

The environmental predictors of statistical significance were NDVI, LST, elevation, and the number of rainy days. Air temperature variables (mean temperature and degree days above 18 °C) were not significant, though their coefficients were positive. Experimentally, *O. ostertagi* has an optimal developmental temperature of 25 °C, nonetheless, larvae develop in fecal cultures from 10 to 35 °C (Pandey, 1972). Temperatures can change the rates of nematode infections, but water is essential for its development

(Stromberg, 1997).

Total precipitation measured at the nearest weather station was not significant in the model (p=0.091), despite the trend towards higher BT ODRs as total rainfall during the growing season increased. The number of days with at least 1 mm of rain, which is deemed enough to penetrate the soil by passing through the surface foliage, was a significant predictor for BT ODR (p=0.027). Water amounts can limit plant growth, and this growth is restricted by both the amount of water in the soil (soil water) and by the potential evapotranspiration (PET), which is the amount of water evaporation and transpiration that would occur if there was enough soil water available (McCall and Bishop-Hurley, 2003). Environmental PET constantly varies according to levels of sunlight (radiation), wind speed, temperatures, humidity, plant density, and soil properties (Denmead and Shaw, 1962). However, the predominant method to replenish soil water, especially on pasture, is rain. Water not only has a direct influence on the vegetation growth (NDVI), as explained above, but water also has an influence on the nematode life cycle. Although sheathed L_3 larvae carry some protection against desiccation, they need water to survive. Not only does rain help in dispersing eggs and larvae away from fecal pats, but it also maintains the larvae on the upper herbage (8-16cm) rather than on the lower herbage (soil to 8cm) (Stromberg, 1997), improving their transmission rate.

The LST's negative correlation with NDVI and precipitation, and positive correlation with elevation is consistent with what is reported in the literature, where LST increases rapidly with water stress, and vegetation also plays an important role (Sandholt *et al.*, 2002). The LST was found to have protective effect on the BT ODRs, and therefore, the effect of LST may be confounded by precipitation or NDVI. It was no surprise to find elevation as a significant predictor, since it was strongly correlated with other significant environmental factors; it is difficult to explain its relationship to BT ODR biologically. However, elevation is likely confounded by other environmental factors, such as, being in a low-lying coastal region with greater precipitation and vegetation versus being in the elevated prairies with decreased precipitation and vegetation growth.

The random effect components for both 'province' and 'ecoregion' models indicated a strong clustering effect of herds (ICC=0.664, and 0.660, respectively), therefore, the BT ODR values depend more on which herd they came from rather than which province or ecoregion the sample was taken from. The 'ecoregion' model made the NDVI-04 variable non-significant, with a slightly decreased coefficient, suggesting that when accounting for ecoregions, the surrounding vegetation index was less important than when accounting for provinces.

The majority of producers marked 'No' to treating milking cows with

anthelmintics in the questionnaire (81/177=45.8%). In another questionnaire, unrelated to this study but performed in Canada (Chapter 5), 73% (27/37) of producers used anthelmintics, and in total, 62% (23/37) of producers treated their milking cows specifically. The higher proportion found in Chapter 5 might be a reflection of the selection process, where cattle in those farms had to have access to pasture, and those producers were more likely to use anthelmintics, or perhaps simply that over time more producers have been using anthelmintics on their farms (2004 compared to 2007).

Within Canada, there have been four published studies investigating on-farm management practices and bulk tank ODRs or titres (Caldwell *et al.*, 2002; Guitián *et al.*, 2000; Hovingh, 1998; Sanchez and Dohoo, 2002). Hovingh *et al.* (1998) had investigated the effects of herd-level predictors on BT milk antibodies against *O. ostertagi*, *Dictocaulus viviparous*, and *Cooperia oncophora* in Prince Edward Island, using in-house ELISA tests developed with researchers in the Netherlands. As percentage of dry matter supplied by stored feeds increased, the OD values for *O. ostertagi* and *C. oncophora* decreased. Age had an effect, where as the percentage of heifers in the lactating herd increased, the ODs for *O. ostertagi* decreased; this was true even though milk yield was positively related to age and negatively related to ODs against *O. ostertagi*. The use of anthelmintics on the farm, and the average milk yield for the herd were protective against *O. ostertagi* ODs. Finally, though not statistically significant, the total pasture area mechanically

mowed, at least once before grazing, had a negative effect on both *O. ostertagi* and *C. oncophora* OD values.

Guitián *et al.* (2000) found a significant effect from treating heifers in the spring, as well as housing methods (e.g. confined, pastured, etc.) for both milking cows and heifers in Nova Scotia. A second model in that study showed an association with spreading manure on pasture and increased bulk-tank ODs, and with mixing heifers and dry cows on the same pasture. Sanchez *et al.* (2002), investigating farms in Prince Edward Island, found an association between housing type and bulk tank ODRs for milking cows only, and also found a significant treatment effect when producers de-wormed the entire herd at least once in the previous year. In Québec, Caldwell *et al.* (2002) circulated a questionnaire to their producers asking many questions regarding pasture rotation, contamination history, mechanical mowing, artificial drainage, and grass proportion in diet; they found associations between several risk factors and bulk-tank titres against *O. ostertagi*. Specifically, heifers rotating on an intensive pasture (mobile fence), or heifers on old pastures (cattle were on the same pasture last year) were associated with higher bulk-tank titres. Models for subpopulations of farms showed different associations, most notably, mechanical mowing of heifer pastures would decrease bulk-tank titres, but only for those farms that allow their heifers on pastures.

Questions in this study were focused on exposure to pasture, mixing of groups on pasture, and anthelmintic treatments for both heifers and milking cows. The results of this study are in accordance with previous findings in Canada, where as pasture exposure increases, so too does the BT ODR values, and there is a negative association between whole-herd treatment with anthelmintics and BT ODRs.

Sharing pastures by different cattle groups was investigated by Sanchez *et al.* (2002), but contrary to this study, they found no significant relationships in Prince Edward Island. The proportions of heifers on pasture/paddock, heifers grazed on pastures shared with dry cows, and heifers grazed on pastures shared with milking cows are virtually the same between the two studies, however, the proportion of milking cows on pasture/paddock (vs. confinement/yard) is very different; Sanchez *et al.* (2002) found that 97% of their herds had milking cows on pasture/paddock, compared to only 56% in this study. While Sanchez *et al.* (2002) had greater exposure to pasture and sample sizes than this study, which should have increased statistical power, their lack of significance may be explained by their lack of an interaction between the two pasturing variables. More specifically in our study, when the interaction term in the 'province' model is removed, both pasturing terms (heifers mixed with milking cows, and heifers mixed with dry cows) are non-significant, and it is only when the interaction is accounted for (heifers, dry cows, and milking cows) that the relationship becomes significant.

Biologically, the statistically significant interaction is difficult to explain. The questions regarding the combinations of pasture mixing (heifers mixed with milking cows and heifers mixed with dry cows) were asked in such a way that it is impossible for the interaction to differentiate between an effect from having all three groups mixed together (heifers, dry cows, and milking cows), or the specific combination of milking cows with dry cows mixed on pasture. Further studies would be needed to properly address this potential effect.

In Europe, two studies (both using the commercially available ELISA test Svanovir®) collected information to compare farm management practices and bulk tank ODRs (Almería *et al.*, 2009; Charlier *et al.*, 2005b). Almería *et al.* (2009) conducted a small study in two Mediterranean islands (Minorca and Girona) and noted that herds with access to pasture had a larger mean individual ODR than those without access to pasture, although the relationship was not significant (p=0.14), and the size of the grazing area was positively correlated with individual averaged ODRs (p<0.02) – there were no significant treatment effects (p>0.123). Charlier *et al.* (2005b) conducted a large study involving 1,032 Flemish dairy herds. Overall, the significant variables were herd size, herd type (dairy or mixed), mean somatic cell counts, average lactation number, and cow exposure to pasture; there were no significant effect from treatment protocols for either heifers or milking cows. When investigating only those farms with milking cows on pasture, Charlier *et al.* (2005b) found additional relationships with daily pasture grazing time,

mechanical mowing of pasture, and turnout month for the season.

2.5. Conclusions

This study expanded on the first four provincial studies in Canada, investigating the effects farm management practices, including anthelmintic treatment, may have on bulk tank ODR measuring *O. ostertagi* antibodies. This study is unique since it included nine of Canada's ten provinces and spanned many geographic ecoregions across Canada, from the east to the west coast, and was the first to include environmental factors in the analysis. It is likely that the large diversity of ecological regions within Canada was partially responsible for the statistically significant influence which environmental factors (climate, vegetation, and elevation) had on the measured parasite exposure; if the study had been carried out within one or two ecoregions, perhaps that significant difference would not have been found.

Overall, the greater the exposure that heifers and milking cows had to pasture, the higher the levels of anti-parasite antibodies were in bulk tank samples. Sharing pastures between heifers, dry cows and milking cows was also associated with higher BT ODRs, although this effect is biologically difficult to explain, and further research is warranted to investigate this potential effect. Treating the entire herd or treating milking cows at calving had reduced BT ODR values. Farms in areas with higher number of rainy

days, higher NDVI values, and lower LSTs, were also likely to have higher BT ODRs. Seasonal variation was such that late summer and early fall, when parasite load was at its highest, yielded larger BT ODRs. Due to the high clustering effect at the herd level, factors at the herd level (e.g. pasturing methods, anthelmintic administration) had a higher potential impact on bulk-tank measurements than the herd's surrounding environmental factors.

2.6. References

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Table 2.1. Summary of BT ODR values, overall, and by provinces.

	Mean	Median	Standard Deviation	Interquartile Range
All Provinces	0.327	0.279	0.219	0.152 – 0.473
British Columbia (BC)	0.237	0.181	0.191	0.114 - 0.311
Alberta (AB)	0.231	0.184	0.162	0.129 - 0.307
Saskatchewan (SK)	0.352	0.311	0.176	0.206 – 0.507
Manitoba (MB)	0.277	0.205	0.224	0.123 – 0.367
Ontario (ON)	0.242	0.206	0.167	0.115 – 0.375
Québec (QC)	0.370	0.344	0.258	0.143 – 0.530
New Brunswick (NB)	0.446	0.461	0.200	0.293 – 0.587
Nova Scotia (NS)	0.514	0.550	0.221	0.343 – 0.678
Prince Edward Island (PE)	0.414	0.374	0.208	0.261 – 0.570

Table 2.2. Pasture sharing practices as derived from the questionnaire. There were a total of 122 herds that completed these two questions.

		Milking Cows mixed with Heifers			
		Yes	No	Missing	
Heifers mixed with Dry Cows	Yes	55	4	18	77
	No	47	16	13	76
	Missing	8	0	34	42
		110	20	65	195

Table 2.3. Questions asked to producers with a summary of answers. There were 195 respondent herds. (Missing)

Questions asked to producers		Total Answered
Heifers in the SUMMER of 2004: (16)	1) totally confined (barn 24hr/d)	28
	2) access to concrete/gravel	13
	3) access to small field	26
	4) spent some time grazing	112
Heifers with access to pasture...same pasture as dry cows? (42)	Yes / No	76/77
Heifers were treated with pour-on/inj. in the FALL 2003? (23)	Yes / No	69/103
Heifers were treated with pour-on/inj. in the SPRING/SUM 2004? (23)	Yes / No	43/129
Heifers were treated with a BOLUS, SUMMER 2004? (23)	Yes / No	5/167
Heifers were treated with pour-on/inj. in the FALL 2004? (23)	Yes / No	43/129
Milking Cows in the SUMMER of 04: (12)	1) totally confined (barn 24hr/d)	71
	2) access to concrete/gravel	10
	3) access to small field	31
	4) spent some time grazing	71
Milking cows with access to pasture... same pasture as Heifers? (65)	Yes / No	20/110
Milking cows were treated with oral de-wormers? (18)	Yes / No	7/170
Milking cows were treated with pour-on/inj. at DRY OFF? (18)	Yes / No	8/169
Milking cows were treated with pour-on/inj. at CALVING? (18)	Yes / No	12/165
Milking cows were treated with pour-on/inj. WHOLE HERD? (18)	Yes / No	59/118

Table 2.4. Correlations and related scatterplot matrices between environmental predictors.

	NDVI						
Total Precip.	0.597	Total Precip.					
Days Precip.	0.454	0.692	Days Precip.				
LST	-0.466	-0.310	-0.246	LST			
Air Temp.	0.574	0.453	0.395	-0.015	Air Temp.		
Degree Days >18°C	0.505	0.413	0.359	-0.013	0.965	Degree Days >18°C	
Elevation	-0.717	-0.538	-0.319	0.366	-0.640	-0.635	Elevation

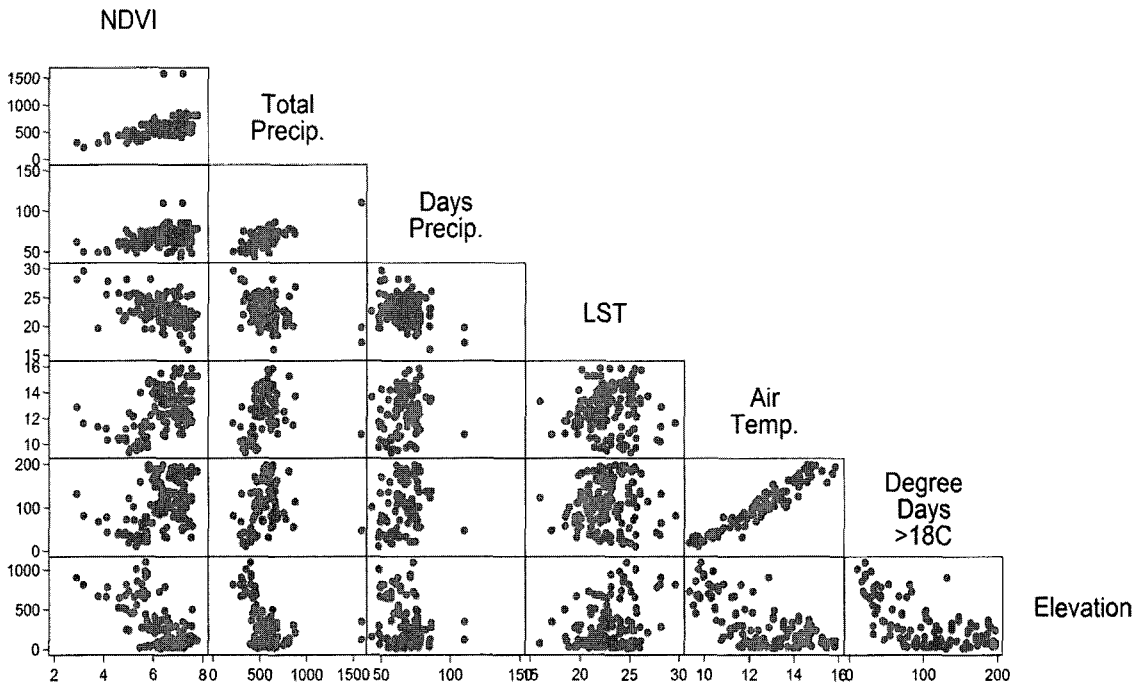

Table 2.5. Multilevel mixed 'province' model. It included province and herd as random effects, and the coefficients are based on transformed ODR values ([(ODR+0.1)*100]^0.5). The model includes 9 provinces, 115 herds, and 412 bulk-tank samples.

Fixed Effect			
Variables	Coefficient	95%CI	*P* value
Season			<0.001
Autumn & Winter (Nov Feb)	Baseline		
Spring (March-June)	-0.305	-0.488, -0.121	0.001
Summer (July-Sept)	0.763	0.552, 0.973	<0.001
Heifer Housing			0.002
Confined	Baseline		
Concrete	0.187	-1.197, 1.571	0.791
Field	0.616	-0.489, 1.721	0.275
Pasture	1.363	0.336, 2.391	0.009
Milking Cow Housing			0.003
Confined	Baseline		
Concrete	0.500	-0.522, 1.521	0.338
Field	0.205	-0.450, 0.861	0.539
Pasture	1.071	0.486, 1.657	<0.001
Interaction Sharing Pastures			<0.001
Heifers shared with Dry Cows	-0.405	-0.854, 0.045	0.078
Milking cows shared with Heifers	-2.393	-3.563, -1.222	<0.001
Heifers shared with Dry Cows and Milking Cows	0.199	-0.449, 0.847	0.547
Averaged surrounding NDVI in 2004	0.418	0.054, 0.783	0.025
Milking Cows got treatment at calving	-0.828	-1.495, -0.160	0.015
Whole herd received treatment	-0.710	-1.139, -0.282	0.001
Constant	2.795		

Random Effect			
Level	Variance	Std.Err.	ICC
Province	0.2996	0.2060	0.169
Herd	0.8750	0.1529	0.664
Residuals (BT ODRs)	0.5934	0.0487	-

Table 2.6. Multilevel mixed 'ecoregion' model. It included herds as random effects, and the coefficients are based on transformed ODR values ([(ODR+0.1)*100]^0.5). The model includes 13 ecoregions, 115 herds, and 412 bulk-tank samples.

Fixed Effect			
Variables	Coefficient	95%CI	*P* value
Season			<0.001
Autumn & Winter (Nov-Feb)	Baseline		
Spring (March-June)	-0.300	-0.483, -0.116	0.001
Summer (July-Sept)	0.760	0.550, 0.970	<0.001
Heifer Housing			0.005
Confined	Baseline		
Concrete	0.344	-1.054, 1.742	0.629
Field	0.663	-0.461, 1.787	0.248
Pasture	1.403	0.357, 2.448	0.009
Milking Cow Housing			0.002
Confined	Baseline		
Concrete	0.395	-0.632, 1.421	0.451
Field	0.042	-0.632, 0.716	0.903
Pasture	1.015	0.408, 1.622	0.001
Interaction Sharing Pastures			<0.001
Heifers shared with Dry Cows	-0.341	-0.787, 0.106	0.135
Milking cows shared with Heifers	-2.560	-3.763, -1.357	<0.001
Heifers shared with Dry Cows and Milking Cows	0.115	-0.554, 0.784	0.736
Averaged surrounding NDVI in 2004	0.312	-0.039, 0.664	0.081
Milking Cows got treatment at calving	-0.828	-1.564, -0.185	0.013
Whole herd received treatment	-0.749	-1.176, -0.321	0.001
Constant	3.403		

Random Effect			
Level	Variance	Std.Err.	ICC
Ecoregion	0.2363	0.1676	0.135
Herd	0.9169	0.1595	0.660
Residuals (BT ODRs)	0.5930	0.0486	-

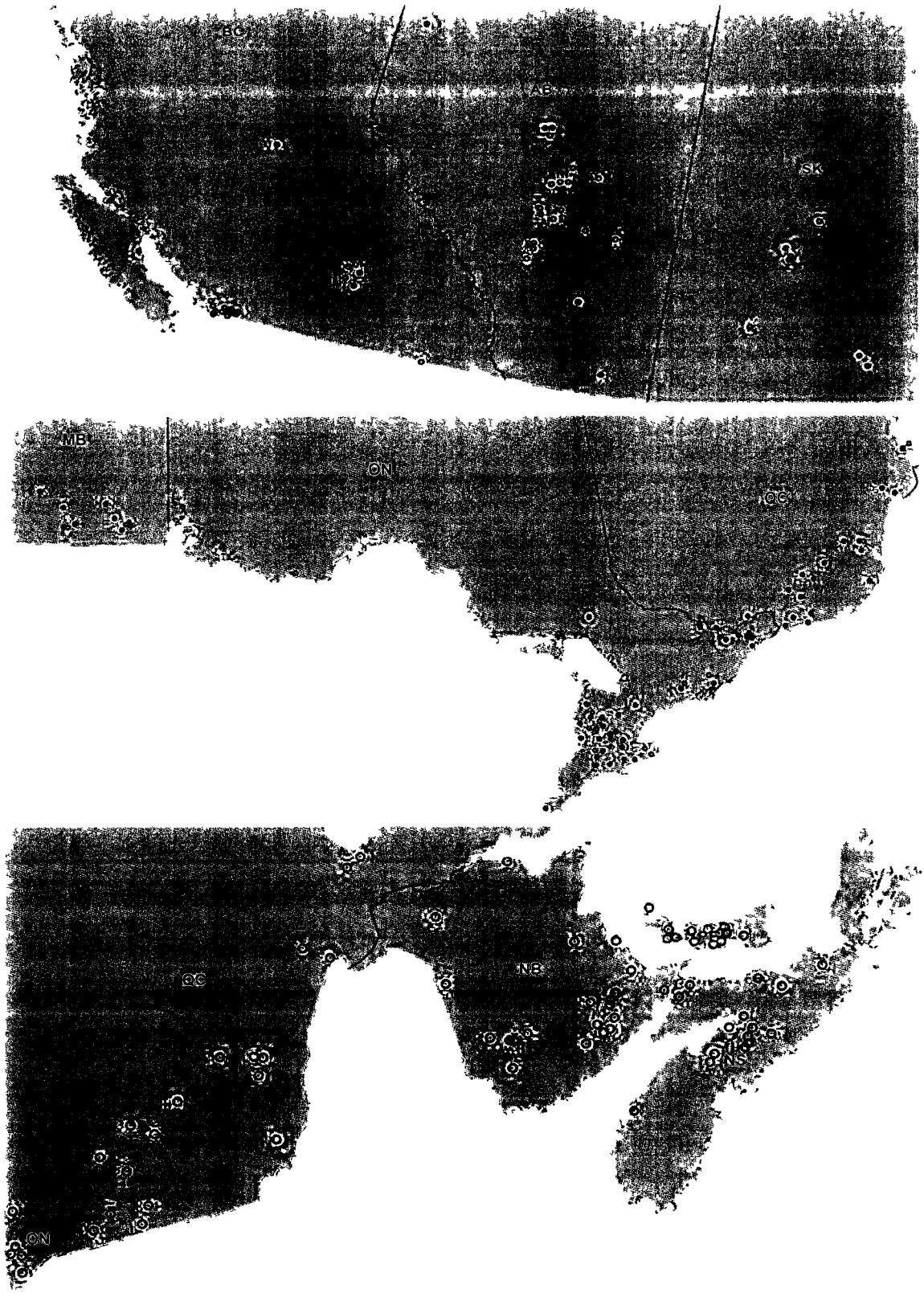

Figure 2.1. Five kilometer radius surrounding participating herds. The coordinates are base on their postal codes.

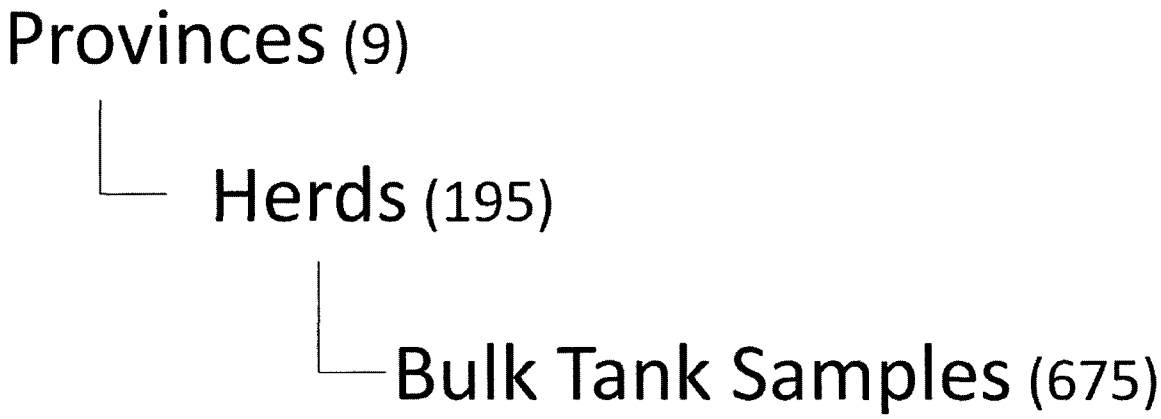

Figure 2.2. Hierarchical structure of the data, showing total number of units and their ranges at each level.

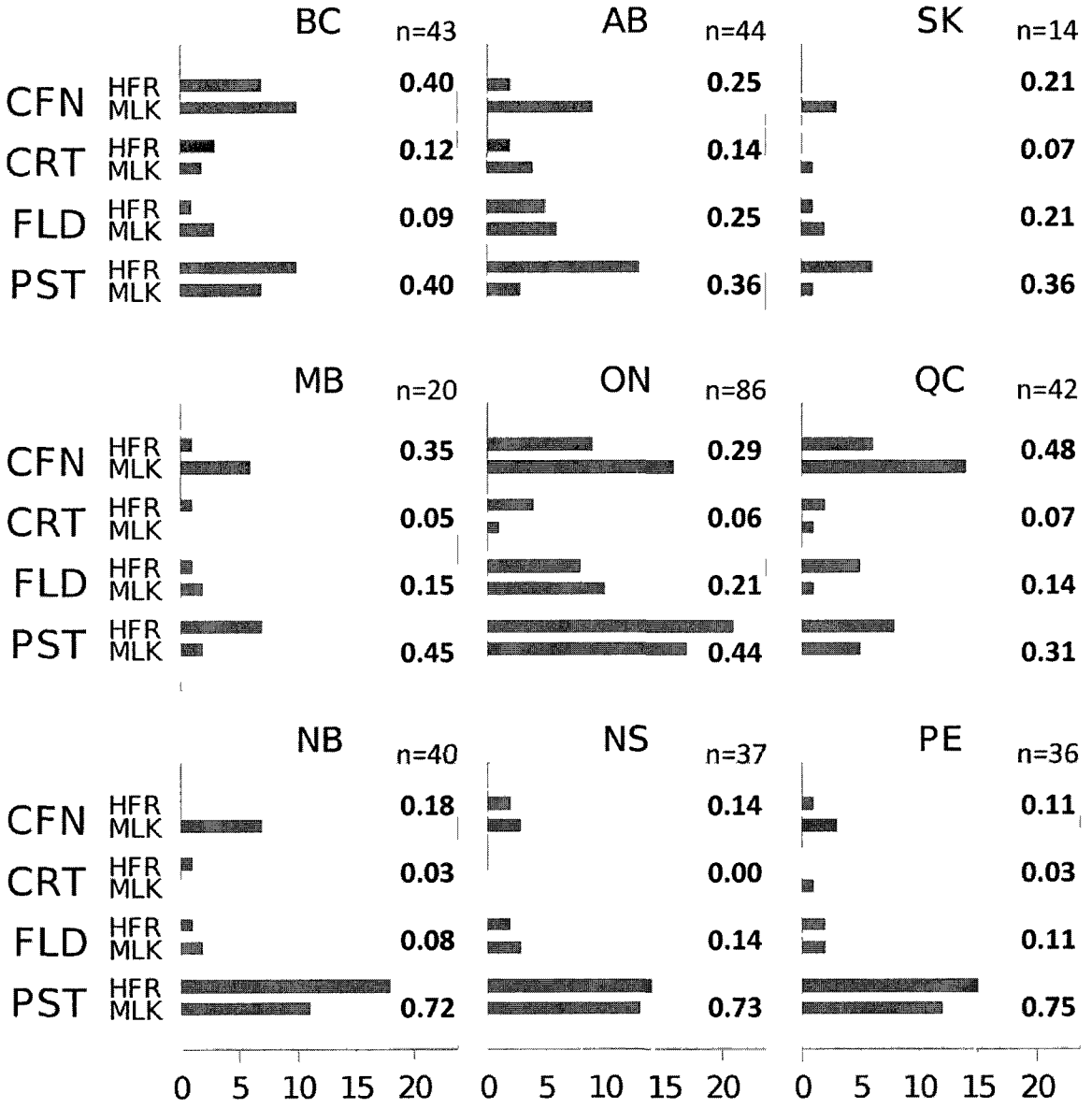

Figure 2.3. Bar graph of pasturing methods (CFN=Confined, CRT=Concrete, FLD=Field, PST=Pastured) **for milking cows** (MLK) and heifers (HFR) **by province.** Proportions in the right margin were calculated for each pasturing method. There were 362 responses in total.

Figure 2.4. Digital raster of averaged NDVI values (Natural Resources Canada scale) **for the 2004 growing season.** Farms, including their 5-km radius, are shown. Each pixel represents NDVI values for each square kilometer. The prairies are demarcated by a yellow line.

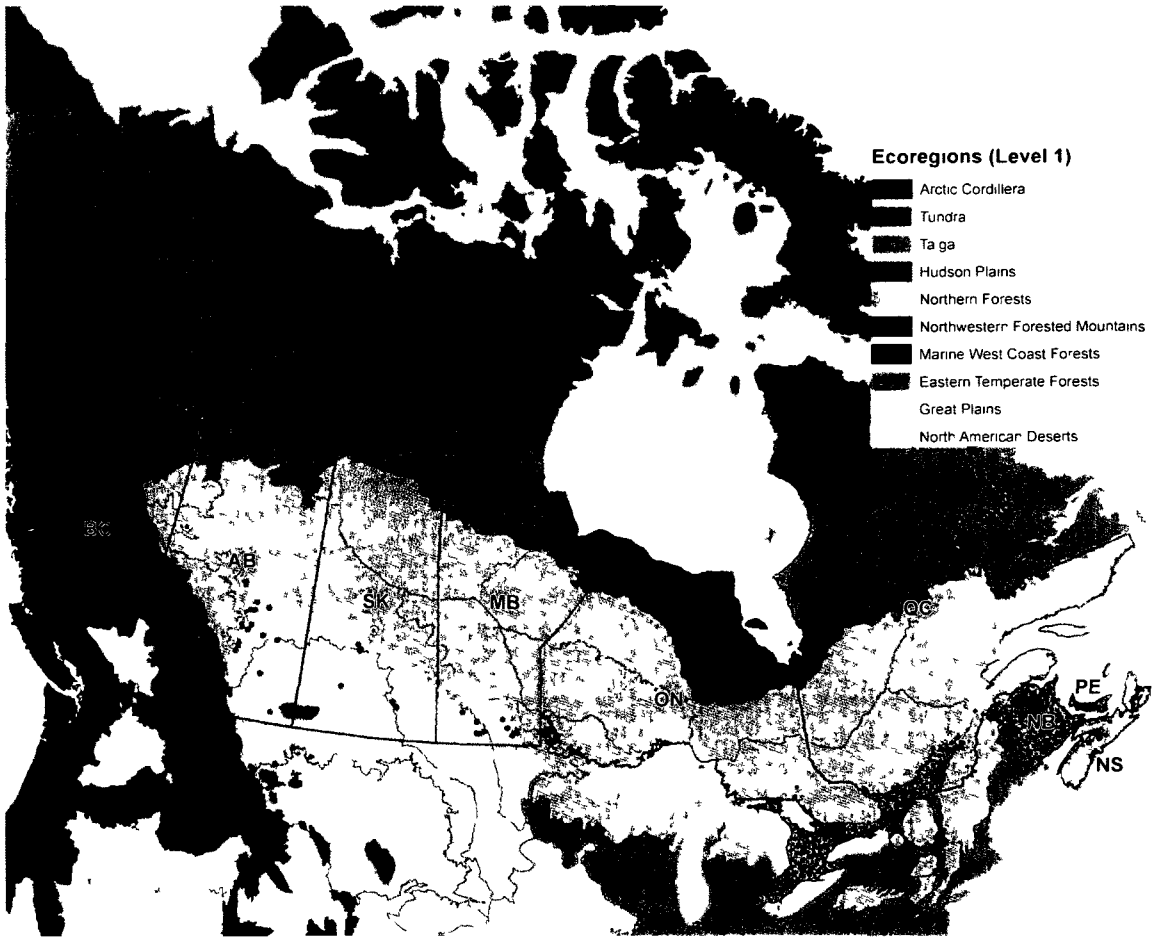

Figure 2.5. Ecoregions of Canada with participating farms. Farms are shown by their surrounding 5km radius. The legend describes Level-1 ecoregions, while polygons outline Level-3 sub-regions.

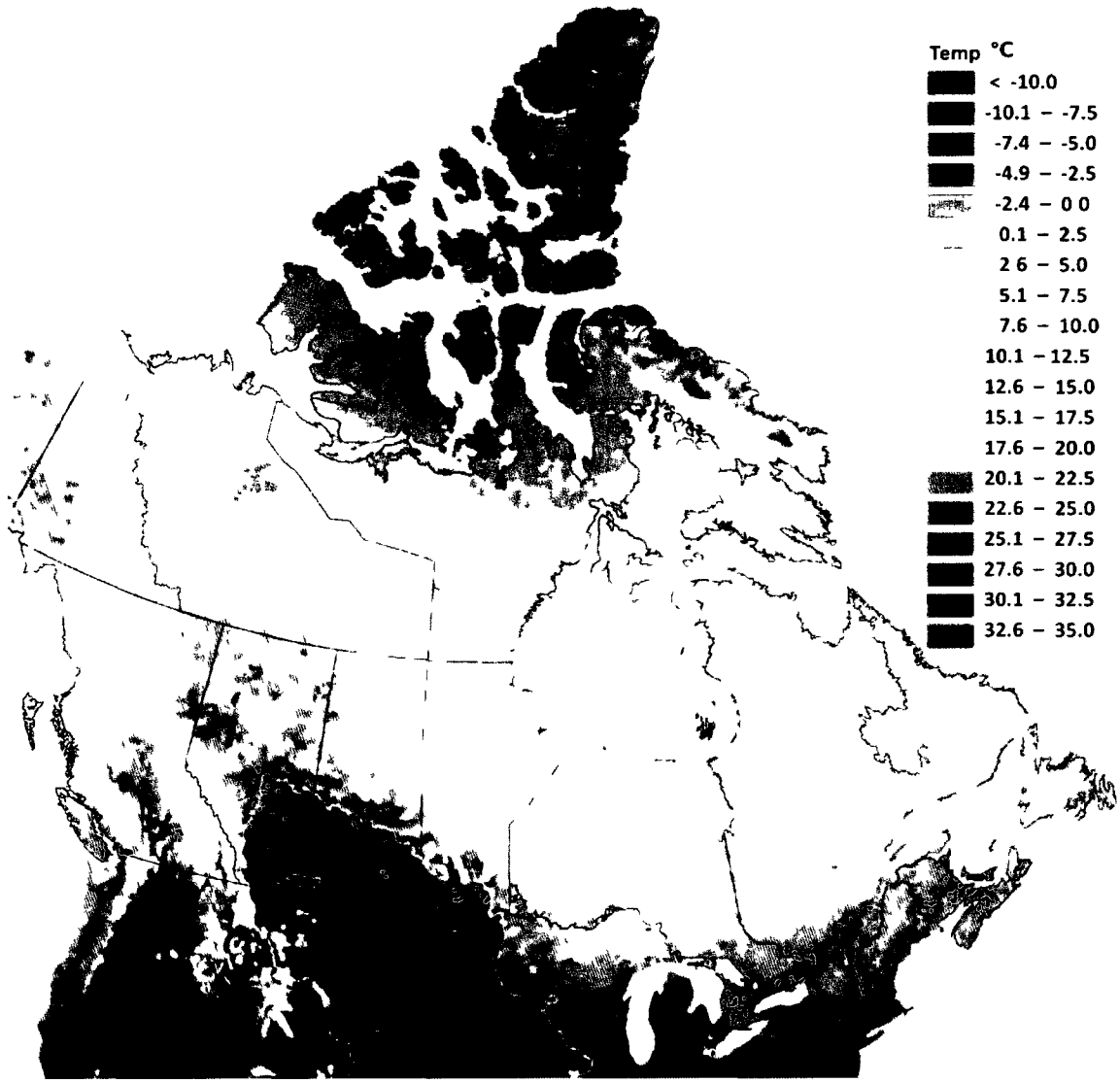

Figure 2.6. Averaged land surface temperatures (°C) for the 2004 growing season (April to October). The prairie boundaries are outlined in yellow.

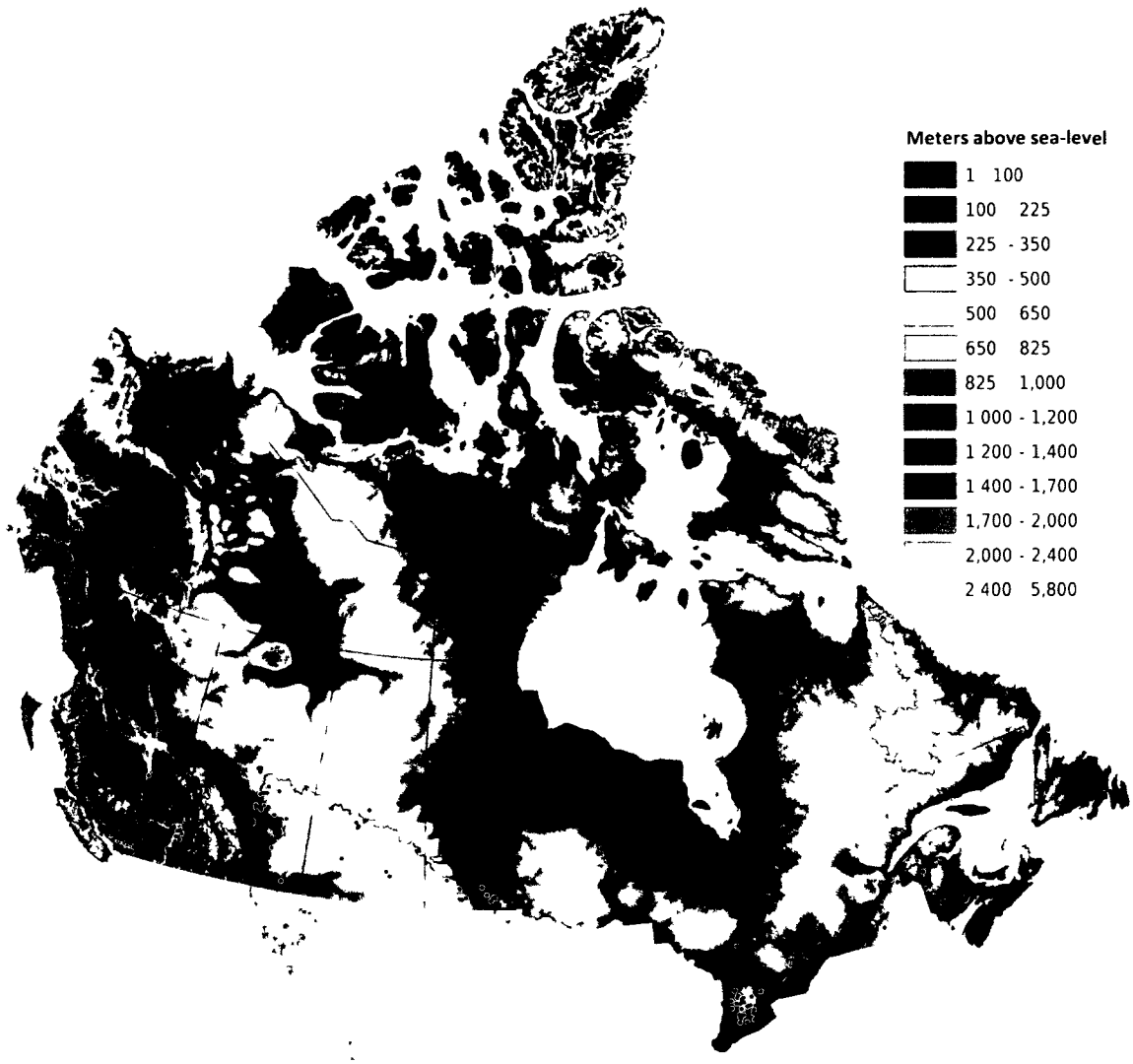

Figure 2.7. Digital elevation model of Canada with participating farms. Farms are shown by their surrounding 5km radius. The prairie boundaries are outlined in blue.

Chapter 3

The Impact of Milk Handling Procedures on *Ostertagia ostertagi* Antibody ELISA Test Results*

* Manuscript based on this chapter: R. Vanderstichel, I. Dohoo, H. Stryhn, 2010. The impact of milk handling procedures on Ostertagia ostertagi antibody ELISA test results. Vet Parasitol. 169(1-2):204-8

3.1. Introduction

Intestinal parasitism can have a negative effect on milk production in dairy cattle (Guitián *et al.*, 2000; Kloosterman *et al.*, 1993; Nødtvedt *et al.*, 2002). An enzyme-linked immunosorbent assay (ELISA) can be used to measure antibodies against *O. ostertagi* (Keus *et al.*, 1981), the most common and economically important nematode in cattle (Gibbs and Herd, 1986). Normalized results from this ELISA test, referred to as optical density ratio (ODR), quantify levels of gastrointestinal nematode infection in dairy cows, and there is a negative association between milk production and ODR values. Cows with elevated ODR have lower milk yields than cows with lower ODR (Charlier *et al.*, 2007; Ploeger *et al.*, 1989; Sanchez and Dohoo, 2002). Svanovir® (Svanova Veterinary Diagnostics, Uppsala, Sweden) is an ELISA test commercially available and currently used in Europe to determine levels of parasitism from milk samples.

Research is underway at the Atlantic Veterinary College, Prince Edward Island, Canada, to determine herd sampling guidelines for ELISA testing using Svanovir® to inform producers if improved parasite control is necessary at the herd level to increase overall milk production. Milk samples for the study were collected across many Canadian provinces (Alberta, Ontario, Québec, New Brunswick, Nova Scotia, and Prince Edward Island) in collaboration with the Canadian Bovine Mastitis Research Network (CBMRN), which is a nation-wide network of veterinary colleges and agricultural research stations engaged in

udder health research. Dairy Herd Improvement (DHI) programs are involved with routine on-farm collection of milk samples to monitor cow and herd productivity, milk quality, and will provide the productivity data for this research effort. In the long run, it is expected that DHI programs will offer parasite ELISA testing as a service to clients so the impact of milk handling procedures on ELISA test results must be evaluated.

The milk samples, acquired from the CBMRN and DHI are subjected to a variety of handling and processing procedures, henceforth referred to as stressors. Milk from CBMRN is frozen, shipped to central laboratories, thawed for bacterial cultures, placed in hot water baths (37-43°C for 20-30 min) for somatic cell count, and refrozen for storage.

Milk collected by DHI during routine farm visits consists of a 40ml composite (all milking quarters) sample from individual cows which is preserved with bronopol (2-Bromo-2-Nitropropane - 1,3 Diol), delivered to a laboratory, placed in a hot water bath at 43°C for 15 to 25 minutes and then processed through automated machines to run component analyses (fat, protein, lactose etc.) and somatic cell counting.

The manufacturers of Svanovir® recommend using a fresh milk sample centrifuged to remove the lipid layer (referred to as defatting). However, routine collection of such samples will be expensive, and samples available for routine screening will likely have been subjected to one or more stressors.

Two previous studies investigated the effect of preserving milk samples on ODR values through an indirect crude ELISA for *O. ostertagi* antibodies, produced in-house (Charlier *et al.*, 2005; Sanchez *et al.*, 2002). Sanchez *et al.* (2002) investigated the effects of bronopol and freezing, while, Charlier *et al.* (2005) investigated the effects of length of time in cold storage, multiple freeze-thaw cycles, and whole vs.defatted milk. No studies have yet looked at the effect of heating milk samples on ODR values or any other stressors using the Svanovir® test.

The goal of this study was to determine if heating, freezing, re-freezing or defatting milk samples had an effect on ODR values from a milk ELISA test (Svanovir®) measuring *O. ostertagi* antibodies.

3.2. Material and Methods

3.2.1. Study Design

One hundred and forty milliliters of milk from each one of 40 individual cows from two dairy herds were collected during regular milking periods. The samples were collected and refrigerated at 4°C for no more than 16 hours prior to testing to ensure milk freshness. Samples were chosen specifically from a larger pool of 87 cows to represent a uniform distribution of the range of ODR values (Fig. 3.1). All milk samples were preserved chemically by adding 6mg of bronopol (2-Bromo-2-Nitropropane - 1,3 Diol and 0.3mg of Pimaricin per 40 ml of milk; Brotab, D&F Control Systems, Inc., Dublin, California, United States of America) to prevent milk spoilage.

Each of the 40 milk samples was subdivided into 6 containers, each container then subdivided into 2 tubes, for a total of 12 aliquots, each aliquot representing a different combination of stressors, as portrayed in Fig. 3.2. Freezing stressors were either 1) freezing for 3 days, 2) freezing for 3 days, thawing, and re-freezing for 1 week, or 3) freezing for 3 days, thawing, and re-freezing for 4 weeks; freezing temperatures were always at -20°C. Heating refers to submerging samples in a hot water bath at 43°C for 20 minutes. Defatting refers to centrifuging milk for 3 minutes at 16,000 x g to remove the lipid layer. This resulted in a data structure with a hierarchical structure shown in Fig. 3.3, with a total of 480 tubes, 40 samples per 12 stressor effects.

3.2.2. ELISA Test

The commercial ELISA kit, Svanovir®, was performed according to the manufacturer's specifications. Positive and negative controls were run in triplicates, and were both supplied in the kit. All samples were tested only once.

3.2.3. Statistical Methods

Summary statistics of distributions and concordance correlation coefficients (CCCs) were determined using Stata10.1 (2008). CCC evaluates the agreement between two series of continuous measurements, where, values close to 1 indicate very good agreement while values approaching zero reflect very poor agreement (Dohoo *et al.*, 2009).

The statistical analysis was based on a linear mixed model with fixed effects of freeze/heat combinations (6 combinations as shown in Fig. 3.2) and defatting (no/yes) as well as their interaction, and with random effects for cows and containers (as shown in Fig. 3.3). The outcome (ODR values) was transformed to normalize residuals (Dohoo *et al.*, 2009); 0.15 units were added to have positive values and then a square root transformation was applied. Model assumptions were evaluated by inspection of residuals at all levels. A significant interaction between the two treatment factors was represented by an interaction plot for back-transformed least square means with 95% confidence limits, and biologically interesting contrasts of the interaction were constructed. Extensions of the random part of the model were assessed by likelihood-ratio tests. The analysis was performed in SAS9.1 (2003) using the MIXED procedure.

3.3. Results

The 40 selected milk samples had a relatively uniform distribution, with a median value of 0.258 and a range of -0.107 to 0.805 (Fig. 3.1).

The residual analysis of the linear mixed model revealed unequal variation between fresh and frozen milk samples (Fig. 3.4); simple descriptive statistics confirmed that the variation was larger among the fresh samples. Consequently, the mixed model was extended to allow for differences in variation between fresh and frozen samples. There was evidence of differences in variation at the container ($p<0.001$) and residual ($p<0.001$)

levels. The variance was 3.64 times greater in the fresh group than in the frozen group at the container level (fresh=$5.32x10^{-4}$, frozen=$1.46x10^{-4}$) and 5.23 times greater in the fresh group than in the frozen group at the residual level (fresh=$16.17x10^{-4}$, frozen=$3.09x10^{-4}$) (Table 1). Figure 3.5 represents strong evidence ($p<0.001$) of an interaction between the 6 stressor combinations at the container level (freeze/heat) and the 2 stressor combinations at the tube level (defatting vs.whole), both of these stressor effects were also strongly significant when assessed individually ($p<0.001$).

A total of 8 interaction contrasts of biological interest were further examined (Table 3.2), and of those, 6 were statistically significant. As an example, the first contrast reported a significant ($p=0.048$) estimated difference of 0.018 between ODR values (original scale) in the fresh, non-heated milk (both defatted and whole) versus the frozen, non-heated milk (both defatted and whole).

CCCs were determined between all twelve possible stressor combinations, for a total of 66 pairwise comparisons. The range of CCC values was 0.646 to 0.992 and the interquartile range was 0.909 to 0.982 for all CCC values. The distribution of the pairwise comparisons was strongly left skewed (Fig. 3.6), with 77% of comparisons having values greater than 0.90. The CCC between the manufacturer's recommendation versus the CBMRN handling scheme (fresh, not heated, and defatted versus frozen, thawed, heated, refrozen for 4

weeks and not defatted) was 0.931.

3.4. Discussion

3.4.1. Optical Density Ratio

The ODR value from the Svanovir® test is on a continuous scale, so there is no specific cut-point indicating a high or low level of parasitism, per se. The range of ODR values between the lowest and highest levels of parasitism is usually between -0.10 and 1.2, although it can vary depending on the exposure to parasites in the population tested and the controls used. Therefore, the estimated average difference in the ODR of 0.062, the difference found between the optimal sample recommended by the manufacturers of Svanovir® and samples submitted to the most extreme stressors (Contrast 6, Table 3.2), would have a relatively small effect on the estimate of parasite burden in the cow.

3.4.2. Bronopol

It is common to add bronopol, a preservative, to milk samples collected on a farm. All samples in this study were preserved using bronopol both because of collection requirements by CBMRN and DHI, and because bronopol has been shown to have no significant effect on the ELISA readings (Sanchez *et al.*, 2002; Sweeney *et al.*, 1994).

3.4.3. Interaction of Stressors

The interaction between the fixed effects makes for a complicated interpretation of the effects of defatting, heating and freezing. Contrasts were

created based on biological interests to help explain the results (Table 3.2). No adjustments were made for the multiple comparisons created by contrasts. It is normally advisable to use an adjustment such as Bonferroni adjustments or Scheffé's method to account for the increasing chance of error as more comparisons are made (Christensen, 1998). The adjustments make the statistical test more conservative (i.e. less likely to be statistically significant). In this study, identifying any possible effect of stressors on the milk ODR was considered desirable, even if the p-value estimates are too liberal.

3.4.4. Freezing

Sanchez *et al.* (2002) evaluated CCC between fresh defatted milk and 1) defatted milk Frozen for 1 week (CCC=0.97), 2) defatted milk Frozen for 6 weeks (CCC=0.98), and 3) defatted milk Frozen for 35 weeks (CCC=0.91). Although we cannot directly compare the two studies, some of the treatments in this recent study are comparable to part of the Sanchez *et al.* (2002) study. The CCC between fresh non-heated defatted samples and the frozen for three days non-heated defatted samples in this study was 0.97. Also, in this study, comparing fresh defatted heated samples to defatted frozen heated & re-frozen for 7 days had a CCC of 0.95. These two CCC are similar to Sanchez' comparison between fresh defatted milk and defatted milk frozen for 1 week.

The mean difference of 0.018 units between defatted fresh and defatted frozen was not significant (Contrast 7, Table 3.2, p=0.099). This difference is

comparable to reported mean differences between defatted fresh, and defatted frozen for 1, 6, and 35 weeks, as 0.04, 0.02 and -0.02, respectively (Sanchez *et al.*, 2002). In conclusion, freezing for short periods has little effect on milk ODR values.

3.4.5. Re-freezing

The ODR values for the samples (both defatted and whole) which were re-frozen for 4 weeks are higher than any of the ODR for the other stressors (Table 3.2, Contrast 3, $p<0.001$). This may have been a true effect of refreezing or may simply be due to a plate effect, as all samples were run on a single plate. It may also be associated with fluctuations in the control solutions. To reduce the variation due to plate effect, we used ODR normalization methods ($ODR = (OD_{sample} - OD_{negative})/(OD_{positive} - OD_{negative})$) throughout the study as recommended by Sanchez *et al.* (2002). This normalization method assumes constant controls for all plates and thus the controls (particularly the positive control) have a larger influence on the ODR values. Even after normalization, however, there may be a true effect from re-freezing milk for 4 weeks.

3.4.6. Heating

To process milk through automated analytical instruments for both component analysis and somatic cell counting, performed by DHI laboratories, the samples must be heated to melt the butter fat content to produce a homogenized sample (Bentley, 2008; Foss, 2008). Butter fat will melt at

40°C; most protocols require milk samples to be placed in a hot water bath (37 - 43°C) for 20-30 minutes. The effect of heating decreased the ODR values by 0.044 units (p<0.001, Contrast 4, Table 3.2). This small effect would not be sufficient to preclude the use of routinely collected DHI samples for ELISA testing once the DHI composition analysis had been carried out.

3.4.7. Defatting

Svanovir® recommends using defatted milk samples, also referred to as whey. After centrifuging milk there are three visible layers: 1) the top layer consists of lipids; 2) the middle liquid portion is the whey; 3) the bottom portion contains the solids. Fat could interact with immunoglobulins, therefore, ELISA tests are usually performed on the whey. As illustrated in Contrast 2 (Table 3.2), the overall difference between whole milk and defatted milk samples was negligible (0.011, p=0.002). Charlier *et al.* (2005) found no significant differences between defatted and whole milk, and furthermore, it didn't seem to matter if the whole milk was shaken to break-up the cream border or if the pipette was plunged through the top layer of cream. Defatting is a labor intensive procedure and appears to be unnecessary.

3.4.8. Svanovir® Recommendation vs.CBMRN/DHI Samples

The effect of the routine milk collection protocol used by the CBMRN, compared to what is recommended by the manufacturers of the Svanovir® (Contrast 6 in Table 3.2), yields a statistically significant increase in ODR by 0.062 (p<0.001). This difference is not biologically important if one considers

that it represents less than 5% of the possible range of ODR (-0.10 to 1.20) between the lowest and the highest levels of parasitism.

Furthermore, differences in optical densities between an ELISA plate with empty wells and the same ELISA plate with wells that have either 100µl of distilled water or 100µl of Phosphate Buffered Saline (supplied in the kits) can have an averaged range of 0.041 units (data not shown).

3.5. Conclusions

In Europe, Svanovir® – an ELISA measuring *O. ostertagi* antibodies in milk, is commercially available, and used in some countries to suggest whether milk production is likely to improve by deworming the herd. Svanovir® manufacturers recommend using fresh defatted milk. To implement a similar monitoring program in North America, samples would most likely be obtained through DHI programs. Milk samples collected through DHI programs are likely to undergo one or many of the transportation, processing and storage stressors replicated in this study. This study found that the effects of the individual stressors on test outcome depend on the combination of stressors present (interaction). Overall, although outcome of the test is slightly influenced by the storage method, length of storage and defattening process, the differences were minimal and would have little effect on the interpretation of the results. Fresh, whole, heated milk, the most likely sample to be used in DHI based surveillance programs, will yield reliable results. So too will frozen, whole milk, the most likely to be used in large scale research projects.

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Table 3.1. Random effects component of the linear mixed model indicating separate estimate variation for fresh and frozen samples.

Random Effects	Estimate	Standard Error
Cow Level		
–	0.058410	0.013240
Container Level		
Frozen	0.000146	0.000043
Fresh	0.000532	0.000261
Tube Level		
Frozen	0.000309	0.000035
Fresh	0.001617	0.000259

Table 3.2. Biologically interesting contrasts selected to explain the effects of the interaction between stressor effects.

Contrast No.		Defat vs Whole	Frozen - Heated Combination	Estimate[1]	Diff.[2]	*p*
1	Freezing 3d	Defat & Whole	Fresh, Not Heated	0.3286	0.018	0.048
		Defat & Whole	Frozen, Not Heated	0.3110		
2	Re-freezing 1w	Defat & Whole	Fresh, Not Heat	0.3286	0.012	0.176
		Defat & Whole	Re-Frozen 1 Week	0.3166		
3	Re-freezing 4w	Defat & Whole	Fresh, Not Heated	0.3286	0.055	<0.001
		Defat & Whole	Re Frozen 4 Weeks	0.3833		
4	Heating	Defat & Whole	Fresh, Not Heated & Frozen, Not Heated	0.3198	0.044	<0.001
		Defat & Whole	Fresh, Heated & Frozen, Heated	0.2754		
5	Defatting	Whole	All 6 combinations	0.3089	0.011	0.002
		Defat	All 6 combinations	0.3198		
6	ELISA vs.CBMRN[3]	Defat	Fresh, Not Heated	0.3101	0.062	<0.001
		Whole	Re-Frozen 4 Weeks	0.3719		
7	ELISA vs.DHI[4]	Defat	Fresh, Not Heated	0.3101	0.046	0.001
		Whole	Fresh, Heated	0.2640		
8	Freeezing[5]	Defat	Fresh, Not Heated	0.3101	0.018	0.099
		Defat	Frozen, Not Heated	0.3284		

1 Estimate = least square means of ODR values, back-transformed into original ODR scale

2 Diff. = absolute least square mean differences between the two groups in the contrast

3 Comparison of milk handled according to manufacturers recommendation with those used in the CBMRN study

4 Comparison of milk handled according to manufacturers recommendation with those used in DHI programs

5 Effect of freezing in defatted samples only for comparison with previous study results (Sanchez *et al* , 2002)

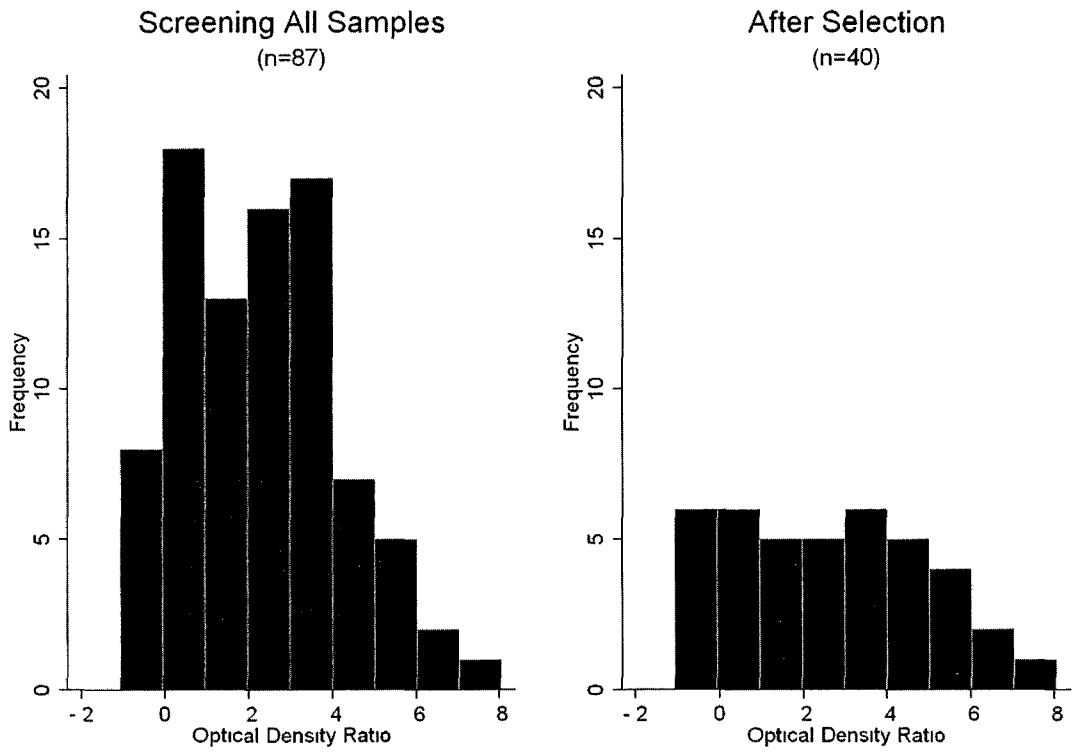

Figure 3.1. Distribution of ODR values from milking cows. Left graph shows both herds, and right graph shows those selected milk samples for the study.

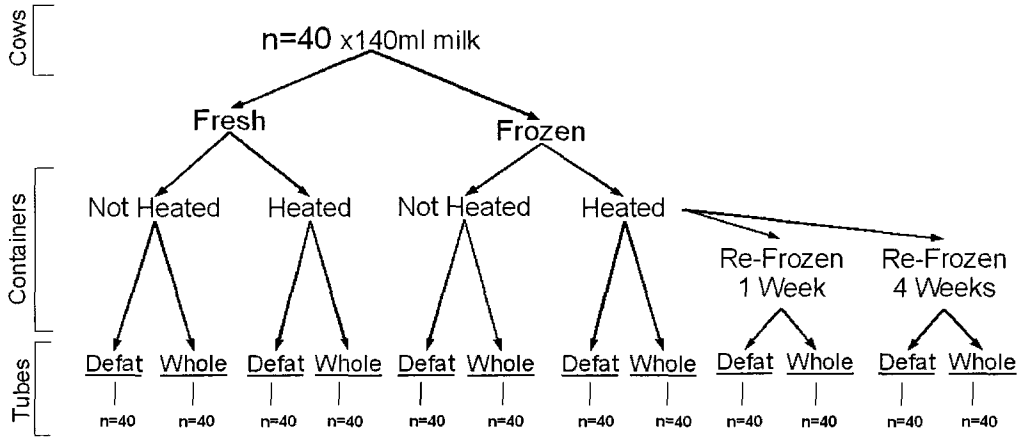

Figure 3.2. Flow chart describing the study design. One hundred and forty milliliters of fresh milk was taken from 40 cows, and subdivided into containers with 6 heat-freeze combinations. Within each combination, the milk was further subdivided into tubes and was either defatted or whole. There were forty ODR values for each of the 12 stressor effects.

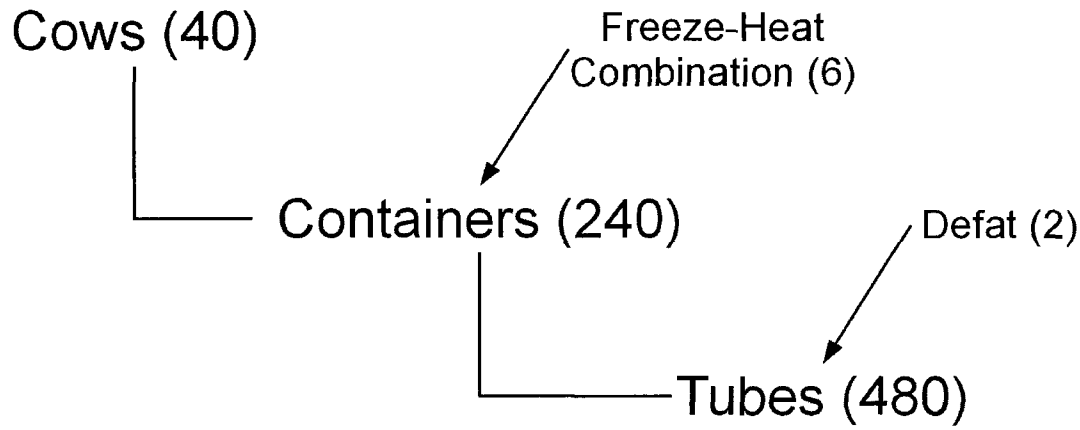

Figure 3.3. Hierarchical structure of the data. There were 40 cows with milk subdivided into 6 containers -- each container underwent a freeze-heat combination. Each combination was subdivided into two tubes either designated to be defatted or left as whole milk.

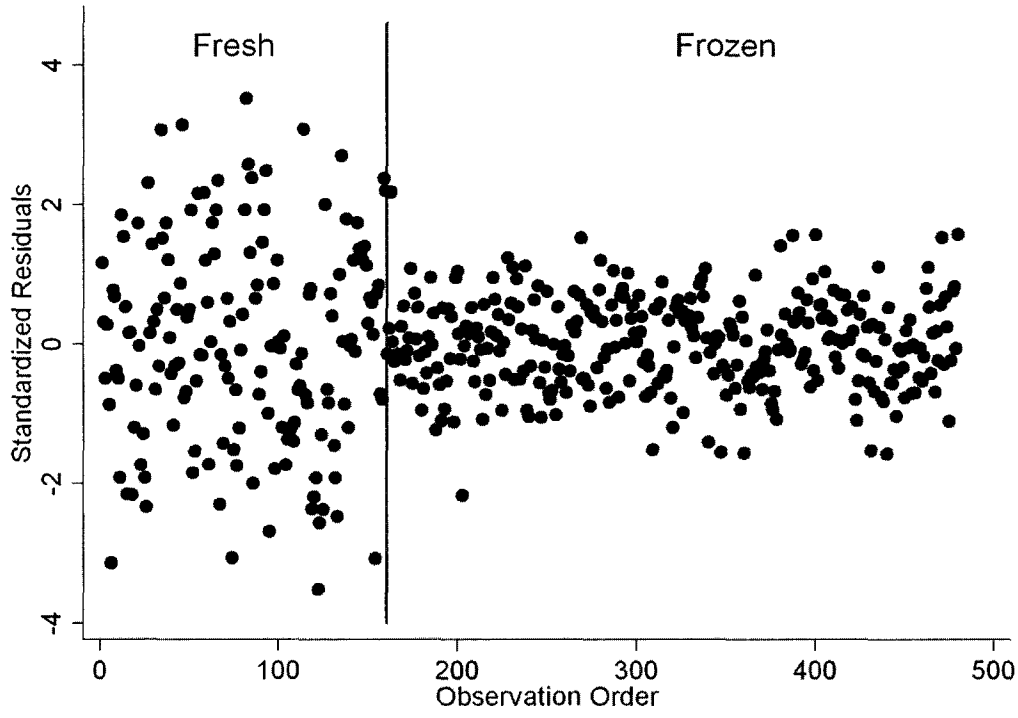

Figure 3.4. Lowest level residuals from the linear mixed model assuming equal variance for both fresh and frozen samples. The observations were ordered such that fresh samples are between observations 1 and 160 (marked by a vertical line), and frozen samples are between observations 161 to 480.

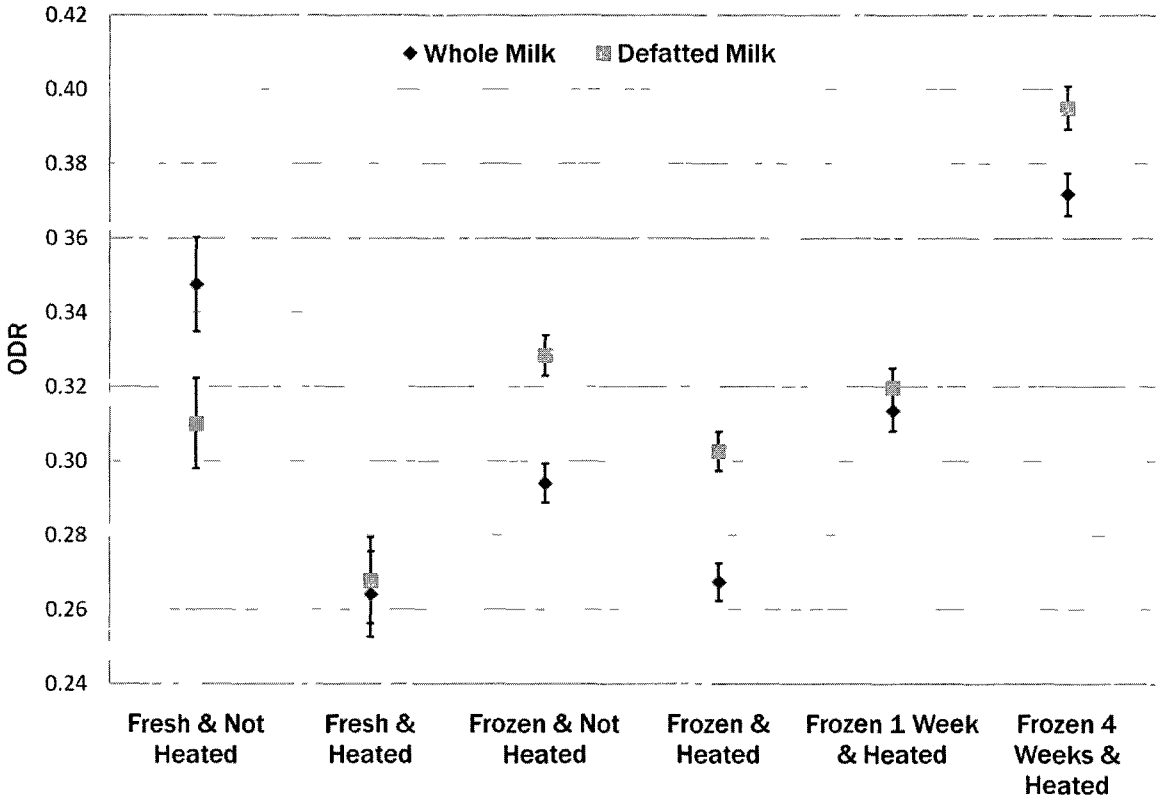

Figure 3.5 Estimated ODR values (with 95% confidence intervals) **from the linear mixed model** for all combinations of stressors in experiment.

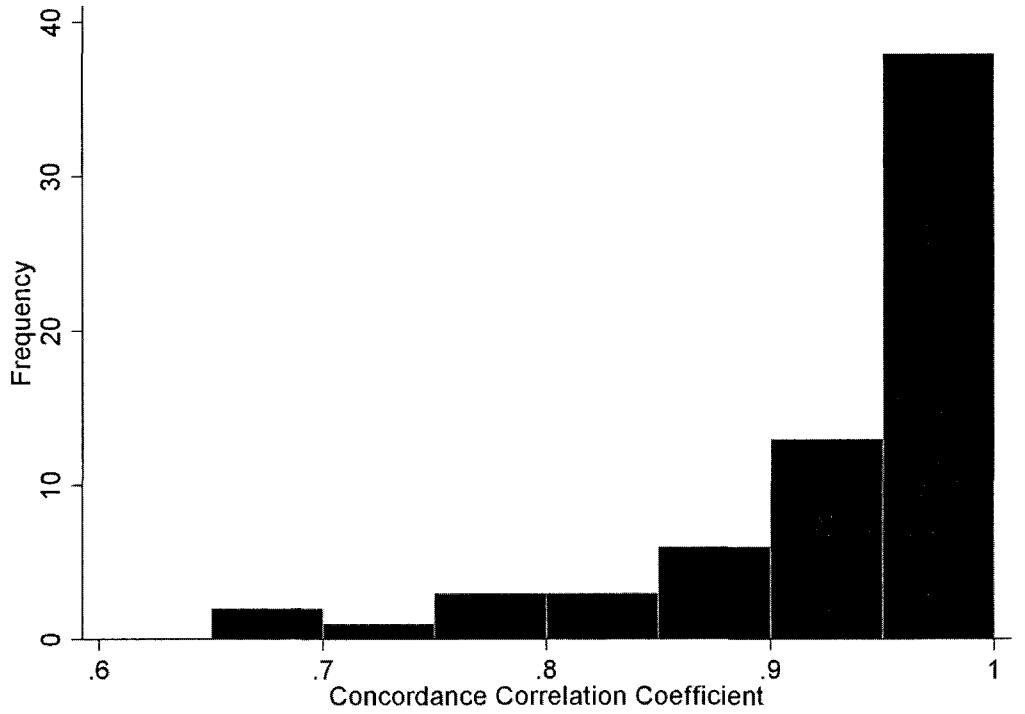

Figure 3.6. Distribution of CCCs between all 66 palrwise comparisons of the 12 stressor effects.

Chapter 4

Applying a Kinetic Method to an Indirect ELISA Measuring *Ostertagia ostertagi* Antibodies in Milk

4.1. Introduction

Svanovir® (Svanova Veterinary Diagnostics, Uppsala, Sweden) is an indirect enzyme-linked immunosorbent assay (ELISA) test commercially available and currently used in Europe to determine levels of parasitism. Svanovir® measures antibodies against *Ostertagia ostertagi* in milk from dairy cattle. *O. ostertagi* is a common intestinal nematode, ubiquitous in the temperate climatic zones of the world (Gibbs, 1988; Louw, 1999; Williams *et al.*, 1993) that has detrimental effects on milk production (Charlier *et al.*, 2005a; Gibbs, 1988; Guitián *et al.*, 2000; Sanchez *et al.*, 2002a). When the antigens used to coat the ELISA wells are derived from crushed whole nematodes, such as in Svanovir®, cross-reactivity can occur with other nematodes such as *Cooperia spp.* (Dohoo *et al.*, 1997; Eysker and Ploeger, 2000; Kloosterman *et al.*, 1984). Thus, a higher result from the Svanovir® test is interpreted as a high level of intestinal parasitism, not an infection with *O. ostertagi* specifically. As such, Svanovir® may be used as a tool to predict the amount of milk production loss due to an undetermined level of parasitism at the individual cow or herd level (Charlier *et al.*, 2005a; Sanchez *et al.*, 2002a).

Svanovir® is an indirect, endpoint ELISA (e-ELISA). An e-ELISA allows the substrate to react and change color for a set amount of time before a stop solution is added, arresting the chromatogenic reaction. The plate is read by a spectrophotometer within a few minutes of adding the stop solution, to determine the samples' optical density (OD).

An e-ELISA has some disadvantages. Firstly, the addition of a stop solution does not necessarily arrest color change. Even though the stop solution terminates the enzymatic reaction, the chemical reaction can continue without functional enzymes (Bullock and Walls, 1977). Additionally, the relationship between endpoint color intensity and antibody level need not be linear, especially for extreme levels, as observed with standard curves (Engvall and Perlmann, 1972; Pesce *et al.*, 1974). Lastly, the chemical reaction is only approximately linear with the enzymatic level in the well during a brief period at the initial phase of the reaction and provided there is an abundant amount of substrate (Tsang *et al.*, 1980). Therefore, an e-ELISA is incapable of distinguishing between a mild and severe increase in antibody level when it lies in the upper regions of the linear scale of the OD, unless the sample undergoes predetermined dilutions. On the other hand, a kinetic ELISA (k-ELISA) can make that distinction.

An e-ELISA can become a k-ELISA if the OD is recorded at regular short intervals (e.g. 45 seconds) starting as soon as the chromatogenic reaction begins. A k-ELISA does not require stop solutions, thus eliminating the problem of continued color change. The measurements in a k-ELISA are taken in real-time, allowing the necessary information to be gathered much sooner than an e-ELISA. In theory, k-ELISA results can quantify the initial approximate linear enzymatic reaction and thus be a truly quantitative test (Tsang *et al.*, 1980).

A normalization method to reduce variation between plates is often used for e-ELISAs (including Svanovir®), and reported as optical density ratios (ODRs) (Charlier *et al.*, 2005b; Sanchez *et al.*; Sanchez *et al.*, 2002b; Sithole *et al.*, 2005; Vanderstichel *et al.*, 2010). ODRs quantify the samples as a percentage between the negative and positive control values. The ODR is calculated as follows:

$$ODR = \frac{(OD_{sample} - OD_{negative})}{(OD_{positive} - OD_{negative})}$$

The ODs measured at predetermined intervals in k-ELISA can be similarly normalized. However, k-ELISA results are usually presented as slopes, not as OD. We propose that the application of a similar normalization equation to the slope will result in a slope ratio (SR).

The advantages of the k-ELISA motivated the investigation into whether Svanovir® can be run as such. The objectives of this study were: 1) determine whether it is possible to run both a k-ELISA and an e-ELISA technique on the same plate without interfering with e-ELISA results, 2) establish an appropriate specific time interval and duration for the k-ELISA measurements in Svanovir®, and 3) understand the relationship between the e-ELISA and the k-ELISA results from Svanovir®.

4.2. Material and Methods

4.2.1. Svanovir® ELISA Test

The milk samples were acquired from on-farm Dairy Herd Improvement programs as part of a larger study described in Chapters 5, and were run according to the manufacturer's specifications, as indicated by Svanovir®'s instructions provided in the kits. The samples were conveniently chosen for this study, from the larger study, by using the milk samples that happened to be the next sequential samples to be tested. Positive, negative and blank controls were run in triplicates. Both the positive and the negative controls were included in the kit. Instructions specific to e-ELISA were: Step 8) add 100µl of ABTS substrate solution (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]- diammonium salt) to each well, and incubate for 30 minutes in the dark at room temperature; Step 9) stop the reaction by adding 50µl of stop solution (1% Sodium Dodecyl Sulfate) to each well and mix thoroughly, and add the stop solution in the same order as the substrate solution in Step 8; and finally Step 10) shake the plate thoroughly and measure optical densities at 405nm and 492nm in a spectrophotometer.

For a k-ELISA, the first seven steps were performed as recommended by the manufacturers, however, the plate was placed directly in the spectrophotometer (405nm and 492nm wavelengths) after the addition of the substrate in Step 8. The spectrophotometer (SpectraMax) and software (SoftMax) were programmed to shake (3 seconds) before every reading and

read the plate every 45 seconds, until the end of the program – shaking homogenizes the color within each well. SoftMax automatically calculates slope values for each well at the end of the k-ELISA program; however, slopes can be derived from regression coefficients between the OD and time for each well.

4.2.2. Study Design

4.2.2.1. Effect of k-ELISA methods on e-ELISA results

To investigate the effect that a k-ELISA method might have on e-ELISA results on the same plate, a total of six plates (3 pairs) with 96 wells were used (n=276 wells), and each pair of plates was identical, it had the same controls and milk samples repeated in their respective wells (i.e. well 1 in the first plate had the same milk sample as well 1 in the second plate). Within each pair of plates, one plate underwent a kinetic process for 15 minutes (including repeated programmed shaking for 3 seconds), and after an additional 15 minutes the stop solution was added and the endpoint ODR (eODR) was recorded (30 minutes after the initial reaction started). The corresponding plate in the pair underwent a standard e-ELISA as described by Svanovir®'s manufacturers, giving a total of 261 paired sample observations.

4.2.2.2. Validity of a 15-minute k-ELISA

In order to confirm that 15 minutes of k-ELISA were sufficient for accuracy, a separate single plate (different to the ones used in 4.2.2.1) with samples was

allowed to undergo the k-ELISA procedure for 28 minutes before the stop solution was added (at 30 minutes) to complete the e-ELISA.

An equation to reduce plate to plate variation (Sanchez *et al.*, 2002b) was used to normalize slope results, yielding a slope ratio (SR):

$$SR = \frac{(Slope_{sample} - Slope_{negative})}{(Slope_{positive} - Slope_{negative})}$$

4.2.2.3 Comparison of Results from k-ELISA and e-ELISA Methods

Finally, to understand the relationship between the e-ELISA and the k-ELISA results from the Svanovir® ELISA test, a total of 27 plates underwent both methods of testing. Each plate started with the k-ELISA procedure for 15 minutes, and ended with the standard e-ELISA procedure at 30 minutes, giving results for both real-time kinetic and endpoint ELISAs. To evaluate whether results of a k-ELISA and an e-ELISA (endpoint 30 minutes) were comparable, the CCC between the slope ratio (SR) of the k-ELISA and eODR of the e-ELISA was calculated. Additionally, to explore other time points for the Svanovir® k-ELISA, a concordance correlation coefficient (CCC) analysis between the eODR and SR at various other time points was performed in these 27 plates.

The coefficient of variance (CV=σ/μ) for the slope ratios in the k-ELISA was graphed by ten percentile groups to explore the variation at the lowest and

highest ends of the endpoint ODs.

4.2.3. Statistical Methods

Concordance correlation coefficients, determined using Stata10.1 (2008), were used to evaluate the agreement between two series of continuous measurements. Values close to 1 indicated very good agreement while values approaching zero reflected very poor agreement (Dohoo *et al.*, 2009). Within the CCC analysis is a Bradley-Blackwood F-test to compare the mean and the variance between the two series (Bradley and Blackwood, 1989), where $p<0.05$ indicates that either the mean or the variance (or both) are unequal between the series.

Simple linear regressions to determine slope estimates for each series of wells during the kinetic analysis were modeled with Stata10.1.

4.3. Results

4.3.1. Effect of k-ELISA Methods on e-ELISA Results

A total of 260 pairs of wells from the 6-paired plates were included in the CCC analysis (one of the wells was contaminated and had to be removed from the study). The CCC between eODRs from kinetic and endpoint series was 0.953 with no significant difference of mean or variance between the two series ($p=0.195$, Bradley-Blackwood F-test). A histogram of the ODR values for the standard e-ELISA plates (Fig. 4.1) shows the right-skewed distribution of the ODR values from the conveniently chosen milk samples. The mean, median, and standard deviation for the ODR values are 0.238, 0.177, and 0.216,

respectively, with a range between -0.188 and 1.157.

4.3.2. Validity of a 15-minute k-ELISA

Figure 4.2 shows the OD in real time over 28 minutes for each individual well and for the average of the plate. After 28 minutes, it appeared the wells had not reached their maximum ODs and would have continued to rise. Based on a linear regression model predicting OD readings and accounting for the quadratic relationship of time, the estimated time to reach a plateau would be approximately 50 minutes. The overall slope decreased with time, for instance, the slopes at 15 and 28 minutes were 1.15×10^{-2} and 0.98×10^{-2} OD units/minute, respectively. This time-dependency in the slopes makes it necessary to normalize the slopes before evaluating the CCC between the values at any time interval and the eODR (30 minutes). Normalization reduces the between-plate variation, and a component of the plate-to-plate variation is probably due to different stop times.

Bland and Altman (1986) plots (Fig. 4.3) were created to compare eODRs with SRs at 15 minutes, the time point presumed for the Svanovir® k-ELISA, and two other extreme time points (3 and 28 minutes). The CCCs between eODR and SR at 3, 15 and 28 minutes were 0.997, 0.999 and 0.999, respectively. Corresponding *p* values for the Bradley-Blackwood F-tests were 0.051, 0.177 and <0.001, respectively.

4.3.3. Comparison of Results from k-ELISA and e-ELISA Methods

The CCC between the SRs of the k-ELISA at 15 minutes and the eODRs of the e-ELISA was 0.946, indicating excellent agreement between the two measurements. The CCCs of the SRs versus eODRs were initially low and increased with time (Fig. 4.4, right). As an example, the CCC values were less than or equal to 0.8560 before 5.25 minutes and greater than or equal to 0.9443 after 10.5 minutes (indicated with arrows in Fig. 4.4, right).

The final plot (Fig. 4.5) demonstrates the CV of slope ratios by percentile categories of the endpoint ODs (divided into 10% increments) for 2.25 and 10.5 minutes. The CV values remained constant for time intervals greater than and equal to 10.5 minutes.

4.4. Discussion

4.4.1. Effect of k-ELISA Methods on e-ELISA Results

The CCC was very high and there were no differences in means or variance between the pairs of series (p=0.195, Bradley-Blackwood F-test). Therefore, it is reasonable to assume that k-ELISA methods have no effect on the endpoint ODR, so that k-ELISA and e-ELISA can be safely performed on the same plate with minimal influence on e-ELISA results.

4.4.2. Validity of a 15-minute k-ELISA

Integrating the fifteen minutes of k-ELISA within Svanovir®'s endpoint protocol was seamless. To confirm that 15 minutes was enough, however, one plate was programmed to run for 28 minutes, allowing 2 minutes to remove the

plate from the spectrophotometer and add the stop solution before the end of the endpoint's 30-minute incubation. SRs from the k-ELISAs at 15 minutes were nearly identical to the eODR values (CCC=0.999) for this plate, thus a k-ELISA program set for 15 minutes should yield satisfactory results. The Bland-Altman plot (Fig. 4.3) shows how the agreement between eODR and SR changed as time progresses. The variation in the difference between eODR and SR decreased with time, as seen with smaller 95% limits of agreement intervals. The 95% limits of agreement interval at 15 minutes was empirically better than at 3 minutes, but only marginally lower at 28 minutes, suggesting that a 15-minute k-ELISA would be an adequate method for Svanovir®.

Although the ODs increase as time passes in a k-ELISA, the slopes actually decreased as time progressed; in other words, the ODs increase at a continuously decreasing rate. Using a regression model, it was calculated that they would continue to decrease until leveling off at approximately 50 minutes. This illustrated that the e-ELISA stops the reaction before its natural completion. Because the stop solution may fail to arrest the chemical reaction or color change, the raw OD will vary somewhat depending not only on when exactly the stop solution is added, but when the reading is performed. This stresses the importance of normalizing the values, as specified in the Svanovir® manufacturer's instructions.

While running the 28-minute plate, it was noticed that ODs recorded at 30 minutes, after the stop solution was added, were lower than ODs at 28

minutes. Given that ODs should have continued rising until 50 minutes had elapsed (based on the regression model mentioned above), the 30-minute ODs would be expected to be higher than the 28-minute ODs. The decrease in ODs at 30 minutes was artificial, due to the addition of 50µl of transparent stop solution. The stop solution diluted the concentration and thus influenced the ODs. One could explain this theoretically by using the Beer-Lambert Law which states that $OD \equiv A_\lambda = k\,c\,l$, where A_λ is the absorbance, k is a constant for the chemical species, c is the concentration of the light absorbing species, and l is the length of the light path. In the case of fixed wells (fixed light distances) and the same chemical species added to all wells (k), the OD would then change in proportion to the concentration of the chemical species (c), and the dilution from the stop solution would change the concentration factor in a uniform fashion, affecting all ODs proportionally. Alternatively, one could remember that normalization equations cancel out any uniform effect on all samples, because the controls are equally affected in the same plate – normalized OD values are ODRs, and normalized slopes are SRs.

4.4.3. Comparison of Results from k-ELISA and e-ELISA Methods

Figure 4.4 shows the comparison of CCCs for ODR versus eODR (left) and SR versus eODR (right) for all time points. The SR appeared more stable than the ODR for each time point recorded, probably because the slope was 'additive', acquiring past information for the calculated estimate as time progresses. This is different to the ODR, where the estimate was derived from a specified

time point, without any past information – an instant in time. For this reason, it is recommended to use SRs rather than ODRs when using k-ELISAs, provided SRs are measured for at least 10 minutes. The data also suggests that k-ELISA could be reduced to as little as 10 minutes without losing information; therefore, switching from an e-ELISA to a k-ELISA in the Svanovir® test could reduce the total time by as much as 20 minutes.

Tsang *et al.* (1980) theorized that k-ELISA quantifies the initial approximate linear enzymatic reaction only when the proportion of substrate concentration greatly surpasses that of the enzymes. This theoretical condition has the potential, in the extreme ends of the OD scale and only initially, to further differentiate between those samples that have very high levels of antibodies with those that have high levels, but which have similar elevated ODs on the linear scale. If this is true, there would be, potentially, no need to dilute samples with high antibody levels if they could be quantified by their initial slopes.

The properties of ODs make it so that variation is dependent on the value of the OD, where larger OD values have larger variation. One method to standardize these values is to categorize them by their percentile groups (increments of 10%) and record the coefficient of variation (CV); this is achieved by taking the ratio between the standard deviation of the OD values within that of the percentile group's mean OD. Assuming the theories proposed by Tsang *et al.* (1980) are correct, it was anticipated that the

extreme percentile categories (<10 and >90) would have the greatest amount of variance for the initial time points (1.5 and 2.25 minutes), however, variation continue to drop after the 20th percentile (Fig. 4.5). By 10 minutes, the overall variance across all percentile groups was dramatically reduced. One reason for a low variance within the highest percentiles of these data may be due to the fact that overall, our sample OD values were not very elevated when compared to their controls or even from samples taken from other populations with higher levels of infection, such as in Europe. Our average eODR for the 27 plates was (0.272) when, depending on the season, it is common to have means above 0.60 in Belgium with the same test (Charlier *et al.*, 2007). Interestingly, serum samples have higher levels of antibodies than milk, and may therefore benefit from k-ELISA readings, although Svanova has designed the ELISA test strictly for milk samples. Though it would be interesting to investigate the initial slopes of samples with much higher ODs and compare them to their eODRs, we would also have to anticipate more variance within the lower OD values. Figure 4.4 shows increased variances of SRs for eOD values in the lower percentile categories for initial time points. This may be due to the nature of small ODs, where tiny changes have a large impact on minuscule values, compounded by the fact that slopes are calculated on very few values. For a slope to be calculated, a minimum of three values are needed, hence the first possible measurement for a slope is at 1.5 minutes, if the k-ELISA is programmed to measure every

45 seconds.

In theory, the initial few readings from a kinetic ELISA have the ability to differentiate between the high OD samples (i.e. low vs. high range within elevated values). This relationship could not be analyzed with these data because our antibody levels were too low. As seen with these data, if initial values were to be used, the lower OD slopes would probably have more variation than expected, though their impact on the overall measurements would be minimal.

4.5. Conclusion

There are certain advantages of using a k-ELISA over a more traditional e-ELISA. Our results show that it is possible to run a real-time k-ELISA and an e-ELISA on the same samples without affecting the final e-ELISA results. Kinetic ELISAs require less time than endpoint ELISAs; in the case of the commercial test Svanovir®, a k-ELISA can reduce test time by 15 to 20 minutes and does not require a stop solution. Our results support using normalized k-ELISA slopes by transforming them into slope ratios (SRs).

Although milk used in this study did not allow for the investigation of the differentiation between large and very large levels of antibodies, the increased coefficient of variance (CV) found in the lower end of the ODs support the suggestion that k-ELISAs would be able to detect subtle differences at both extremes of the OD scale.

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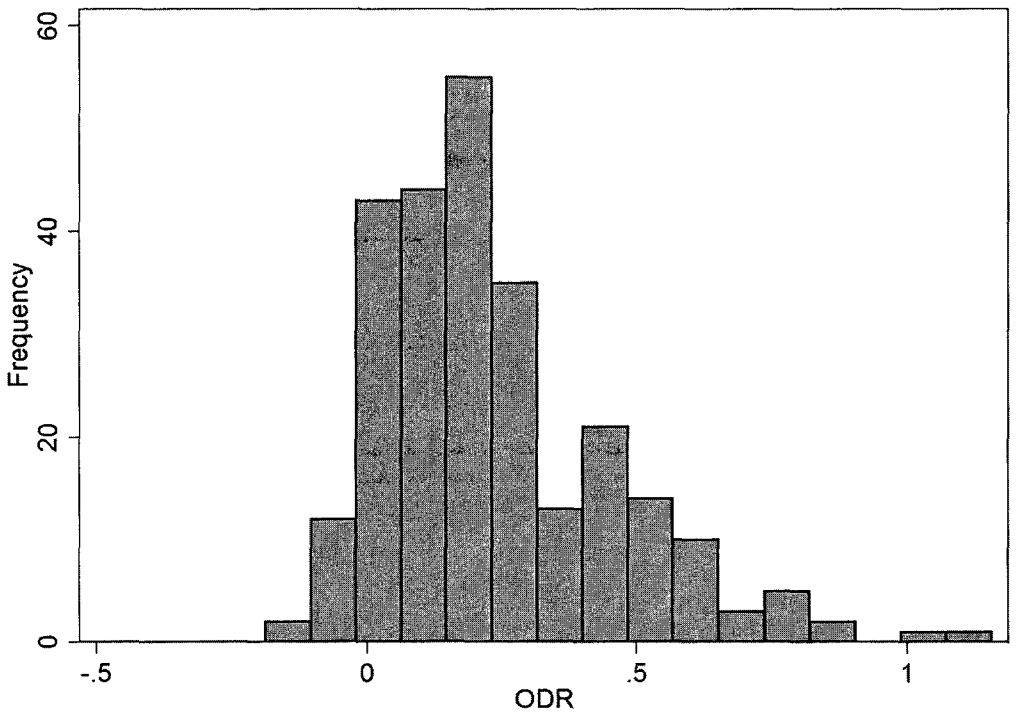

Figure 4.1. Histogram of the ODR values for the standard e-ELISA procedure *to assess the effect of k-ELISA methods on e-ELISA results.*

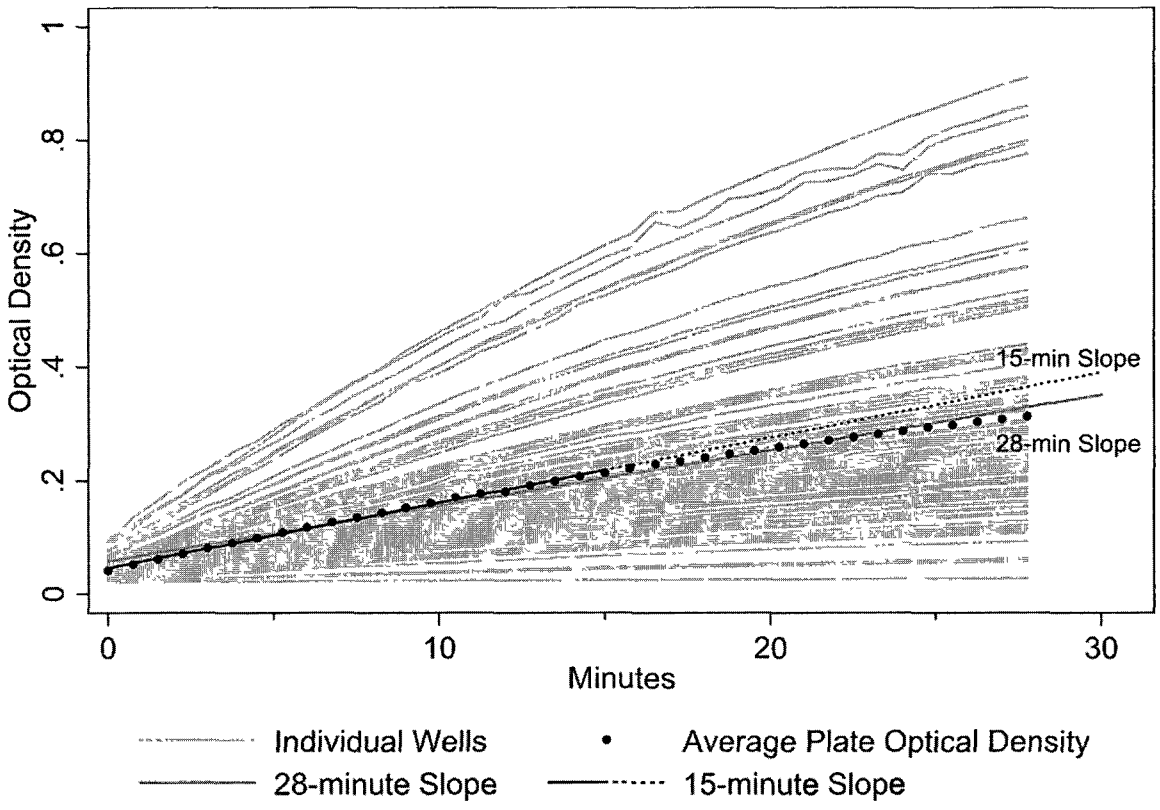

Figure 4.2. Optical densities from k-ELISA procedures for all wells within the 28-minute plate.

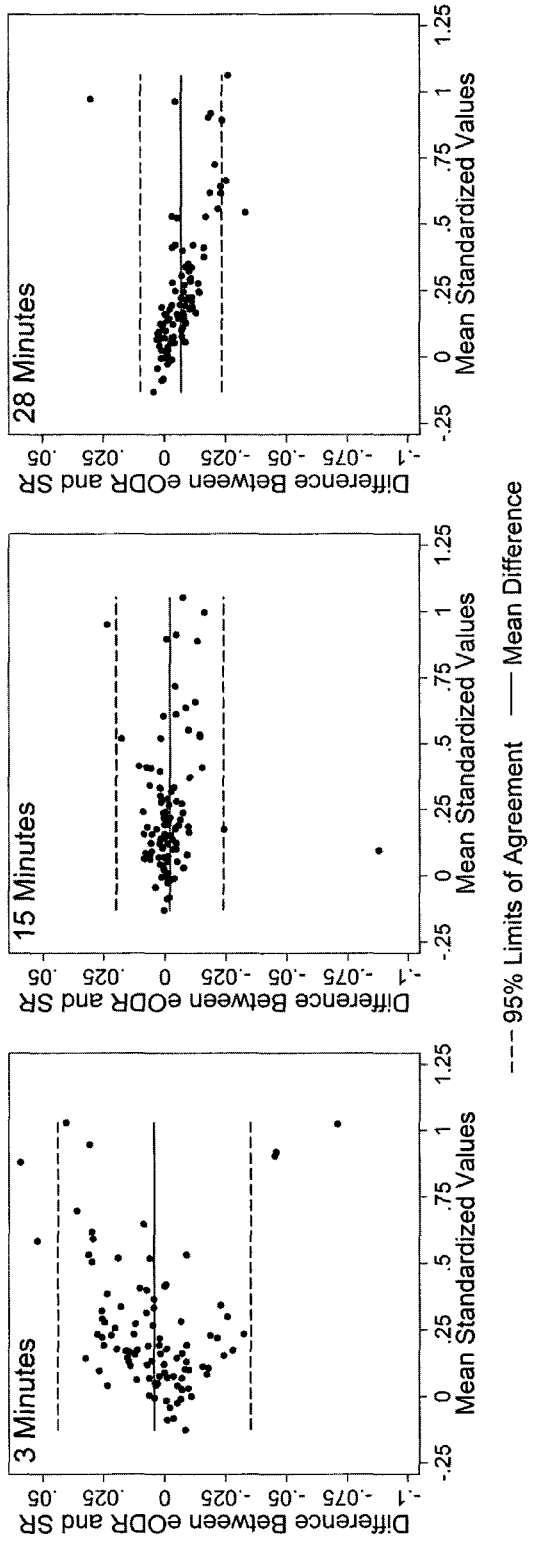

Figure 4.3. Bland-Altman plots of the limits of agreement between SR and eODR for 3, 15, and 28 minutes.

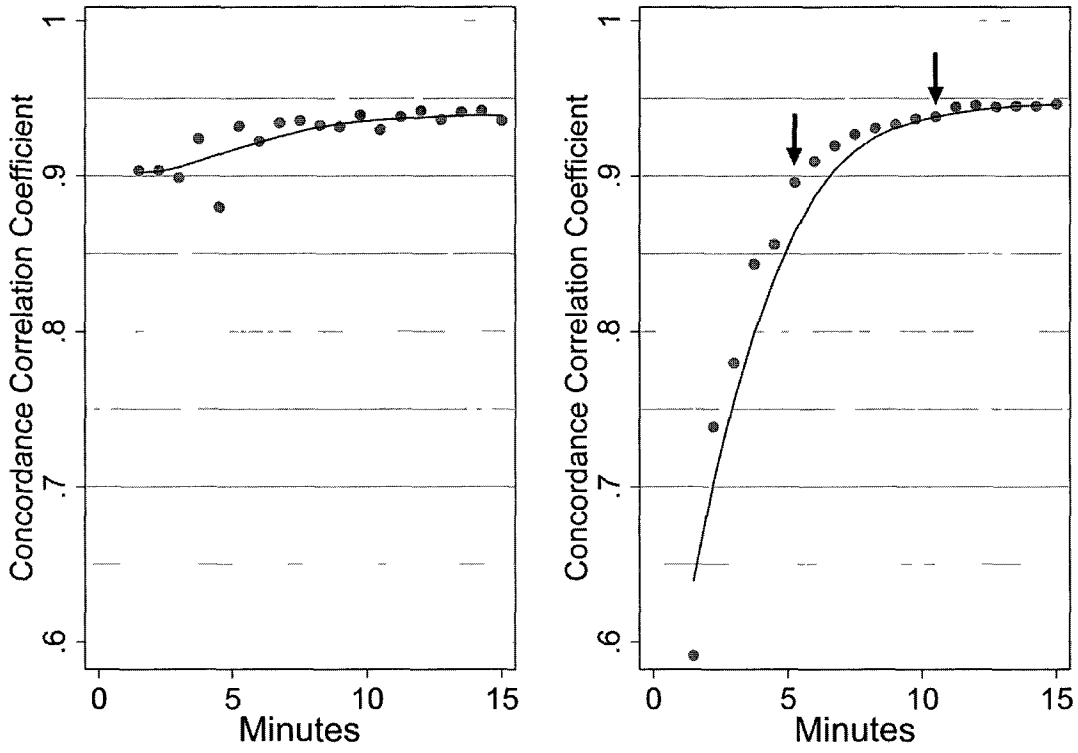

Figure 4.4. Concordance correlation coefficients between ODRs and eODRs (left) **and between SRs and eODRs** (right) for readings from 1.5 to 15 minutes for 27 plates (arrows point to CCCs for 5.25 and 10.5 minutes).

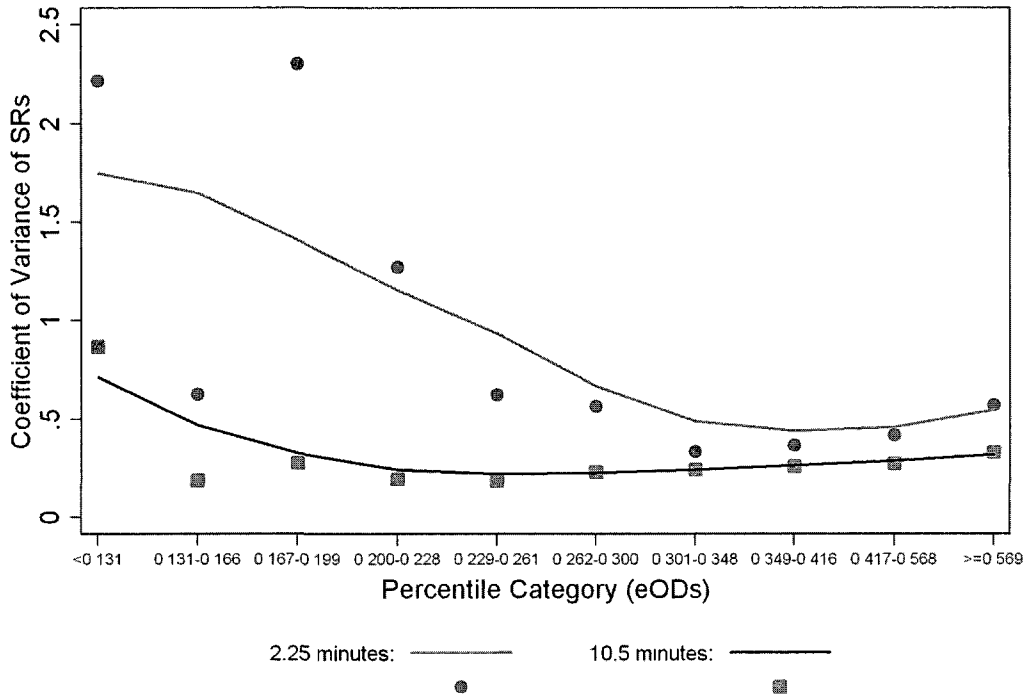

Figure 4.5. Coefficient of variance of SRs by eOD percentile category (in 10% increments) **for 2.25 and 10.5 minutes.** Lowess lines are shown for both times (2.25 and 10.5 minutes).

Chapter 5

Predicting the Effect of Anthelmintic Treatment on Miik Production of Dairy Cattle in Canada Using an *Ostertagia ostertagi* ELISA from Individual Milk Samples

5.1. Introduction

Gastrointestinal nematodes, such as *Ostertagia ostertagi* and several species of *Cooperia*, are ubiquitous in temperate climates (Gibbs, 1988; Louw, 1999; Williams *et al.*, 1993). They have been primarily considered as a production limiting disease in first-season grazing animals, though in the past decade, more evidence has demonsted their detrimental effects on production in adult dairy cattle. In a recent meta-analysis, Sanchez *et al*. (2004) demonstrated that overall, producers lose approximately 0.35 kg of milk per parasitized cow per day. The study included 75 studies worldwide with many different drugs, and levels of pasture exposure. The fifteen studies evaluating the use of ivermectin, moxidectin or eprinomectin averaged 301 cows and a beneficial improvement of 0.8 kg/cow/day. Of those studies, seven had significant differences with an overall estimated difference of 0.97 kg/cow/day.

Svanovir® (Svanova Veterinary Diagnostics, Uppsala, Sweden) is an indirect enzyme-linked immunosorbent assay (ELISA) test commercially available and currently used in Europe to determine levels of gastro-intestinal parasitism. The ELISA measures antibodies against *Ostertagia ostertagi* in milk from dairy cattle using whole crushed worms, which inherently increases cross-reactivity with other nematodes (i.e. *Cooperia spp.*) (Dohoo *et al.*, 1997; Eysker and Ploeger, 2000; Kloosterman *et al.*, 1984).

The ELISA test has demonstrated some predictive abilities as a tool to estimate the amount of milk production loss due to an unknown level of

parasitism at the individual and herd level(Charlier *et al.*, 2007; Sanchez *et al.*, 2002a; Sithole *et al.*, 2005). The objective of this study was to use Svanovir® ELISA results on individual milk samples from cows, in both semi-confined and pastured dairy herds in Canada, to predict milk production response following anthelmintic treatment of individual cows.

5.2. Material and Methods

5.2.1 Comparable Studies in Literature

In the literature, the study designs and methods for comparable studies appear to differ, displaying large heterogeneity amongst studies. To compare and discuss studies in the literature with findings from this study, a search for all clinical trials (anthelmintic vs. placebo) using anti-parasite antibodies (individual or bulk tank) as a predictor for the effect of milk production in dairy cattle was performed. References were derived from the meta-analysis performed by Sanchez *et al.* (2004), and updated with an online literature search using CAB Abstracts and Medline databases. The search included articles from 2002 to the present (February 2010) with the following sets of keywords: *(anthelmintic dairy cattle), (milk production nematodes), (milk production anthelmintic), and (dairy cows dairy herds anthelmintics).*

5.2.2. Herd and Animal Selection

Producers in the National Cohort of Dairy Farms (NCDF) study carried out by the Canadian Bovine Mastitis Research Network (CBMRN), a network of farms, laboratories, and researchers investigating mastitis in Canadian herds,

were contacted. Herds participating in the NCDF represented typical Canadian commercial dairy farms, based on provincial milk production levels, specified distributions of bulk tank somatic cell count (SCC), and housing types (Reyher *et al.*, 2010). Farms were included in this study if either milking cows, dry cows or heifers had access to pasture or a grassed paddock during the year, to ensure some levels of exposure to infective nematode larvae. Within the NCDF, forty farms allowed their cattle access to pasture or paddock. The desired sample size was to include approximately 45 herds with an estimated 3,000 cows; the sample size was determined from simulations based on two previous studies (Sanchez *et al.*, 2002a; Sithole *et al.*, 2005), and accounted for clustering effects from the herds. Specifically, the partial correlations for the interaction terms (ODR and treatment) in the final model from the two studies were 0.07 for Sanchez *et al.* (2002a), and 0.06 for Sithole *et al.* (2005). A simulation using a hypothetical dataset with two variables correlated at r=0.07 was generated. Random samples of 1,000 cows were obtained and the 5th and 95th percentile of the regression coefficient were obtained – this process was repeated ten times, and the results were averaged. The entire process was repeated for random samples of 2000, 3000, and 4000 cows. The simulation determined that 2,000 cows would provide adequate estimates, however, 3000 cows would increase the precision of the estimate and account for likely subject drop-outs.

5.2.3. Sampling and Milk Collection

The procurement of on-farm milk samples came from both CBMRN and routine Dairy Herd Improvement (DHI) collections, between March 1, 2007 and April 30, 2008. DHI programs provide producers monthly records of milk production, milk quality, and reproduction parameters. Samples were either frozen (-20°C) or refrigerated (2 to 4°C). A previous study found that milk handling procedures, necessary for transportation, storage, and DHI testing, did not have any impact on ELISA results (Vanderstichel *et al.*, 2010). Samples were identified and barcoded by either the CBMRN or the DHI companies – Valacta (formerly ADLIC in the Maritimes) and CanWest. These samples were later matched with their corresponding cow, collection date and respective production data from the Canadian DHI database, Vision2000.

5.2.4. Measuring Parasitism (ELISA)

Milk collected from cows during their late lactation period (>200 days in milk) were processed to quantify *O. ostertagi* antibodies using a commercial ELISA kit, Svanovir®, and were tested according to the manufacturer's specifications. Samples were tested once as recommended by Sanchez *et al.* (2002b), however, positive, negative and blank controls were run in triplicates for each plate – both positive and negative controls were supplied. The spectrophotometer (SpectraMax) and software (SoftMax) were programmed as recommended by Svanova. Optical densities (ODs) were exported into electronic text files, matched with their corresponding barcodes, and finally merged with their respective cow data. All ODs within ELISA plates were

normalized, producing optical density ratios (ODRs), using the mean from the triplicate positive and negative controls from their respective plate. This commonly used normalization method reduces plate-to-plate variation, and relates values from samples to their standardized controls (Charlier *et al.*, 2005; Sanchez *et al.*, 2002b; Vanderstichel *et al.*, 2010):

$$ODR = \frac{(OD_{sample} - OD_{negative})}{(OD_{positive} - OD_{negative})}$$

5.2.5. Treatment

All cows calving between May 1, 2007 and May 31, 2008 received one dose of either eprinomectin (Eprinex®, 65ml = 325mg) or mineral oil (65ml, acting as placebo) applied along the backline from the withers to the tail head, near the time of parturition (2 weeks pre- to 3 days post-parturition). Each bottle was numbered and the treatment allocation was randomized using systematic randomization to assign odd or even bottle numbers to a treatment group. Producers administered the treatments sequentially, unaware of the contents within the bottle, and recorded the bottle number, cow identification, and calving date. This information was later merged with the production records and respective ELISA results. Producers were asked, when possible, to keep treated cows apart for as long as they could after treatment (ideally more than 24 hours); there is evidence that macrocyclic lactones (such as ivermectin, doramectin, moxidectin, and eprinomectin) can be transferred mechanically from cow-to-cow via grooming and licking (Barber and Alvinerie, 2003; Laffont

et al., 2001). Specifically, Barber and Alvinerie (2003) described mechanical transfer in all treatment groups in their study, including eprinomectin treated cattle. It is worth noting that Alvinerie *et al.* (1999) found systemic absorption, as measured by the half-life of absorption ($t_{½}$ ka) for eprinomectin to be twice as fast than what is documented in literature for ivermectin and doramectin. Although mechanical transfer is possible for eprinomectin, it is likely to be less than what would be expected for other macrocyclic lactones because of the faster absorption, however, there are no published studies describing this specific difference.

Only the primary investigators involved had knowledge of the randomization protocol.

5.2.6. Questionnaire

All participating dairy producers completed a 'parasite' questionnaire about anti-parasitic treatments (before and during the study), pasturing techniques, housing of milking cows, dry cows and heifers, and the length of time treated cows were kept apart after treatment (see Appendix B). This questionnaire was part of a larger questionnaire (CBMRN questionnaire) for all herds within the NCDF. One question included in the parasite questionnaire was used as a validation tool for the CBMRN questionnaire, as described by Dufour *et al.* (2010); the questions in each questionnaire were posed, on average, 262 days (SD 75) apart. These validation results from the CBMRN questionnaire also reflect the validity of the parasite questionnaire.

5.2.7. Statistical Analyses

Unless otherwise stated, all statistical analyses, including summary and descriptive statistics, were performed in Stata11 (2009).

Heifers and cows were categorized according to their treatment status, and their completeness of information for ELISA test results. A flow chart was created to summarize their status within the study and subsequently for the analysis (Fig. 5.1).

5.2.7.1 Multivariable Mixed Analysis

5.2.7.1.1 Repeated Measures and Random Effects

Statistical models included only those cows for which all information was collected (Groups F and I in Fig. 5.1). A multilevel mixed-effects linear regression, with test day milk yield (kg/cow/day) as the dependent variable, was fit using maximum likelihood methods (Stata 11, xtmixed; SAS, Proc MIXED), with structured residual errors between repeated milk measurements. Unstructured (UN), auto-regressive (AR1), and moving-average (MA) residual structures were computed with Stata, while auto-regressive moving-average (ARMA), and ante-dependent (ANTE) residual structures were computed with SAS9.1 (2003). There were two random effect variables (herd and cow), and a residual structure between DHI test dates, creating a 3-level hierarchy to the analysis – herds, cows, and test dates (repeated cow milk yields within lactations).

5.2.7.1.2. Fixed Effects

Milk production variables were included to control for herd, cow, and test date effects on milk production. The variable for time period and seasonal effects was divided into trimesters throughout the study period ('Housed' = January 15th to May 14th; 'Grazed' = May 15th to September 14th, and 'Shoulder' = September 15th to January 14th), starting May 1, 2007 and ending December 31, 2008, giving a total of 5 trimesters. This allowed for differences in milk production due to varying seasons and years. The dates used to define the three trimesters (Housed, Grazed, and Shoulder) were based on grazing seasons in Canada, where cows are typically turned out to pasture in the middle of May and the first frost usually occurs after the middle of September; the 'shoulder' period is a transitional period between fully grazed and fully housed. Cow-level variables included both calving season (three categories, using the same yearly trimesters as the seasonal effects), and lactation number (separated into three categories; 2nd, 3rd, and ≥4th lactations). Variables related to testing dates and affecting milk yields were milk somatic cell counts (SCCs), and days-in-milk (DIM). SCCs were transformed to a natural logarithm scale (lnSCC) to linearize their effect (Dohoo *et al.*, 2009). The relationship between DIM and milk yield was assumed to follow Wilmink's function (Schaeffer *et al.*, 2000): $Y=DIM+DIM^{-0.05}$, where Y is the 24-hour milk yield in kg/cow/day; the first DIM term was centered to reduce collinearity between the two terms, and the second DIM was computed from the original DIM variable.

The responses from the 'parasite' questionnaire were also added as potential fixed effect variables. A stepwise selection process, including any variables with a *p* value <0.15, was used to identify possible significant predictors.

5.2.7.1.3. Fractional Polynomial and Treatment Effects

The treatment effect of eprinomectin was expected to depend on the level of parasitism in the cow, where low ODR values indicated low levels of parasitism (Kloosterman *et al.*, 1993; Ploeger *et al.*, 1989). Therefore, the estimates from the interaction between ODR and treatment on milk production were used to determine how well the ODR predicted the response to treatment. For simplicity, the latest ODR values from the cows' previous lactation were dichotomized by their median value to investigate the residual structures (UN, AR1, MA, ARMA, or ANTE). The relationship between milk production and ODR is unlikely to be linear (Sanchez *et al.*, 2005), so subsequently fractional polynomials were applied to the continuous ODR values. Fractional polynomials also allowed flexibility in the interaction terms, not simply in the ODR terms.

A 2-degree fractional polynomial (FP), using two terms, is likely the most parsimonious method to obtain a good fit to the data (Royston and Sauerbrei, 2008). The FP analysis (fracpoly, Stata 11) generated two new centered terms, the first being the variable to the power of a calculated constant (one of the following: -3, -2, -1, -0.5, 0, 0.5, 1, 2, and 3, where 0 refers to a natural logarithm transformation) and the second being either a second power term

from the same series or the product of the natural log of the variable and the variable raised to the same power. For example, ODR will become ODR^{p1} and ODR^{p2}, where if the power selected to produce the best-fit for both terms is -2, $ODR^{p1} = ODR^{-2}$, and $ODR^{p2} = ODR^{-2}\ln(ODR)$. Royston and Sauerbrei (2008) recommend to test a fractional polynomial interaction with a likelihood ratio test between the nested model and the full model using maximum likelihood (ML) methods (not restricted-ML methods). The difference in deviance is compared with a χ^2 on two degrees of freedom. In this study, the FP analysis of ODR generated two new terms (ODR^{p1} and ODR^{p2}); four new variables were subsequently generated, two FP terms for each eprinomectin and placebo groups, giving: $ODR_{tx=0}{}^{p1}$, $ODR_{tx=0}{}^{p2}$, $ODR_{tx=1}{}^{p1}$, $ODR_{tx=1}{}^{p2}$. If x* represents all other fixed explanatory variables, the full model included x*, treatment, $ODR_{tx=0}{}^{p1}$, $ODR_{tx=0}{}^{p2}$, $ODR_{tx=1}{}^{p1}$, and $ODR_{tx=1}{}^{p2}$, while the reduced model included x*, treatment, ODR^{p1}, and ODR^{p2}. The overall treatment effect was derived by subtracting the estimates of $ODR_{tx0}{}^{p1}$, and $ODR_{tx0}{}^{p2}$ from $ODR_{tx1}{}^{p1}$, $ODR_{tx1}{}^{p2}$, and treatment. The treatment effect and its confidence interval were plotted against ODR to visualize the relationship between ODR and treatment effect.

5.2.7.1.4. Model Diagnostics

Best linear unbiased predictions (BLUPs) were calculated for both random effects (herds and cows), and were subsequently plotted against the predicted outcome to verify the assumption of heteroscedasticity. The residuals from

the repeated measures were also evaluated by plotting the standardized residuals against the predicted outcome. Normality assumptions were verified by plotting the quantiles of the BLUPs, for both random effects, against quantiles of normal distribution. Similarly, the quantiles from the residuals (repeated measures) were plotted against quantiles of normal distribution. (Dohoo *et al.*, 2009)

5.2.8. Reporting Clinical Trial Findings

Reporting of the clinical trial followed the REFLECT statement (Reporting Guidelines For Randomized Control Trials) (O'Connor *et al.*, 2010) as closely as possible.

5.3. Results

5.3.1. Comparable Studies in Literature

Medline and CAB Abstracts searches produced 87 and 170 articles, respectively. Of those, 5 recent studies evaluated how measuring anti-parasite antibodies could predict milk production response to anthelmintic treatments (Charlier *et al.*, 2007; Charlier *et al.*, 2010; Sanchez *et al.*, 2005; Sanchez *et al.*, 2002a; Sithole *et al.*, 2005); three studies from the previous meta-analysis were also included (Kloosterman *et al.*, 1996; Ploeger *et al.*, 1990; Ploeger *et al.*, 1989). Table 5.1 provides a summary of these studies.

5.3.2. Herd and Animal Selection

There were 98 herds in the NCDF and of those 40 herds met the selection

criteria and agreed to participate in the study. Two herds failed to record cow treatments and were removed, leaving a total of 38 herds.

5.3.3. Sampling and Milk Collection

Between May 1, 2007 and June 1, 2008, there were 3,006 dairy cattle that calved from the 38 participating herds (see Fig. 5.1). There were 997 heifers (first-time-calvers), limiting the number of possible ODR reading to 2,009 cows. One thousand and five hundred cows were recorded as having received a treatment (either Eprinex® or Placebo), however 35 failed to receive their treatment within the recommended time near calving. For the analysis, there were 1,088 cows with an ODR value from the previous lactation, and treated within the recommended time near calving. There were on average 34.8 cows (range 6 to 80) from each herd that contributed to the study.

Overall, there was an average of 9.0 DHI milk tests per cow (median=9, standard deviation=2.6), and the average 24-hour milk yield for all cows from all test dates was 32.2 kg/cow/day (median=31.6, standard deviation=9.5).

5.3.4. ELISA and Milk Production

There was on average, 2.2 individual ODR samples per tested cow-lactation (range 1 to 9). ODR values from late lactation periods were recorded as the latest ODR value for that cow, regardless of the sample date. The median day between the test date and the calving date for the latest ODR values was 88 (IQR 68 to 133). The overall average latest ODR value for all cows with ODR values (Groups F, I, & L, Fig. 5.1) was 0.307.

5.3.5. *Treatment*

A total of 2,117 treatments (either placebo or anthelmintic) were dispensed, with 2,058 doses applied during the correct time interval (2 weeks pre- to 3 days post-parturition). The placebo group (I) had an averaged latest ODR value of 0.303 (IQR 0.111 to 0.448) while the eprinomectin group (Fig. 5.1, Group F) had 0.297 (IQR 0.098 to 0.427). There were no reported adverse reactions to any of the treatments.

5.3.6. *Questionnaire*

Thirty seven of the 38 enrolled herds completed the parasite questionnaire (see Appendix B). A question about pasturing practices for milking cows, to validate the CBMRN and parasite questionnaire, was provided to seventeen of the 37 producers (Dufour, 2010). The kappa value was 0.90 (95%CI 0.71 – 1.00), indicating excellent agreement between two answers that producers gave nearly a year apart (average 262 days).

Between May 2006 and April 2007, prior to the commencement of the study, 73% (27/37) of the producers in the study used medications for deworming and/or external parasite control. Eighty six percent of the producers who used anti-parasitic drugs, treated their milking cows. Treating milking cows prior to calving was the most popular period to treat (11/28 = 39%), followed by treating all milking cows in the Fall (10/28 = 36%). Again, those producers who used anti-parasitic drugs, 75% treated their heifers. Fall treatment of heifers was the most popular (12/28 = 43%), followed by spring treatment of

heifers (9/28 = 32%). During the summer of 2007, approximately half (54%) of the producers in the study had their milking cows on pasture, while the other half kept them confined. The vast majority of producers placed their dry cows on pasture (27/38 = 71%), and a similar number of producers also kept their heifers on pasture (26/38 = 68%). Half (15/30 = 50%) of those who did place either milking cows, dry cows or heifer on pasture, kept them within their respective groups (i.e. dry cows with dry cows only), while 37% mixed their dry cows with their heifers on the same pastures.

During the study, 21 herds could not keep their cows apart after treatment, while only 4 herds were able to keep them apart for more than 24 hours (recommended); the remaining 12 herds varied between one and 24 hours.

5.3.7. Multivariable Mixed Analysis

5.3.7.1. Repeated Measures

Different residual structures from models with the same fixed and random effects were investigated along with their respective correlation structures for the repeated milk yields over time within cow lactations. Figure 5.2 shows the estimated unstructured correlations, and the stationary correlations for various structures. The model for unstructured correlation would not converge if >9 test dates were included, and the model for MA would not converge if >8 test dates, therefore all correlation structures, for comparison, were estimated with 8 test dates. The unstructured correlation matrix (Fig. 5.2, a) revealed the data structure, and required the addition of 28

covariance terms and 8 variance terms. The correlation for the MA structure (Fig. 5.2, c) did not allow a sufficient decrease in the coefficients as the interval between tests increased; it required and additional 7 terms. The model for the ARMA(1,1) correlation structure (Fig. 5.2, d) was more parsimonious, however, the coefficients decreased too quickly as intervals between tests increased. The most parsimonious, and similar to the unstructured matrix, was the AR1 correlation structure (Fig. 5.2, b) – it became the residual structure of choice for the remaining analyses.

5.3.7.2. Random Effects in the Model

There were two random variables included in the final model (Table 5.2) – a herd and a cow variable. The estimated variances at the herd, cow, and test date (residual) levels were 6.545, 16.505 and 30.713, respectively. The correlation between any test order interval, indicated by time, is calculated with the intra-class correlation coefficient (ICC) value derived from the AR1 structure (ρ), as demonstrated in Fig. 5.3. The ICCs reveal similarities between any milk record in relation to the cow or herd. The final model estimated the ICC between any two milk tests within a cow, but taken far apart in time, to be 0.429, and between any milk test within a herd to be 0.122.

5.3.7.3. Fixed Effects

All of the fixed effects selected for their biological merits to explain milk yields, were statistically significant at p=0.05 (or nearly so). The largest change in

milk production due to seasonal and yearly differences was between the second and fourth period (Shoulder 2007 and Grazed 2008) with an increase of 1.34 kg milk/cow/day (95%CI 0.68 – 1.99). The largest difference between calving seasons was between the summer and the fall, where cows calving in the fall were estimated to produce 1.69 kg milk/cow/day (95%CI 0.91 – 2.47) more than cows calving in the summer. Cows in their second lactation period (first recorded lactation with ODR values) produced on average 2.10 kg milk/cow/day (95%CI 1.47 – 2.74) less than cows in higher lactation periods. It was estimated that for every increase of one lnSCC unit, the milk production dropped by nearly one kilogram per day (-0.95, 95%CI -1.06 to -0.84). As expected, days-in-milk was a strong predictor of milk production during the lactation period of a cow ($p<0.001$).

5.3.7.4. Fractional Polynomial and Treatment Effects

The fractional polynomial relationship between milk production and ODR, when controlling for x* fixed effects, is shown in Fig. 5.4. As ODR increases up to 0.8, the daily milk yield decreases, however, for values greater than and equal to 0.8, the daily milk yield increases slightly – this may be due to the limited number of observations (279 out of 4365 placebo cows = 6.4%) with extreme values.

The final model containing the two centered FP terms for the latest ODR values were: $ODR^{p1}=(ODR^{-2})-0.5775$ and $ODR^{p2}=(ODR^{-2} \times \ln(ODR))-0.1585$. The maximum log likelihood for the model with the main effects for ODR was -

25594.08, while it was -25592.10 for the model with all four FP terms for the treatment by ODR interaction. The χ^2 statistic from the likelihood ratio test was 4.05 for two degrees of freedom, giving a *p*-value of 0.138 for the interaction. Fig. 5.5 shows the interaction plot for ODR values from both placebo and treated cows, against milk production. As ODR values rise from 0 to 1 for placebo cows, milk production declines by approximately 0.8 kg milk/cow/day. When ODRs are greater than 0.04, milk yield values are positive for eprinomectin-treated cows, and peak to approximately 0.63 kg milk/cow/day when ODR is equal to 0.37. Of greater interest was the effect of treatment, which was derived from the differences in milk yields between placebo and treated cows, as shown in Fig. 5.6. The estimated treatment effects were positive when ODR values were above 0.12, however, the lower bound of the 95% CI never went above zero. The maximum estimated treatment effect occurred when ODR was equal to 0.46, where it was estimated to increase milk production by 0.73 kg/cow/day. There was a negative effect of treatment when ODR values were below 0.12, though the confidence interval was relatively large for these values. The confidence interval range also rose quickly as ODR values increased above 0.6. The decline in the difference between treatment and placebo for ODR values above 0.6 is due to the apparent decline in production in treated cows above this ODR level. There are relatively few observations with ODR values greater than 0.6 in the treated cows (593 out of 4096 observations from treated

cows = 14.48%)

5.3.7.5. Questionnaire Predictors

Twenty seven producers answered 'yes' to giving any medications for deworming and/or external parasite control between May 2006 and April 2007; this was the only variable that made it through the stepwise elimination. Those producers who answered 'yes' to this question had an estimated 2.03 kg milk/cow/day more than producers who answered 'no' (p=0.050).

5.3.7.6. Model Diagnostics

The model assumptions of heteroscedasticity and normality were verified. There was no visual indication of any model assumption violations, and no transformations of the outcome were deemed necessary.

5.4. Discussion

Based on the sampling design used to select the NCDF producers, herds available to this study represented the current distribution of commercial dairy farms in Canada. Herd selection was founded on milk production, bulk tank somatic cell counts, and housing type. CBMRN recorded that 34% of the NCDF farms were housed in 'freestall', which was comparable to estimated national (region-weighted) averages of 36% (Reyher *et al.*, 2010). It is possible that housing types of study herds do not accurately reflect farm pasturing protocols, however, it is likely these farms represented the average pasture exposure of dairy cattle across Canada. Only those farms which

allowed cattle to have access to pasture or paddock at some time during their production cycle were included in this study, thus representing a subset population of the typical Canadian dairy farms.

The pasturing method inclusion criteria were deemed important to ensure a certain level of exposure of infective nematode larvae to increase the statistical power of the study. Variables describing the producer's pasturing techniques were derived from the parasite questionnaire. Treatment effects were expected to vary according to the pasturing techniques, though no significant or interesting relationships were observed during the analysis.

The average ODR value in this study was lower than anticipated (0.262, SD 0.243) when compared to other studies from similar regions. Sithole *et al.* (2005) reported an average herd bulk tank ODR of 0.41 (SD 0.13) for 65 herds with limited outdoor exposure during a one year period, however, bulk tank ODR values are usually larger than averaged individual ODRs (Charlier *et al.*, 2010). Sanchez *et al.* (2005), had similar, though slightly larger, results from individual ODRs; the recorded average was 0.297 (SD 0.251) with a larger range from -0.051 to 1.558. It is worth noting that Sanchez *et al.* (2005) and Sithole *et al.* (2005) used in-house ELISA kits with different controls than those used by Svanova, which would influence their ODR values, and could also account for different ODR values between studies.

The majority of the producers were unable to follow the specified instructions to keep their cows apart for more than 24 hours after treatment. This request

was to prevent mechanical transfer (licking) of anthelmintics because it is possible for cows to receive a sufficient dose of anthelmintic from licking/grooming neighboring treated cows (Barber and Alvinerie, 2003; Laffont *et al.*, 2001). Unfortunately, as reported, only four herds managed to keep their cows apart for more than 24 hours.

5.4.1. Final Model

The auto-regressive residual structure (AR1) was chosen based on preliminary models using simplified interaction terms (treatment and dichotomized ODR), where the AR1 correlation matrix was the most similar to the unstructured matrix and yielded the most parsimonious model. Sithole *et al.* (2005) found the ARMA residual structure to have a better fit to their data, however, the AR1 residual structure was the best choice in both studies by Sanchez *et al.* (2005; 2002a). When fitting the more complicated interaction with FP terms from continuous ODR values, the AR1 structure was assumed to be present for up to and including ten test dates; all the information for an expected lactation period (10 months) could be computed. As test dates got further apart, the milk yield correlations between the test dates decreased, however, after the 6th test, there were very little differences and the correlations stayed very close to the constant 0.43; this relatively elevated constant correlation indicates a moderate amount of clustering within a cow, no matter when the milk was taken. The ICC for any milk test between cows within a herd (0.122) indicates some level of clustering within a herd.

The only significant predictor to arise from the questionnaire was whether or not producers had used anthelmintics on their farm within a year prior to the study. Using anthelmintics was estimated to increase milk production by 2.03 kg milk/cow/day (p=0.050), which is a biologically substantive amount. What could not be determined from this study was whether or not this predictor was confounded by 'type' of producer, where producers who strive to increase productivity might also have been more prone to treat their cows with an anthelmintic before the study. Only one quarter of the producers did not treat with anthelmintics, and there was no obvious over-representation from any province (p=0.063, Fisher's exact). On-farm treatment prior to the study may have also contributed to lower ODR values than were originally expected. It is, however, more likely that a combination of both factors (type of producer, and earlier on-farm treatment) were responsible for the statistical trend and magnitude of the estimate.

The interaction explaining the treatment effect was not statistically significant (p=0.138), and there were three potential contributing factors. (1) Many of the herds with 'access' to pasture had limited access, such as a paddock, or only allowed a group of animals (e.g. dry cows or heifers) to graze. This limited access to pasture would have reduced the exposure of cows to the parasite's infective stage, reflected in low ODR values. (2) Three quarters of the herds had been using an anthelmintic on the farm within one year of the study. Therefore anthelmintic treatment before the study (and throughout the

study), may have reduced the overall parasite exposure on the farm. It is therefore more difficult to investigate the interaction if there were few larger ODR values (Bailar and Mosteller, 1988). (3) Although the producers were asked to keep their cows apart for at least 24 hours after treatment, only four farms managed to do so. It is difficult to estimate the importance of mechanical transfer of eprinomectin treated cattle between animals. However, some amount of mechanical transfer is plausible. Each of those three factors could have individually biased the interaction term towards the null hypothesis. Furthermore, interaction terms require additional statistical power to be detected when compared to the ability to detect main effects (Greenland, 1993).

The estimated treatment effect peaked at 0.73 kg milk/cow/day when the latest ODR value was 0.46; this positive treatment effect continuously declined after 0.46, though it always remained positive. The FP terms for the ODR plotted against milk production (Fig. 5.5) looked very similar to the quadratic terms Sanchez *et al.* (2005) found when looking at individual cow ODR values from confined or semi-confined Canadian dairy herds; they found a greater treatment effect at the upper ODR range. Ploeger *et al.* (1989) had an estimated treatment effect at the upper antibody level range of approximately 1.5 kg milk/cow/day (estimated from both the graph and regression coefficients, converted from standardized 305 day production). Charlier *et al.* (2010) ran several statistical models, and the final model

(including all parameters and the interaction) estimated the treatment effect to be > 3.58 kg milk/cow/day when ODR was greater than 1. Sanchez *et al.* (2002a) investigated individual ODR dichotomized (high/low, 0.5 cutpoint) and estimated the interaction effect to be 2.99 kg milk/cow/day – the remaining studies investigated bulk tank milk samples. The studies evaluating the treatment effects on milk production as individual anti-parasite antibodies increase (serum or milk on a continuous scale) found consistent positive results, although there has been variation in the estimated magnitude of the response.

5.5. Conclusion

The ability to predict the effect of anthelmintic treatment on milk production depends on the level of parasitism quantified by an ELISA test measuring milk antibodies against *Ostertagia ostertagi*. The interaction showed a trend (p=0.138) towards a beneficial treatment effect when the individual ODR values, measured in late lactation, were greater than 0.12. Interestingly, there was an estimated negative treatment effect for ODR values less than 0.12, emphasizing the potential need to determine the parasite load within cows and herds prior to anthelmintic treatments. Several factors could have contributed to the interaction being biased towards the null, such as combinations of overall lower ODR values (from generally low levels of pasture/paddock exposure, and either previous treatment or treating half the herd throughout the study), and the high probability of mechanical transfer of

anthelmintics between cows after treatment. Although not statistically significant at p=0.05, the study findings were consistent with previous studies analyzing the interaction between anthelmintic and anti-parasite antibody levels on milk production, and of particular interest, the similarities in the shape of these plotted relationships.

5.6. References

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Table 5.1. Studies, found in the literature, evaluating how quantifying anti-parasite antibodies could predict the milk production response to anthelmintic treatments.

First Author (Year)	Anti-parasite antibody (Ind. or BT)[A]	Anthelmintic Treatment	Effect of Tx kg/cow/day (SE)	at Ab value	*P* value
Charlier (2010)	Ind. (whole herd) Continuous[C]	Whole Herd Eprinomectin	Reduced LMM[B]: 6.2 (3.1) Full LMM[B]: 3.58 (3.24)	>1[C] >1[C]	(Interaction) 0.047 0.27
Charlier (2007)	Bulk Tank Categorized[C]	Whole Herd Eprinomectin	Largest 10th percentile: 4.0 (1.53)	10th decile[C]	0.03
Sithole (2005)	Bulk Tank Dichotomized[C]	Individual Near Calving Eprinomectin	Final LMM[B]: 0.385 (0.366)	>0.5[C]	(Interaction) 0.149
Sanchez (2005)	Ind. (whole herd) Continuous[C]	Individual Near Calving Eprinomectin	Plotted quadratic terms (LMM[B]): ~3[E]	>0.5[C]	(Interaction) <0.05
Sanchez (2002)	Ind. (partial herd) Dichotomized[C]	Individual Near Calving Eprinomectin	Final LMM[B]: 2.99 (1.66)	>0.5[C]	(Interaction) 0.07
Kloosterman (1996)	Bulk Tank Dichotomized[D]	Individual Dry Period Ivermectin	Least square means: 0.57[F]	High[D]	0.21
Ploeger (1990)	Ind. (5 random/h) Continuous (Range 4.0-8.5)[D]	Individual Near Calving Albendazole	Reported as: "No significant correlations were found between the treatment response per herd and the serological parameters measuring nematode infection." [D]		
Ploeger (1989)	Ind. (5 random/h) Continuous[D]	Individual Near Calving Ivermectin	Linear Regression: 0.528 (0.251)‡	Increments of 1[D] (range 3.8-8.0)	(Interaction) <0.05

[A] Ind.=Individual; BT=Bulk Tank
[B] LMM: Linear Mixed Model
[C] Optical Density Ratios (ODRs)
[D] Serum is initially diluted 1:20; then titre value was the highest dilution that gave a positive (e.g. 1=1/20, 2=1/40, 3=1/80, etc.)
[E] As described in conclusions, and estimated from graph (kg milk/cow/day vs. ODR, by treatment)
[F] Converted from standardized kg/305 d as reported in The Netherlands

Table 5.2. Final multilevel mixed model predicting milk loss, containing herd, cow, and test date as random effects, with fixed effects accounting for milk production, questionnaire predictors, and interaction terms (fractional polynomials). The assumed residual structure was AR(1), and the model contains 37 herds, 1088 cows, and 8254 observations for milk yields (kg/cow/day).

Fixed effects

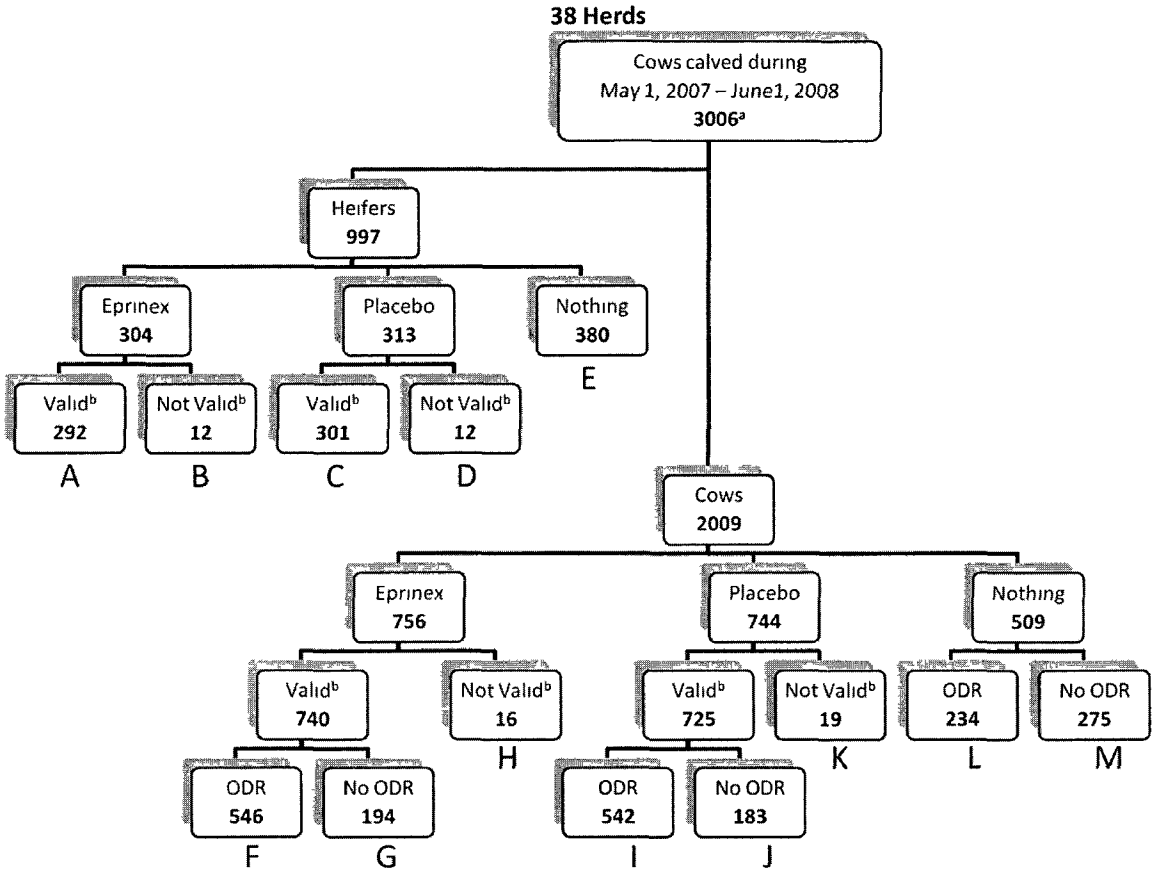
Variable	β	Standard Error	95% CI	P
Intercept	144.311	3.393	137.660, 150.961	0.000
Time Period				<0.001
Shoulder 2007	Baseline			
Housed 2008	-0.622	0.370	-1.347, 0.104	0.093
Grazed 2008	0.622	0.436	-0.232, 1.476	0.153
Shoulder 2008	0.714	0.515	-0.294, 1.723	0.165
Housed 2009	-0.308	0.611	-1.506, 0.890	0.614
Calving Season				<0.001
Grazed	Baseline			
Shoulder	1.692	0.397	0.915, 2.470	<0.001
Housed	1.234	0.451	0.351, 2.118	0.006
Lactation Group				<0.001
2nd	Baseline			
3rd	2.193	0.398	1.413, 2.973	<0.001
4th and greater	2.014	0.372	1.284, 2.744	<0.001
Days in milk				<0.001
DIM centered	-0.119	0.002	-0.123,-0.114	<0.001
$DIM^{-0.05}$	-139.806	4.058	-147.760,-131.852	<0.001
Log Somatic Cell Count	-0.950	0.054	-1.057,-0.844	<0.001
Anti-parasite Before?	2.027	1.036	-0.004, 4.057	0.050
ODR and Treatment Interaction§				0.138*

Random Effects

Level	Variance	Standard Error	Rho (ρ)
Herd	6.545	1.758	
Cow	16.505	1.306	
Residual (AR(1))	30.713	0.901	0.462

§ FP structure of treatment and ODR are described in text and displayed graphically

* Likelihood Ratio Test between the full (displayed in this table) and the reduced model (not shown)



[a] Counts represent one cow-lactation
[b] Valid refers to receiving the treatment within 2 weeks pre- and 3 days post-parturition

Figure 5.1. Flow chart of subjects within the study evaluating the effect of parasite load on milk production. The diagram shows the final allocation of cows and heifers grouped by treatment and data completeness. (n= bold number in each cell)

A) Unstructured $corr(Milk)$ =

Test	1	2	3	4	5	6	7	8
1	1							
2	0.630	1						
3	0.550	0.726	1					
4	0.487	0.624	0.703	1				
5	0.461	0.590	0.705	0.781	1			
6	0.375	0.530	0.604	0.716	0.777	1		
7	0.395	0.488	0.612	0.644	0.764	0.803	1	
8	0.297	0.407	0.512	0.597	0.662	0.735	0.857	1

B) AR(1) $corr(Milk)$ =

Test	1	2	3	4	5	6	7	8
1	1							
2	0.689	1						
3	0.563	0.689	1					
4	0.511	0.563	0.689	1				
5	0.490	0.511	0.563	0.689	1			
6	0.481	0.490	0.511	0.563	0.689	1		
7	0.478	0.481	0.490	0.511	0.563	0.689	1	
8	0.476	0.478	0.481	0.490	0.511	0.563	0.689	1

C) MA(7) $corr(Milk)$ =

Test	1	2	3	4	5	6	7	8
1	1							
2	0.787	1						
3	0.695	0.787	1					
4	0.623	0.695	0.787	1				
5	0.613	0.623	0.695	0.787	1			
6	0.642	0.613	0.623	0.695	0.787	1		
7	0.633	0.642	0.613	0.623	0.695	0.787	1	
8	0.616	0.633	0.642	0.613	0.623	0.695	0.787	1

D) ARMA(1,1) $corr(Milk)$ =

Test	1	2	3	4	5	6	7	8
1	1							
2	0.678	1						
3	0.485	0.782	1					
4	0.352	0.529	0.760	1				
5	0.297	0.420	0.578	0.864	1			
6	0.247	0.331	0.439	0.630	0.858	1		
7	0.217	0.276	0.352	0.483	0.641	0.863	1	
8	0.205	0.251	0.310	0.409	0.529	0.691	0.927	1

Figure 5.2. Correlation matrices from various correlation structures for the residuals from 8 test dates, with random herd and cow effects. The correlation represents the expected level of correlation between two milk production values taken at specified tests.

$$
AR(1)_{Corr(Milk)} = \begin{array}{c|cccccccccc}
Test & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 \\
\hline
1 & 1 & & & & & & & & & \\
2 & 0.693 & 1 & & & & & & & & \\
3 & 0.550 & 0.693 & 1 & & & & & & & \\
4 & 0.485 & 0.550 & 0.693 & 1 & & & & & & \\
5 & 0.455 & 0.485 & 0.550 & 0.693 & 1 & & & & & \\
6 & 0.441 & 0.455 & 0.485 & 0.550 & 0.693 & 1 & & & & \\
7 & 0.434 & 0.441 & 0.455 & 0.485 & 0.550 & 0.693 & 1 & & & \\
8 & 0.431 & 0.434 & 0.441 & 0.455 & 0.485 & 0.550 & 0.693 & 1 & & \\
9 & 0.430 & 0.431 & 0.434 & 0.441 & 0.455 & 0.485 & 0.550 & 0.693 & 1 & \\
10 & 0.429 & 0.430 & 0.431 & 0.434 & 0.441 & 0.455 & 0.485 & 0.550 & 0.693 & 1
\end{array}
$$

Figure 5.3. Correlation matrix for the final model with random herd and cow effects, fixed effects accounting for milk production, questionnaire predictors, and interaction terms (fractional polynomials). The correlation represents the expected level of correlation between two milk production values taken at specified tests.

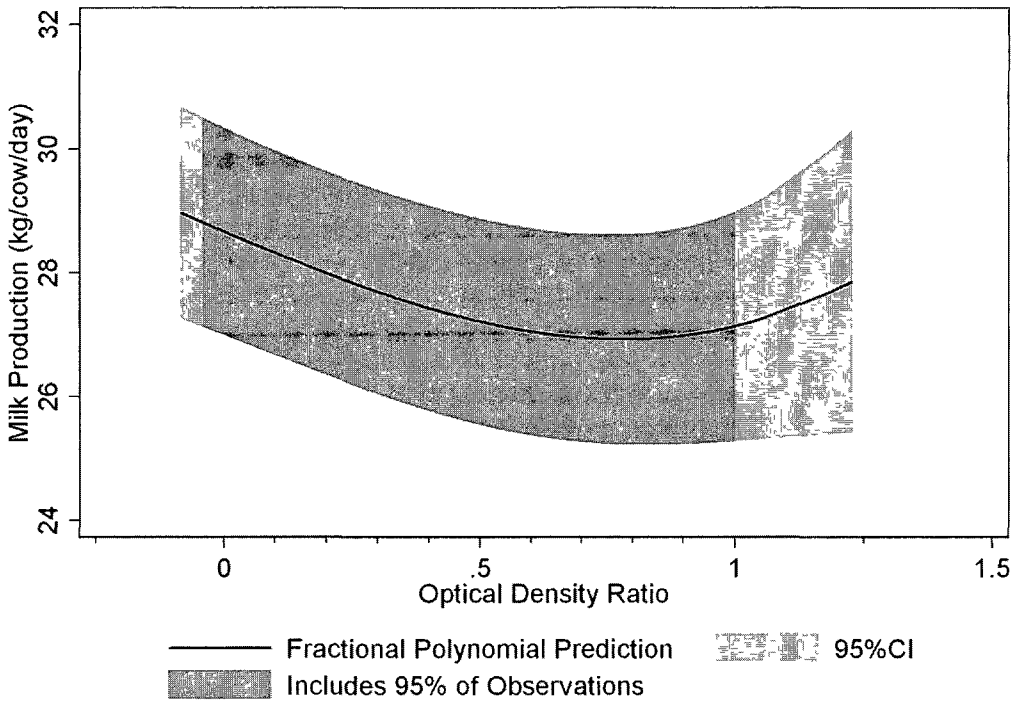


Figure 5.4. Fractional polynomial terms for ODR values, plotted against milk production (kg/cow/day) **for placebo cows.** These estimates account for fixed explanatory variables (time period & season, calving season, lactation group, DIM, lnSCC, and the questionnaire predictor) without random effects for herd and cow.

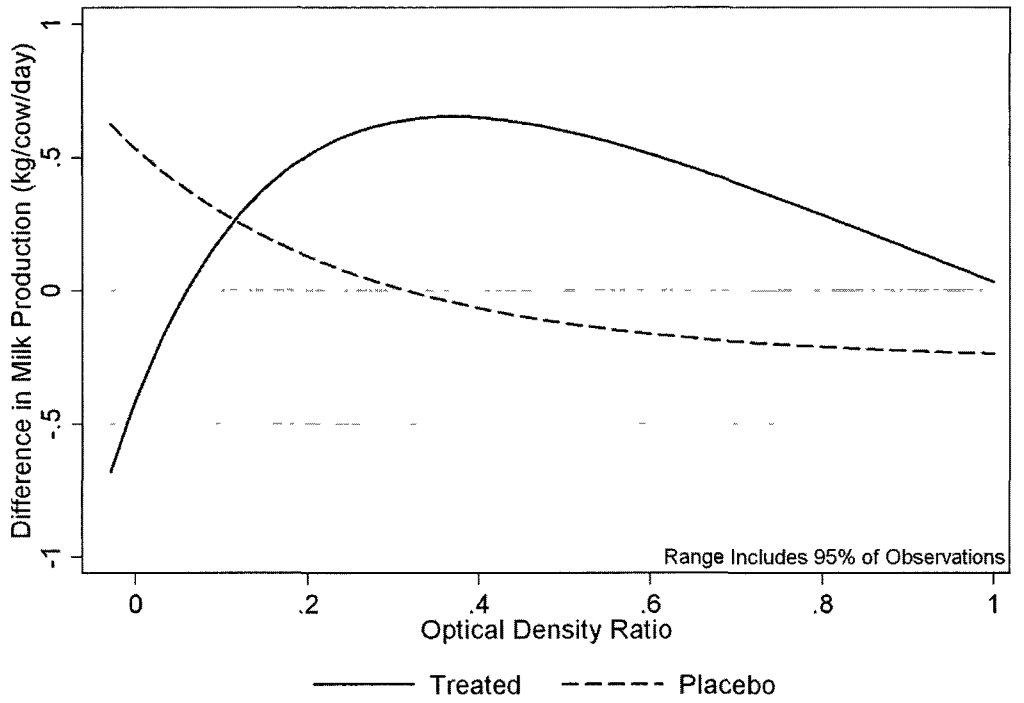


Figure 5.5. Interaction plot for treatment (n=546) **and placebo** (n=542) **predictions of milk production versus ODR**, demonstrating how the differences in expected milk production changes as ODR values change. Predictions are calculated from treatment and ODR coefficients, where the intercept and fixed effects are removed. Zero difference in milk production represents what would be expected from an average placebo cow from any farm at any time. The range of the ODR values include 95% of the modeled observations.

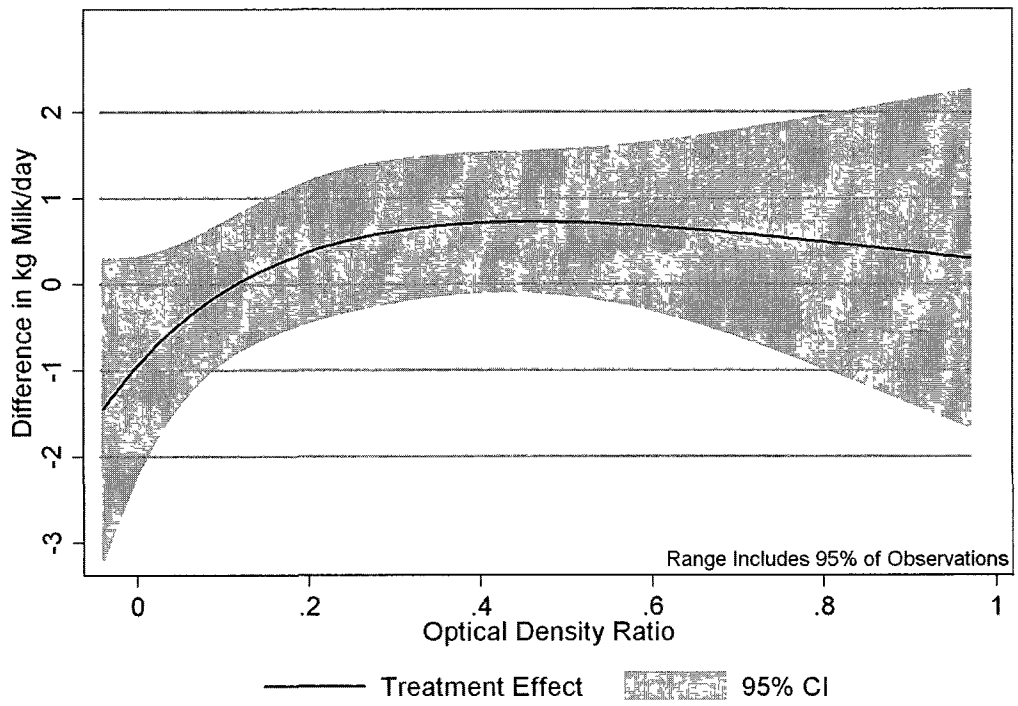


Figure 5.6. Treatment effect showing the difference between expected treatment and placebo milk production (kg milk/cow/day) **versus ODR.** 95% Confidence Interval bands were calculated and plotted. The range of the ODR values include 95% of the modeled observations.

Chapter 6

Predicting the Effect of Anthelmintic Treatment on Reproductive Parameters of Dairy Cattle In Canada Using an ELISA Test from Individual Milk Samples

6.1. Introduction

Gastrointestinal nematodes in temperate climates, such as *Ostertagia ostertagi* and several species of *Cooperia*, are associated with milk production losses in dairy cattle (Sanchez *et al.*, 2004). Unlike infections in first-season grazers, which are manifested by weight loss and diarrhea, adult dairy cattle do not typically show clinical signs (Eysker and Ploeger, 2000). Quantifying levels of parasitism in cattle, with the use of an enzyme-linked immunosorbent assay (ELISA), has the ability to predict milk loss due to parasite infections (Charlier *et al.*, 2010; Ploeger *et al.*, 1990; Ploeger *et al.*, 1989; Sanchez *et al.*, 2005; Sanchez *et al.*, 2002a).

When investigating the effect parasites have on reproductive parameters, studies involving beef cattle are more prevalent since fertility determines the profitability of the cow-calf system. Anthelmintic treatment in beef cows has been shown to improve body condition scores (ensuring proper body condition for re-breeding), increase conception and calving rates, reduce calf mortality and calving-to-breeding intervals; these findings, however, have not been reported consistently (Hawkins, 1993).

In dairy cattle, there are few studies investigating the effect of parasitism on fertility. Walsh *et al.* (1995) found that treated cows had a significantly reduced calving-to-conception interval, however, there were no significant differences in calving-to-first service intervals. Two studies investigated the predictive ability of an ELISA test on reproduction parameters. Sanchez *et al.*

(2002a) found that treated cows with high levels of parasite antibodies had a significant reduction of breeding-to-conception intervals when compared to the placebo group carrying elevated parasite antibodies. Sithole *et al.* (2006) failed to show any beneficial treatment effect – it is worth noting that the study suffered from low parasite exposure and statistical power, therefore, the results regarding reproductive performance were not conclusive.

Svanovir® (Svanova Veterinary Diagnostics, Uppsala, Sweden), an indirect ELISA test, is commercially available and currently used in Europe to determine levels of intestinal parasitism (Almería *et al.*, 2009; Charlier *et al.*, 2007; Forbes *et al.*, 2008). The objective of this study was to evaluate the ability of Svanovir® results from individual milk samples from cows, in both semi-confined and pastured dairy herds in Canada, to predict the effect of anthelmintic treatment on reproductive parameters.

6.2. Material and Methods

Reproduction data were collected simultaneously with a study predicting the effect of anthelmintic treatment on milk production of dairy cattle in Canada, using *O. ostertagi* ELISA from individual milk samples. The methods for herd and animal selection, sampling design, milk collection, ELISA tests, and treatment protocols are described in Chapter 5 under the 'Materials and Methods' section.

Briefly, on-farm milk samples of individual dairy cows were collected from both the Canadian Bovine Mastitis Research Network (CBMRN) and routine Dairy

Herd Improvement (DHI) programs between March 1, 2007 and April 30, 2008. Monthly electronic records of milk production, milk quality, and reproduction parameters were made available from DHI programs. Milk samples from cows in their late lactation (>200 days in milk) were processed to quantify *O. ostertagi* antibodies using a commercial ELISA kit, Svanovir®. The results from ELISA tests are reported as optical density ratios (ODRs) and represent a percent positivity between the supplied negative and positive controls (Sanchez *et al.*, 2002b; Vanderstichel *et al.*, 2010). All cows calving between May 1, 2007 and May 31, 2008 received one dose of either eprinomectin (Eprinex®, 65ml = 325mg) or mineral oil (65ml, acting as a placebo) applied along the backline from the withers to the tail head, near the time of parturition (2 weeks pre- to 3 days post-parturition). Participating producers were asked to complete a questionnaire pertaining to anti-parasitic treatments, pasturing techniques, and housing-type for their milking cows, heifers and dry cows (see Appendix B).

6.2.1. Reproduction Parameters

There were 4 possible reproduction outcomes that were recorded or calculated; 1) Number of services per conception (NSC), 2) First-service conception risk (FSCR), 3) Days to first-service (DFS), and 4) Days to conception (DC). The lactation start date, first breeding and last breeding dates, the number of services, and the following calving date were made available from DHI records.

The recorded dates for the last service and the following calving were used to calculate the approximate conception date. The approximate conception date was compared with the last service date in order to accept the last service date as the true conception date. To calculate the approximate conception date, two hundred and eighty days were subtracted from the date of calving, with an additional 34 day grace period (15 days were allocated for the gestation variation and an additional 19 days for the minimum estrous cycle duration, assuming an estrous cycle with 2 follicular waves) (Adams *et al.*, 2008; Norton, 1956). Last service dates falling outside of the 280 +/- 34 days range were assumed to have been erroneously recorded and were marked as missing.

Variables with potential predictive abilities on reproduction parameters were the number of days in the dry period, calving season (January 15th to May 14th, May 15th to September 14th, and September 15th to January 14th), parity (2, 3, and 4+ years), lactation milk yield standardized for 305 days (simply referred as milk yield) and averaged somatic cell counts throughout the lactation period (Dohoo *et al.*, 2001; Sithole *et al.*, 2006).

To understand the effect of anthelmintic treatment on the dairy cow's reproduction, ODR values, treatment allocation (Eprinex® or Placebo), and their interaction, were forced in each model as fixed effects, regardless of their statistical significance. The remaining fixed effect variables were derived from the questionnaire.

6.2.2. Statistical Analyses

Each reproduction outcome (NSC, FSCR, DFS, and DC) was analyzed separately. Unless otherwise stated, all statistical analyses, including summary and descriptive statistics, were performed in Stata11 (2009). The inclusion criteria was $p<0.15$ for unconditional associations and statistical significance was set at $p<0.05$ in the multivariable models.

6.2.2.1. Number of Services per Conception (NSC)

In keeping with the hierarchical structure of the data, herds were added as a random variable to a multilevel negative binomial regression, which was fit in MLwiN (Rasbash *et al.*, 2009). To simplify calculations for the intraclass correlation coefficients (ICCs), as described by Stryhn *et al.* (2006), random herd effects were assumed to follow a normal distribution. The correlation between individual cow's number of service-to-conception is measured by the ICC, and reveals similarities between any two cows within a herd; values closer to zero indicate that most of the variation is within a herd, and therefore has very little clustering, while higher ICCs (closer to 1) are associated with higher amounts of clustering within a herd (Dohoo *et al.*, 2009).

A negative binomial regression was used to explain fixed effects without accounting for herds. Model diagnostics included the evaluation of Cook's deviance and Anscombe residuals. The deviance residual was used to compute the Deviance χ^2 Goodness of Fit test. (Dohoo *et al.*, 2009)

6.2.2.2. First-Service Conception Risk (FSCR)

Multilevel logistic regression, with maximum likelihood estimates for herds as a random variable, explained the effects of treatment and ODR interactions on FSCR. The risk variable was generated so that cows that conceived on their first-service were marked as 'yes' and those which conceived on a later service were marked as 'no'. Model diagnostics for the multilevel logistic regression included the assessment of residuals at the herd level. The ICC was calculated from the approximated fixed error variance, set at $\pi^2/3$, for the latent variables. (Dohoo *et al.*, 2009)

6.2.2.3. Days to First-Service (DFS) and Days to Conception (DC)

The number of days to first-service were analyzed using a Cox proportional hazards model with a random effect for herd. Adding a random variable to a survival model to describe the excess risk or 'frailty' for distinct categories, in this case herds, is an acceptable method to control for clustering within herds (Therneau and Grambsch, 2000); these models are referred as frailty models. The hazards for herds were assumed to follow a gamma distribution, and the statistical test to include a frailty model involves a likelihood-ratio test for theta=0, where theta is the estimated scale parameter for the gamma distribution. The ICC for frailty models, also referred as Kendall's tau, can be calculated for gamma frailty models from the theta parameter such that $ICC=\theta/(2+\theta)$ (Therneau and Grambsch, 2000).

Days to conception was similarly analyzed using frailty models. The

conception date was assumed to be the last service date, provided this date fell within a plausible range based on the next calving date (explained in section 6.2.1).

Model diagnostics included the verification of the assumed proportional hazards using Schoenfeld and scaled-Schoenfeld residuals, checking for outliers with deviance residuals, and assessing goodness-of-fit with Cox-Snell residuals (Dohoo *et al.*, 2009). Time-varying effects (linear) were added when the hazard for a variable was not proportional and there was biological merit to including such an effect.

6.3. Results

Summary statistics and results for herd and animal selection, milk collection, ELISA ODRs, treatments, and questionnaire results are described in Chapter 5 under the 'Results' section. Since more information was required to assess the effect of anthelmintics on reproduction, compared to measuring milk production, there were some additional losses to the total number of cows in this study. Figure 6.1 shows the allocation of cows in the two studies, and explains how the cows in this study, used to assess reproduction parameters, are a sub-population of the study in Chapter 5. From the original 1,088 cows with milk production information, 685 had all the necessary reproduction information. There were 93 cows, including an entire herd, that had no breeding information (missing), and 126 cows were not bred. Of the cows that had information and were bred, 6 died, 135 were sold, and 43 were not

pregnant. When considering those cows which did not receive a treatment (either placebo or anthelmintic) as part of the placebo group, an additional 154 cows were used in the multivariable analyses to increase statistical power.

6.3.1. Number of Services per Conception (NSC)

Since it is impossible to have zero breeding before conception, the variable for the number of times a cow was bred before conception was transformed, by subtracting all values by one, to allow zeroes in the count data and facilitate the use of conventional count data distributions. After the transformation, the mean number of services were 1.31 with a variance of 2.55, indicating that a negative binomial model was more appropriate than a Poisson model.

A multivariable negative binomial model with herd as a random variable (normally distributed) was fit, however, the variance at the herd-level was very small (0.098) which transcended into very small ICCs for herds, ranging between 0.0048 and 0.0095. The low ICCs supported other studies which also found that reproduction parameters in dairy cattle do not cluster within herds (Dohoo *et al.*, 2001), and therefore the random herd effect was removed.

The final multivariable negative binomial model for the number of services per conception (Table 6.1) shows the interaction between ODR and treatment. Adding the interaction between treatment and ODR did not significantly contribute to the model (p=0.772), and nor were ODR, or treatment

significant.

Both the days to first-service and the lactation milk yield were included in the model as significant predictors of the number of services per conception. As days to first-service increased, the number of services was predicted to decrease (coefficient=-0.006), therefore, the longer the producer waited to start breeding a cow, the lower the number of services were required for conception. An average placebo cow with low a low ODR value, with an average milk yield (10,557 kg) would be predicted to require 1.40 services (*SE*=0.088) to conceive if breeding started in her 50th day in lactation, compared to only 1.16 services (*SE*=0.079) to conceive if breeding had otherwise started in her 90th day in lactation.

Milk yield was estimated to have a detrimental effect on the number of services; an average placebo cow with a low ODR value and an average breeding start date (days to first-service=87.1) was predicted to require 1.06 services (*SE*=0.085) to conceive if she had a milk yield of 9,450 kg, compared to 1.30 services (*SE*=0.081) for conception if she had produced 11,705 kg of milk during her lactation (9,450 and 11,705 kg milk represent the 25th and 75th percentiles for milk yield, respectively).

Diagnostics for the negative binomial model identified one cow with a high residual (Anscombe residual = 4.494) and high influence (Cook's Deviance = 0.050); the model was re-fit without that observation with minor changes to the coefficients (model not shown). None of the changes affected the

direction or significance of the coefficients.

6.3.2. First-Service Conception Risk (FSCR)

There were 342 cows that conceived on their first-service, out of 834 (41%) cows that eventually conceived. A multilevel logistical regression model, with herd as a random variable, was fit to predict the FSCR. The interaction terms for ODR and treatment were not significant (Table 6.2). The ICC was very low (3.1%) and borderline non-significant (p=0.053), therefore, the random herd effect could have been removed with little impact to the coefficients or their significance.

The same two fixed effect variables that were significant for the number of services per conception, namely, days to first service and the lactation milk yield, were the only two significant predictors for the FSCR model. Days to first-service had a beneficial effect on first-service conception risk, where the longer the producer waited to start breeding, the higher the risk of conception in the first service (p=0.027). If a producer waited to start breeding an average cow (placebo with low ODR and milk yield=10,557), from the 50th to the 90th day in lactation, the odds of conception on the first service would increase from 0.58 to 0.72. In other words, the cow was 1.24 times more likely to conceive if she was bred for the first time on her 90th day in lactation, rather than on her 50th day in lactation.

Milk yield had a detrimental effect on first-service conception risk, where an average cow (placebo with low ODR and days to first-service=87.1) with a milk

yield of 9,450 kg was predicted to have a first-service conception odds ratio of 0.88, compared to 0.57 for an average cow with a milk yield of 11,705 kg. A cow was 1.54 times more likely to conceive is she was in the 25th percentile for milk yield, rather than being in the 75th percentile (9,450 vs. 11,705 kg milk, respectively).

The model diagnostics revealed normally distributed and homoscedastic residuals at the herd-level with no obvious outliers.

6.3.3. Days to First-Service (DFS) and Days to Conception (DC)

The days to first-service was rightly skewed, where the average number of days being 87.1 (median=77.5, IQR=67–101). Days to conception was also rightly skewed, though with a thicker right tail, where the mean number of days was 139.6 (median=125, IQR=83–177.5). Frailty models were used to explain the effect of the treatment and ODR interaction on DFS and DC for cows that conceived.

The lactation milk yield was the only significant variable for both frailty models, however, it consistently failed to be proportionally hazardous over time and thus violated the most important assumption for a frailty model. To circumvent the issue of non-proportionality, milk yield was added as a time-varying covariate for both models predicting DFS and DC.

Looking specifically at the model to predict DFS (Table 6.3), the treatment and ODRs along with their interaction was not significant. The survival model was such that time to first-service was the event of interest, therefore, a placebo

cow with a high ODR would be protected from a first-service compared to a placebo cow with a low ODR, however, this effect was borderline significant (p=0.066). None of the other terms in the interaction (including the interaction itself) were statistically significant.

The milk yield variable was significant (p=0.001), and was also included as a time-varying covariate, on biological merit, even though this effect was borderline significant (p=0.061).

The random variable for herd was significant (p<0.001), despite being small and yielding such a low ICC (0.031).

The frailty model for DC (Table 6.4) was nearly identical to the DFS model. DC was the only reproduction outcome that utilized the conception date. The discrepancies between the last-service date and the approximate conception date (as explained in section 6.2.1) yielded 26 erroneous observations that were subsequently recorded as missing for the DC model.

Again, the survival model was such that time to conception was the event of interest, therefore, a placebo cow with a high ODR would be protected from a conception compared to a placebo cow with a low ODR; this particular effect was only borderline significant (p=0.083). The overall interaction and their individual terms were not statistically significant.

Both the fixed and time-varying effect for the milk yield variable were highly significant (p<0.001); the coefficients were such that as milk yield increased, the hazard of conception decreased, however, as days into the lactation

period increased, the hazard of conception did not decrease as much. For example, the hazard for a cow (in the placebo group with a low ODR and producing an average milk yield of 10, 557 kg) to conceive is different early in the lactation (hazard = 0.05628 when DIM=50) than later in the lactation period (hazard = 0.0573 when DIM=200). The estimated linear time varying effect would predict the milk yield hazard for conception at 50, 100, 150, 200, 250, and 300 days in milk, for a placebo cow with low ODR, to be 0.0563, 0.0566, 0.0570, 0.0573, 0.0576, 0.0580, respectively. The hazard of conception is the probability for a cow to conceive, given that the cow has not yet conceived at that specified time, therefore, the milk yield for a cow has a lower hazard for conception early in the lactation than later in the lactation. The theta parameter in the DC model was much smaller than that for the DFS model, however, it remained statistically significant (p=0.01) and produced a smaller ICC (0.017).

6.4. Discussion

There were no statistically significant effects of anthelmintics on reproduction parameters (NSC, FSCR, DFS, and DC) from pastured and semi-confined herds across Canada, even when accounting for parasite load measured by an ELISA test (Svanovir®). There were, however, borderline statistically significant effects of high ODR (vs. low ODR) in the placebo the group for both DFS and DC (p=0.066 and p=0.083, respectively). The effect of ODR on the placebo group indicates a potential role that intestinal parasitism may play on

fertility.

Milk yield was consistently significant for the reproduction parameters, and generally, higher milk yields were associated with detrimental reproductive results. Higher yielding cows had a greater NSC, lower risk of conceiving on the first service, and a decreased hazard for both first-service and conception; the milk yield hazard for both first-service and conception was, however, not decreasing as much as the lactation period progressed (linear time-varying effect).

The predicting variable of days to first-service was significant for the NSC, and FSCR (Table 6.1 and 6.2, respectively). It was estimated that the longer it took a producer to commence breeding for a cow, the greater the odds of conception on the first-service, and generally, the fewer the number of services were required for that conception.

The closest interaction coefficients from being significant were the comparison of high ODRs vs. low ODRs in the placebo group for both DFS (p=0.066) and DC (p=0.083) (Table 6.3 and 6.4, respectively). The negative coefficients for 'Placebo & High ODR' in both models indicated that higher ODR values in placebo cattle were associated with decreased hazards of detecting a first heat and of conception.

Overall, there was very little clustering of cows within herds when investigating the reproduction parameters, and the highest ICC (0.031) was found for both FSCR and DFS.

The first study to investigate the effect of anthelmintics on reproduction parameters in dairy cattle (Walsh *et al.*, 1995) reported that cows treated with Ivomec®, during the dry period, had reduced DC intervals compared to those cows which did not receive any injections (p=0.018), however, they did not see any differences for DFS intervals. Both analyses used a one-way ANOVA, without accounting for herd effects, and each pair of treated and non-treated cow was matched according to their parity, body condition, expected calving date, and previous milk production.

Both Sanchez *et al.* (2002c) and Sithole *et al.* (2006) performed clinical trials in North America, using eprinomectin. The differences in their study designs were the number and locations of herds, and their inclusion criteria. Sanchez *et al.* (2002c) had 28 herds (549 cows) from two regions (Prince Edward Island and Québec, in Canada) and required cows to have some of their nutritional needs from pasture, while Sithole *et al.* (2006) had 35 herd (2,381 cows) from three regions (Ontario and Québec, Canada, and Minnesota, USA) and only included totally or semi-confined herds.

Sanchez *et al.* (2002c) investigated the NSC, DFS, and DC intervals. They found that treated cows with high ODRs (greater than 0.5) had a significant reduction in the number of services (if conceived, p=0.04) and an increased hazard of conception (p=0.05) when compared to the placebo group with high ODRs.

Similarly to this study, Sithole *et al.* (2006) failed to show any beneficial

treatment effect, although their study suffered from low parasite exposure due to the selection process and, therefore, their results regarding reproductive performance were not conclusive.

This study had similar inclusion criteria to that of Sanchez *et al.* (2002c), where cows needed some parasite exposure from pasturing, however, we had a greater number of herds from across Canada to increase the statistical power. It is likely that there were higher proportions of herds treated with anthelmintics prior to the commencement of this study than there had been in previous studies, as suggested in Chapter 2 when comparing questionnaire responses between two studies; the first in 2005 (Chapter 2) and the second in 2008 (Chapter 5). Using anthelmintics within a herd prior to a study would have reduced the overall parasite burden, and therefore show lower ODR values. Sanchez *et al.* (2002c) reported an average ODR value of 0.49 (median = 0.47), compared to 0.30 (median = 0.24) found in this study. Regardless of the reasons, a reduction of parasite exposure would decrease the overall statistical power of the treatment/ODR interaction.

No effect of anthelmintic treatment on reproduction parameters was found. As was mentioned in the introduction, there are conflicting results, in the literature, on the effect that anthelmintics have on reproduction in beef and dairy cattle. It is, therefore, difficult to know whether this study suffered from a lack of statistical power, or simply that anthelmintics do not strongly affect (if at all) the reproduction of cows.

One variable that consistently affected reproduction, in a negative fashion, was milk yield. There is strong evidence, in the literature, that anthelmintics improve milk production (Sanchez *et al.*, 2004), and perhaps this benefit may play a small negative effect on reproduction in dairy cattle. Sanchez *et al.* (2002c) did not find milk yield to be a significant predictor for reproduction, however, they found a significant effect of treatment on reproduction. Sithole *et al.* (2006), on the other hand, found that during the early lactation period (40-61 days after calving), the hazard of inseminating a cow decreased as peak milk production increased, and there were no significant effect of treatment on reproduction.

It is possible that milk yield played an important intervening effect on reproduction in this study. However, when milk yield is removed from the models, there were no substantial differences in the significance for the interaction terms and no changes in the impact on factors of interest. There were also no significant three-way interactions between ODR, treatment, and milk yields, although, this may be a reflection of low statistical power to detect differences in a three-way interaction.

6.5. Conclusions

This study had more herds from more regions across Canada than other similar studies, and ensured some level of exposure to intestinal parasites from pasturing methods (pasture, paddock, or field access). There was no significant effect of anthelmintic treatments on reproduction, even when

accounting for the level of parasite exposure.

In the literature, there are conflicting results about the effect of anthelmintics on reproduction in beef and dairy cattle, and it is therefore difficult to account the lack of significance to a deficiency in statistical power, or simply to a weak or non-existent effect. This study also found that milk yield had a consistently negative influence on reproduction. It is well accepted that anthelmintics have a positive effect on milk yield, which perhaps contributed to some of the negative reproduction effect. Overall, there was little clustering within herds, which supports findings from other studies, where herd-level variables have little influence on reproduction, rather, it is the cow-level variables that are more important.

6.6. References

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Table 6.1. Number of services if conceived, modeled with a multilevel negative binomial model, and including 831 observations from 36 herds. No random effects for herds were included.

Variable	β	SE[b]	95% CI	P
Intercept	-0.437	0.283	-0.992,0.119	0.123
Days to First Service	-0.006	0.001	-0.009,-0.003	<0.001
Milk Yield[a]	0.011	0.002	0.006,0.016	<0.001
Interaction				0.772[c]
Placebo & High ODR	0.052	0.112	-0.168, 0.272	0.642
Tx & Low ODR	0.122	0.117	-0.107, 0.351	0.297
Tx & High ODR	0.073	0.123	-0.169, 0.314	0.556

Variance Parameters

Alpha (α)	SE*
0.680	0.085

[a] Lactation Milk Yield/100
[b] standard error for the β coefficient
[c] Overall *p*-value for treatment and ODR variables

Table 6.2. First-service conception risk, modeled with a multilevel logistical model, and including 831 observations from 36 herds with herds as random effects.

Fixed effects

Variable	β	SE[b]	OR[c]	OR 95% CI	P
Days to first-service	0.005	0.002	1.005	1.001, 1.010	0.027
Milk Yield[a]	-0.019	0.004	0.981	0.977, 0.989	<0.001
Interaction					0.390[d]
Placebo & High ODR	0.144	0.194	1.154	0.789, 1.689	0.460
Tx & Low ODR	-0.227	0.210	0.797	0.528, 1.204	0.281
Tx & High ODR	-0.046	0.214	0.955	0.628, 1.454	0.831

Random Effects

Level	Variance	SE	Rho (ρ)[e]
Herd	0.104	0.086	0.031

[a] Lactation Milk Yield/100
[b] standard error for the β coefficient
[c] Odds Ratio
[d] Overall *p*-value for treatment and ODR variables
[e] Approximation based on fixing the error variance at $\pi^2/3$ for the latent variables

Table 6.3. Days to first-service, modeled with a Cox proportional hazards model with random effects. There are 831 observations from 36 herds with herds as random effects.

Main effects					
Variable	β	*SE*[b]	HR[c]	HR: 95% CI	*P*
Milk Yield[a]	-0.022	0.007	0.978	0.966, 0.991	0.001
Interaction					0.321[d]
Placebo & High ODR	-0.181	0.098	0.835	0.688, 1.012	0.066
Tx & Low ODR	-0.048	0.103	0.953	0.780, 1.165	0.640
Tx & High ODR	-0.100	0.107	0.905	0.734, 1.115	0.349

Time-Varying Covariates				
Variable	β	*SE*	95% CI	*P*
Milk Yield	1.41×10^{-4}	0.75×10^{-4}	-0.067×10^{-4}, 2.90×10^{-4}	0.061

Frailty	β	*SE*	*P*	ICC
Theta	0.290	0.080	<0.001[e]	0.031

[a] Lactation Milk Yield/100
[b] standard error for the β coefficient
[c] Hazard Ratio
[d] Overall *p*-value for treatment and ODR variables
[e] *P*- value calculated from LRT χ^2=119.51

Table 6.4. Days to conception, modeled with a Cox proportional hazards model with random effects. There are 805 observations from 35 herds, with herd as a random effect, after removing 26 observations with erroneous conception dates.

Main effects

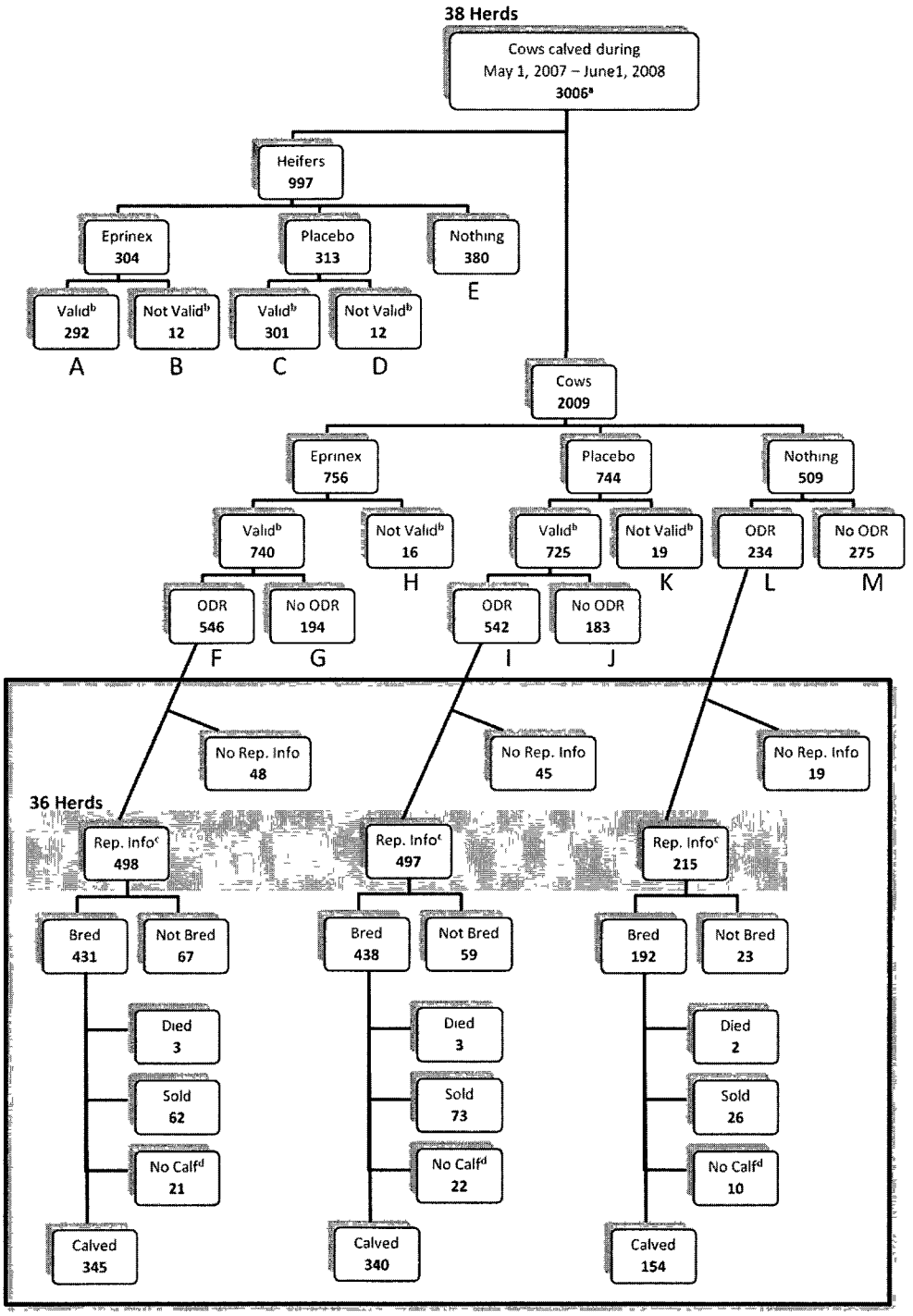
Variable	β	*SE*[b]	*HR*[c]	HR: 95% CI	*P*
Milk Yield[a]	-0.027	0.005	0.973	0.964, 0.982	<0.001
Interaction					0.278[d]
Placebo & High ODR	-0.166	0.096	0.847	0.701, 1.022	0.083
Tx & Low ODR	-0.144	0.103	0.866	0.708, 1.059	0.162
Tx & High ODR	-0.154	0.106	0.857	0.697, 1.055	0.145

Time-Varying Covariates

Variable	β	SE*	95% CI	*P*
Milk Yield	1.18×10^{-4}	0.34×10^{-4}	0.52×10^{-4}, 1.84×10^{-4}	<0.001

Frailty	β	SE*	*P*	ICC
Theta	0.034	0.021	0.010[e]	0.017

[a] Lactation Milk Yield/100
[b] standard error for the β coefficient
[c] Hazard Ratio
[d] Overall *p*-value for treatment and ODR variables
[e] *P*- value calculated from LRT χ^2=5.39



[a] Counts represent one cow-lactation
[b] Valid refers to receiving the treatment within 2 weeks pre- and 3 days post-parturition
[c] Reproduction Information consists of breeding counts, breeding dates (if bred), and calving dates (if calved)
[d] Missing data for calving date

Figure 6.1. Flow chart of subjects within the study evaluating the effect of parasite load on reproduction parameters. The diagram shows the final allocation of cows and heifers grouped by treatment and data completeness.

Chapter 7

Guidelines for the Use of an *Ostertagia ostertagi* ELISA to Predict Production Losses from Gastrointestinal Parasites in Dairy Cattle

7.1. Introduction

It is well accepted that gastrointestinal parasites, such as *Ostertagia ostertagi* and several species of *Cooperia*, adversely affects milk production in dairy cattle (Charlier *et al.*, 2007b; Charlier *et al.*, 2010; Sanchez *et al.*, 2004; Sanchez *et al.*, 2005; Sanchez *et al.*, 2002a). These gastrointestinal nematodes are ubiquitous in temperate climates (Gibbs, 1988; Louw, 1999; Williams *et al.*, 1993), however, even with their associated production losses, clinical signs of infection are rarely seen in adult cattle.

Diagnostic techniques to identify and/or quantify intestinal parasites have improved over the years. Fecal egg counts have traditionally been utilized to identify and quantify infections, however, this test is plagued with false negatives, high variability between consecutive tests (low repeatability), and overall underestimation of the level of infection (Agneessens *et al.*, 2000; Borgsteede *et al.*, 2000; Eysker and Ploeger, 2000; Gross *et al.*, 1999). Serum pepsinogen tests have had some success with quantifying levels of parasitism in first-season grazers, but were less reliable when used to quantify infections in adult cattle (Agneessens *et al.*, 2000; Borgsteede *et al.*, 2000; Gross *et al.*, 1999). Unfortunately, the quality of the pepsinogen test depends on the current life cycle stages of the nematodes in the host (Berghen *et al.*, 1993).

The first ELISA test to quantify nematode infections in cattle was developed in 1981 (Keus *et al.*), and since then, many in-house ELISA tests have been

developed for studies to quantify the infections and their related production losses (Canals and Gasbarre, 1990; Dohoo *et al.*, 1997; Eysker and Ploeger, 2000; Kloosterman *et al.*, 1984; Sanchez *et al.*, 2001; Sithole *et al.*, 2005b). More recently, Svanova (Svanova Veterinary Diagnostics, Uppsala, Sweden) developed a commercial ELISA test (Svanovir®) available in Europe (Almería *et al.*, 2009; Charlier *et al.*, 2005a; Forbes *et al.*, 2008; Guiot *et al.*, 2007). One major advantage of having a commercial ELISA test is the standardization that is now available for the test controls. Results are normalized, using control samples, and reported as optical density ratios (ODRs) (Charlier *et al.*, 2005b; Sanchez *et al.*, 2002b; Vanderstichel *et al.*, 2010), thus permitting the ELISA results to be compared between plates, kits and, to a certain extent, studies and regions. For example, Svanovir® values derived from Spanish dairy herds can be compared to those values found in Belgian herds.

The effect that intestinal parasites have on milk production in dairy cattle is well established. A meta-analysis of milk production response after anthelmintic treatments (Sanchez *et al.*, 2004) reported an overall average treatment effect of approximately 0.35 kg milk /cow/day, and an even higher effect of 0.8 kg/cow/day when evaluating three specific anthelmintics (ivermectin, moxidectin, and eprinomectin), although, it is worth noting that there was significant variation among studies. The effect of parasitism on fertility in dairy cattle has been reported inconsistently between studies (Sanchez *et al.*, 2002c; Sithole *et al.*, 2006). In addition, studies investigating

the effect of anthemintics on fertility in beef cattle, have had conflicting results (Hawkins, 1993). Therefore, the guidelines presented in this chapter will only include milk yield (kg milk/day/cow) as the measure for production losses in dairy cattle.

Study designs and investigation methods to predict the effect of intestinal parasitism on dairy production have varied between studies and regions of the world. One of the major differences between the studies was the 'unit of concern' for the analysis. When selecting the herd as the unit of concern, bulk tank (BT) milk samples were used to measure the level of antibodies against parasites, and production losses were measured at the herd-level (averaged milk production). The treatment intervention, however, could be administered either individually (cow-level) (Kloosterman *et al.*, 1996), as cows calved (Sithole *et al.*, 2005a), or to the entire herd (Charlier *et al.*, 2007b). When selecting the cow as the unit of concern, individual milk samples were taken for the ELISA test, and the individual milk yield was recorded. Once again, the intervention could be either at the individual (Ploeger *et al.*, 1990; Ploeger *et al.*, 1989; Sanchez *et al.*, 2005; Sanchez *et al.*, 2002a) or herd level (Charlier *et al.*, 2010).

Bulk tank samples are much easier to collect from farms, compared to collecting a milk sample from each cow. Running one test for the herd is financially more appealing than having to run, potentially, dozens of cow samples, especially if the producer plans to treat the entire herd. There are,

unfortunately, some disadvantages to BT samples.

Bulk tank samples report higher ELISA values than the average (arithmetic mean) of the individual ELISA values in the herd (Charlier *et al.*, 2010). A general rule for the distribution of parasites in a herd is that approximately 60-80% of the parasites are found in 20% of the herd's individuals (Leighton *et al.*, 1989; Yazwinski and Tucker, 2006), and since the ELISA test is calibrated for the linear portion of the standardized curve, values in the extreme ends of the scale are not necessarily linear. In other words, the ELISA test will give relatively similar elevated readings between samples with a high and very high antibody level. When the milk from few individuals with extremely high levels of antibodies is mixed in a bulk tank with the milk from the majority of individuals with normal or low levels of antibodies, the ELISA result from that BT will be higher than the average of the results from individual samples that contributed to the bulk tank. As such, BT measurements are susceptible to a few extreme individuals, and thus increase the variability of measurements and make prediction of milk production losses more difficult.

In an attempt to help those herds that are using Svanovir® results on bulk tank samples, Forbes *et al.* (2008) created a chart to estimate the amount of production loss in dairy herds associated with intestinal parasitism. This chart should be viewed as a rough guide for producers, since some of the inputs to generate this chart were very crude. The authors arbitrarily chose the value of 0.5 as a cutpoint for BT ODRs, which was derived from the 25th

percentile of measurements from bulk tank samples of many studies, including theirs. The authors also suggest, from the chart, that if BT ODR values are less than or equal to 0.5, gastrointestinal parasites had no effect on milk production, and if BT ODR values are greater than 0.5, milk yields will decline according to an amount determined by an estimated coefficient from another study (Charlier *et al.*, 2005a). It is not clear from their manuscript how the error bars were derived, and the relationship between milk yield and BT ODR was assumed to be linear.

The objective of this study was to utilize information and/or data from both individual milk and herd BT samples from several studies in both North America and Europe, to develop guidelines for the use of a commercial ELISA test (Svanovir®) to predict production losses (milk yield in kg/cow/day) associated with gastrointestinal parasites in dairy cattle.

7.2. Material and Methods

Combining data from three studies with similar designs (Sanchez *et al.*, 2002a; Sithole *et al.*, 2005a, Vanderstichel, Chapter 5) would increase statistical power to accurately predict the amount of milk loss (kg/cow/day) from an ELISA test result taken from an individual cow. While individual milk samples yielded better predictions of milk loss due to parasitism, it is obvious that testing a single BT milk sample would be easier and more economical.

In order to compare BT ELISA results with individual ELISA results, a series of analyses were performed, shown as boxed letters (A, B, C, and D) in Figure

7.1, where:

A. Estimates an annual herd BT ODR value from several BT ODR values (1, 2 or 3) collected within different seasons
B. Estimates the representative cow ODR from the estimated annual BT ODR
C. Estimates the representative cow milk loss (kg/cow/day) from the estimated representative cow ODR (model based on Chapter5)
D. Estimates the average individual milk loss (kg/cow/day) for the herd from the estimated representative cow milk loss

Each process ('A', 'B', 'C', and 'D') will be explained separately, with further details, below.

7.2.1. Estimating Annual BT ODR (Process 'A')

Monthly bulk tank milk samples were collected from herds participating in the 'parasite' study (Chapter 5), and analyzed with Svanovir®, to quantify the amount of anti-parasite antibodies in the milk, as described in Chapter 5. Locally weighted smoothed scatterplots (lowess) demonstrated seasonal patterns in BT ODR values.

A variable to account for time was created to capture the decreasing trend of BT ODR values over time. To quantify the decreasing trend in the BT ODR, a multilevel mixed-effects linear regression, with test day BT ODR as the dependent variable, was fit using maximum likelihood methods (Stata11, xtmixed), with structured residual errors (auto-regressive, first order [AR1])

between repeated milk measurements. Herd was set as a random variable, and the only fixed variable in the model was time. The adjusted BT ODR variable, accounting for the change over time using the estimated coefficients from the multilevel mixed-effects linear regression model for time, will henceforth be referred to as BT ODR.

To determine the number of samples required to reduce variation and increase accuracy for the estimate of the annual herd BT ODR value, a simulation with 1,000 iterations was undertaken for each sampling protocol. The options were 1, 2, or 3 BT samples from one of three seasons. Seasons were defined in trimesters, based on grazing timelines; Housed=Jan15-May14, Grazed=May15-Sept14, and Shoulder=Sept15-Jan14. Each iteration randomly chose (without replacements) 1, 2, or 3 BT samples within a specified season and ran a simple linear regression between the average annual BT for the herd and the averaged BT ODR from the randomly selected samples taken within one season. The R^2 was stored to report the correlation. The same linear regression was re-run a second time, but without a constant; the coefficient of the averaged randomly selected BT ODRs thus became a percentage of the annual BT ODR. The respective coefficients and correlations from the regression models were stored, and later their means and standard deviations were calculated and reported. Running individual model diagnostics on the regressions from each iteration (n=1000) for each combination of sampling numbers (1, 2, or 3) and seasons (grazed, shoulder,

or housed) was not feasible; as such, no model diagnostics were performed on the linear regressions.

7.2.2. Estimating the Representative Cow ODR (Process 'B')

As was previously mentioned, BT samples report higher ELISA values than the average of the individual ELISA values in a herd. Consequently, a process is required to convert a BT ODR value into an estimate for ODR for a representative cow in the herd. Data from herds containing all the individual cow's ODRs and a BT ODRs were available from two studies. Some of the herds that participated in the 'parasite' study (Chapter 5), and most of the herds that participated in a Belgian study (Charlier *et al.*, 2010) had the necessary data to quantify the difference. A simple linear regression, with and without a constant, was used to analyze the correlation and the coefficient, respectively. The coefficient, without a constant, represented the percent difference between BT ODRs and the averaged individual ODRs for each herd. Model diagnostics evaluated the residuals for heteroscedasticity and normality.

7.2.3. Estimating the Representative Cow Milk Loss (Process 'C')

The analysis to predict milk loss was nearly identical to that performed in Chapter 5, with a few exceptions to accommodate the extra data from the two other studies. Briefly, a multilevel mixed-effects linear regression, with test day milk yield (kg/cow/day) as the dependent variable, was fit using maximum likelihood methods (Stata 11, xtmixed), with structured residual

errors (AR1) between repeated milk measurements. There were two random effect variables (herd and cow), and a residual structure between test dates, creating a 3-level hierarchy to the analysis – herds, cows, and test dates (repeated cow milk yields within lactations). To calculate the treatment effect, the interaction between the treatment (placebo vs. anthelmintic) and ODR values (using a 2-degree fractional polynomial transformation) was analyzed, as explained in Chapter 5.

The major difference, between the model in Chapter 5 and the current model, was to allow the fixed 'season' variable, from Chapter 5, to span between years within studies and between studies. This variable accounted for the differences in seasons, years and studies. Also, the fixed effect variable asking producers if they had treated their herds with anthelmintics prior to the study (from the questionnaire in Chapter 5) was removed since the other two studies did not have this information. Model diagnostics were performed as described in Chapter 5.

The predicted milk loss was calculated from the coefficients of the model for each ODR unit from -0.2 to 1.2. The predicted milk loss and its associated ODR values were used to create a simpler approximated function (hyperbolic function), to allow for the milk loss to continuously increase as ODR increased. This simplified hyperbolic function, created with Advanced Grapher (2010), was needed to construct the final nomogram (section 7.2.6). The nomogram was built with software which could not computer fractional

polynomial equations, but could compute hyperbolic functions.

7.2.4. Average Individual Milk Loss (Process 'D')

This process served as both a validation process and a way to apply a final correction factor to convert 'Representative Cow Milk Loss' values into 'Average Individual Milk Loss' values. Those herds from the 'parasite' study which had the necessary data in section 7.2.2, namely that they contained all of the individual cow ODRs during one sampling period with the corresponding BT ODR, contributed to the analysis 'D'. The BT ODRs were converted to 'Representative Cow Milk Loss' values, using processes 'A', 'B', and 'C', and were compared to the average for all of the individually predicted milk losses, using process 'C', for each herd ('Average Individual Milk Loss'). Any negative milk losses, derived from low ODR values in process 'C' (ODR<=0.17, as seen in Fig. 7.5), were replaced with zero, since only positive milk loss was to be quantified and averaged for the purposes of these guidelines; process 'D', serving as a validation process, was able to account for the slight potential bias introduced from replacing negative values with zero after process 'C'.

A concordance correlation coefficient (CCC) analysis was performed to assess correlations, and using the Bradley-Blackwood *F*-test, the equality of means and variances between the two variables was tested. A simple linear regression, without a constant, assessed the percent difference between the two variables, and model diagnostics evaluated the residuals for heteroscedasticity and normality.

7.2.5. Stochastic Prediction for 'Average Individual Milk Loss'

The prediction for 'Average Individual Milk Loss', using outcomes from each analysis ('A', 'B', 'C', and 'D'), was constructed from an empty dataset, exploiting stochastic processes in Stata11.

Specific values used in the simulation, such as means, standard errors (*SE*), and standard deviations (*SD*), are available in the results section (7.3.5). The stochastic process followed the sequence, as depicted in Figure 7.1; however, it incorporated the precision parameters (such as standard errors or standard deviations) from each analysis into the estimates. Each estimated parameter was assumed to follow a normal distribution, $N(\mu,\sigma)$, however, the standard error estimate (σ) was adjusted by a Student's t distribution (where df is the degrees of freedom [n-1]) when the estimated parameter was derived from less than 40 observations.

Specifically, a dataset was created to contain 120 observations, for each season (Grazed, Shoulder, and Housed), with an 'Original BT ODR' variable starting at 0, increasing by 0.01 units, and ending with 1.2. For simplicity, the simulation process for the "Grazed season" is explained. However, the same process was repeated for each season respectively.

The 'Original BT ODR' variable was multiplied by a random variable with parameters matching those derived during the Grazed season, from analysis 'A', to generate a new variable representing the 'Annual BT ODR'. This new estimated annual BT ODR variable was further multiplied by another random

variable, with parameters determined from analysis 'B', to generate the 'Representative Cow ODR' variable.

Values from the 'Representative Cow ODR' variable were rounded to the nearest 0.01 unit in order to match correctly with the fractional polynomial predictions (and respective standard errors) for milk loss from the model in section 7.2.3. A new random variable, using respective predicted milk loss values and standard errors, was created and any negative milk yield values were replaced with zeros; the resulting variable was the 'Representative Cow Milk Loss'.

The final step for the stochastic prediction was to multiply the 'Representative Cow Milk Loss' random variable by a randomly generated variable derived from the analysis 'D'. The process was repeated 5,000 times to create 5,000 averaged individual milk loss values for each unit (0.01) from the 'Original BT ODR' values (range = 0 to 1.2).

An inherent assumption that was chosen for the stochastic model was that each process ('A', 'B', 'C', and 'D') were independent of each other, therefore, correlations and dependencies were not added to the stochastic methods. There were no biological reasons to believe correlations or dependencies existed between each process – as an example, the average of the ODR values from individual cows within a herd compared to their BT ODR should have no bearing on how individual cow ODRs influence the amount of milk loss.

The summary of the simulation results for each ODR unit during each season (a total of 360 output distributions) included: 1) the percentile value for every percentile from the 1st to the 99th, 2) the arithmetic mean, and 3) the standard deviation. With the summary data, cumulative probability plots were created, and the 5th, 50th (median), and 95th percentiles along with the mean and standard deviations were also plotted.

The sensitivity of the stochastic model was analyzed using 'spider' plots (Vose, 2008) to visually identify influential random variables (parameters in the stochastic model). Specifically, the stochastic model had four parameters, one for each process ('A', 'B', 'C', and 'D'), an initial input ('Original BT ODR'), and a recorded outcome ('Average Individual Milk Loss'). To create a 'spider' plot (adapted from Vose (2008)): (1) select the cumulative probabilities for evaluation (i.e. 1%, 5%, 25%, 50%, 75%, 95%, and 99%), (2) select a parameter distribution (i.e. from processes A, B, C, or D), and replace the distribution with one of its specified cumulative probabilities, (3) run a simulation (i.e. 1,000 iterations) and record the mean for the outcome ('Average Individual Milk Loss'), (4) select the next cumulative percentile for the parameter, and repeat step 3, (5) repeat until all selected percentiles have been run for that parameter, then put back the original distribution for that parameter and proceed to the next parameter; repeat steps 2 - 5 until all parameters have been run for the analysis. The recorded means for the outcome ('Average Individual Milk Loss') are plotted against the cumulative

probabilities for each given parameter ('A', 'B', 'C', or 'D'). The result is a line for each parameter, and the vertical range produced by that parameter shows the range of expected outcomes if the values within the parameter were fixed somewhere between the minimum and maximum of the selected percentiles; parameters with horizontal lines, therefore, have very little influence on the outcome.

7.2.6. Simplified Deterministic Nomogram

The definition of a nomogram is "a diagram representing the relations between three or more variable quantities by means of a number of scales, so arranged that the value of one variable can be found by a simple geometrical construction" (Oxford English Dictionary, 2010). One clear advantage of a nomogram is that it allows the solution for complicated calculations of various functions with simple straight lines on paper. The nomogram predicting milk loss and calculating its associated economic loss, from an average of two BT ODRs, was constructed with PyNomo (2010). The GNU General Public Licensed software could not (at the time) accommodate fractional polynomial curves, therefore, the predicted milk loss had to be simplified into a hyperbolic function, as explained in section 7.3.6. The nomogram was created in a deterministic approach, and therefore, the results are assumed to be an asymptotic approximation without specifying precision.

7.3. Results

A general summary of the results for each process ('A', 'B', 'C', and 'D') is

shown in Table 7.4, to highlight the differences and bring together the individual analyses, as they were initially displayed in Fig. 7.1. Results for each process will be presented individually.

7.3.1. Estimating Annual BT ODR (Process 'A')

There were 38 herds that contributed, on average, 12.7 BT samples between May 2007 and December 2008 (range 3 to 18). Figure 7.2 shows the lowess curves for the ODR values for each herd, categorized by percentile groups. There was a downward trend to the ODR values. The multilevel mixed-effects model demonstrated that over time (every 100 days), the ODR values would decrease by 0.0164 units. The adjusted ODR variable ('adj_ODR') followed this equation: (adj_ODR = ODR+(0.0164*100days)). The seasonal variation, using 'adj_ODR' values, can be seen in Fig. 7.3, and results from the simulation, with 1,000 iterations, are summarized in Table 7.1. For example, the annual BT ODR for a herd would be 92.8% (*SD*=3.1%) of the average of two BT samples taken during the grazing season (May 15 to September 14). The estimated correlation between the annual BT ODR and averaging one, two, or three BT samples within the grazing season were 0.818, 0.882, and 0.908, respectively.

7.3.2. Estimating the Representative Cow ODR (Process 'B')

Ten herds from the 'parasite' study (Chapter 5), and 24 herds from the study conducted by Charlier *et al.* (2010) were used to evaluate the relationship between a BT ODR and the average of individual ODRs within a herd. Figure

7.4 is a scatter plot of the BT ODRs versus the averaged individual ODRs, and results from the simple linear regressions are summarized in Table 7.2. For example, if the BT sample was taken within 10 days of the individual ODR values for the entire herd, the correlation between the BT ODRs and the averaged individual ODRs was estimated to be 0.848, and the averaged individual ODR values would be approximately 67.3% (*SE* = 2.4%) of the BT ODR values. A final value of 0.6667 was chosen as the constant for that process. Model diagnostics revealed heterscedastic and normally distributed residuals.

A variable to identify herds from both studies was added to evaluate the potential difference between studies, and it was non-significant (p=0.886). The number of days between the BT ODR and the individual ODRs ranged between 0 and 15 days with the average being 6.5 days.

7.3.3. Estimating the Representative Cow Milk Loss (Process 'C')

When combining data from the three similar Canadian studies (Sanchez *et al.*, 2002a; Sithole *et al.*, 2005a, Vanderstichel, Chapter 5), there were 87 herds, 2,018 cows with 12,524 milk samples. Sanchez *et al.* (2002a) included many herds in their study (28 herds), however, randomly chose a sub-population to undergo the clinical trial; as such, only 101 cows were included in the analysis with a total of 846 milk samples. Sithole *et al.* (2005a) had 29 herds with 829 cows, giving a total of 3,424 milk samples; the manuscript describes BT ODRs, however, individual ODRs were taken but not reported. All 37 herds

with 1,088 cows from Chapter 5 were included, giving a total of 8,254 milk samples.

The interaction term, responsible to describe the treatment effect, was statistically significant (p=0.009). The direction and magnitude of the predicted milk loss was similar to those found from the data in two of the three studies, specifically the 'parasite' study (Chapter 5) and Sithole *et al.* (2005a); Sanchez et al. (2002a) had a similar direction, however, the magnitude was much larger.

To incorporate all three studies into one model, the 'season' variable was modified; it had 14 categories starting in the 'Shoulder' period of 1999 from the study carried out by Sithole *et al.* (2005a) and ending in the 'Shoulder' period of 2008 in the 'parasite' study (Chapter 5).

It is worth noting that while the ODRs were all determined in a similar fashion, the time intervals to include ODR values during the previous late lactation period for cows did vary between studies. Sanchez *et al.* (2002a) averaged the ODRs within 90 days of calving; if ODRs were not available before 90 days, then the ODRs from the last 120 days were accepted. Sithole *et al.* (2005a) were less strict and included all ODRs within 150 days from calving (approximately the last 3 months in lactation). Only the latest ODR value was included in the 'parasite' study, and milk samples were collected after 200 DIM from the previous lactation; the median day between the test date and the calving date for the latest ODR values was 88 days (IQR 68 to 133). The

studies also had different control samples, where both Sanchez *et al.* (2002a) and Sithole *et al.* (2005a) used in-house sample controls, while Vanderstichel *et al.* (Chapter 5) used Svanova's sample controls. Since ODRs depend on the controls, it is possible that different controls could influence ODR values. The multilevel mixed-effects model of milk production, using data from all three studies, and predicting milk loss is shown in Table 7.3, however, due to the complexities of fractional polynomial coefficients, the predicted milk loss values are plotted in Figure 7.5. The equation for the fractional polynomial prediction is:

$$\begin{aligned} &Representative\ Cow\ Milk\ Loss\ (\mathrm{kg/cow/day}) \\ &\quad = \{[(ODR^{-2} - 0.5754) * -0.0068] \\ &\quad + [((ODR^{-2} * \ln(ODR)) - 0.1590) * 6.2274] + 0.4572\} \\ &\quad - \{[(ODR^{-2} - 0.5754) * -0.5892] \\ &\quad + [((ODR^{-2} * \ln(ODR)) - 0.1590) * -6.7896]\} \end{aligned}$$

The model assumptions of heteroscedasticity and normality were verified, and there was no visual indication of any violations; no transformations of the outcome were deemed necessary.

7.3.4. Average Individual Milk Loss (Process 'D')

This process ('D') required individual ODR values for all the cows in the herd, with a concurrent BT ODR measurement. The same 10 herds from the 'parasite' study (Chapter 5), that were included in section 7.3.2, also contributed to this analysis. However, Charlier *et al.* (2010) only reported the average of the individual ODR values, without providing the individual ODR

values; therefore, this data was not used for this analysis. Overall, there were ten observations generated for both the 'Representative Cow Milk Loss' and 'Average Individual Milk Loss' variables. The Bradley-Blackwood *F*-test from the CCC analysis revealed that the two variables had different means and variances (p=0.019). The CCC was much lower than the Pearson's correlation (0.743 vs. 0.878), and Fig 7.6 shows the scatterplot for 'Average Individual Milk Loss' vs. 'Representative Cow Milk Loss' from the CCC analysis. The linear regression estimated the percent difference between the two variables at 0.8747 (SE=0.1199), therefore, the 'Average Individual Milk Loss' was ~87.5% of the 'Representative Cow Milk Loss', for those ten herds. Model diagnostics revealed heterscedastic and normally distributed residuals.

7.3.5. Stochastic Prediction for 'Average Individual Milk Loss'

The results from the analyses for the four processes (7.3.1 to 7.3.4) were used to create the stochastic predictions. Specifically, the parameters for each season, from process 'A', were: Grazed, mean=0.9281 with a SE=0.0309; Shoulder, mean=0.9500 with a SE=0.0256; Housed, mean=1.005 with a SE=0.0363. The parameters from process 'B', to convert 'Annual BT ODR' to 'Representative Cow ODR', were: mean=0.6667 (approximation from Table 2), with a SE=0.0270 (largest standard error in Table 2), and the standard errors were adjusted using the Student's t distribution (t(33)) to account for the small sample size of 34 herds in the parameter estimate.

To convert the 'Representative Cow ODR' to 'Representative Cow Milk Loss'

(kg/cow/day), in process 'C', the estimate and standard errors from the model in section 7.3.3 (and shown in Fig. 7.5) were used to match with the 'Representative Cow ODR' (rounded to the nearest 0.01 unit). For the final step in the stochastic process ('D'), the 'Representative Cow Milk Loss' was multiplied by another randomly generated variable derived from the analysis 'D' (mean=0.8747, *SE*=0.1199); the standard error was also adjusted using the Student's t distribution (t(9)) to account for the small sample size of 10 herds in the parameter estimate.

The summary of the stochastic results for the 'Grazed' season is plotted in Figure 7.7, showing the arithmetic mean, +/- one standard deviation, 5th, 50th (median), and 95th percentiles for the averaged individual milk loss at each original ODR value. The summary plots for the 'Shoulder' and 'Housed' seasons (not shown) were very similar to the 'Grazed' season, however, their subtle differences are better shown with a cumulative probability plot (Fig. 7.8). The cumulative probability plot was calculated to show percentile values for each season when the treatment effect was greater than zero, therefore, the y-axis represent the probability of a positive treatment effect, while the x-axis shows the ODR values.

The results for the sensitivity analysis ('spider' plots) are shown in Fig. 7.9. An individual 'spider' plot could have been created for each 'Original BT ODR' value (range from 0 to 1.2 with 0.01 increments), however, only four are shown to demonstrate how the influence of parameters change as the

'Original BT ODR' values increase.

7.3.6. Simplified Deterministic Nomogram

PyNomo is a software running on the powerful programming language, Python. The software is still in its infancy, having released its first version in 2007, and maintained by a programmer as a hobby.

To convert the average of two BT ODRs within a season to an 'Average Individual Milk Loss' (kg/cow/day), several calculations and transformations were needed. Following the sequence from Fig. 7.1, the average of two BT ODRs, using Svanovir®, from one season (Housed, Grazed, or Shoulder) became the starting value 'Seasonal BT ODR'. The first transformation (process 'A') was to adjust the 'Seasonal BT ODR' from one season to reflect the 'Annual BT ODR' for that herd by multiplying 'Seasonal BT ODR' by one of three constants, depending on the season (as found in Table 7.1); for 'Housed' (Jan15-May14), the constant was 1.005, for 'Grazed' (May15-Sept14), the constant was 0.9281, and for 'Shoulder' (Sept15-Jan14), the constant was 0.9500. The 'Annual BT ODR' was converted to a 'Representative Cow ODR' by multiplication with an approximated coefficient derived from analysis 'B' (0.6667). The resulting 'Representative Cow ODR' then needed to be converted to a 'Representative Cow Milk Loss' value (kg/cow/day), using process 'C'. The model from section 7.3.3 was applied, except that the fractional polynomial function was replaced by the hyperbolic function ((-0.181/ODR)+1.017). Figure 7.5 shows the difference between the fractional

polynomial and the hyperbolic prediction; the major difference was that the hyperbolic function assumed that the treatment effect continuously increased, while the fractional polynomial peaked when ODR=0.58, and then gradually decreased. The 'Representative Cow ODR' was thus converted to a 'Representative Cow Milk Loss' using the hyperbolic function.

The final adjustment was to multiply the 'Representative Cow Milk Loss' values by 0.8747 (derived from process 'D') to determine the outcome ('Average Individual Milk Loss', kg/cow/day).

The graph on the left in the nomogram performs the following calculation, summarizing all four processes into one equation:

$$Avg\ Ind\ Milk\ Loss\ (\text{kg/cow/day}) = \left[\left(\frac{-0.181}{BT_{ODR} * SC * 0.6667}\right) + 1.017\right] * 0.8747$$

Where: BT_{ODR} *= Average of 2 BT ODRs taken within one Season*

SC ≡ Seasonal Constant = 1.0005 for Housed

= 0.9281 for Grazed

= 0.9500 for Shoulder

Note: Only Positive Milk Loss values are multiplied by 0.8747

The nomogram, which converts the estimated 'Average Individual Milk Loss' to an estimated economic cost, is shown in Fig. 7.10. The Python script used in PyNomo to produce the nomogram is available in Appendix C.

A second y-axis was added to the nomogram (labeled as C in Figure 7.10) to include the cumulative probability of having a positive treatment effect, as derived from the stochastic methods explained above. These approximated probabilities should help the interpreter to understand the amount of

uncertainty that is inherently present with these estimates.

7.4. Discussion

The purpose of the 'parasite' study (Chapter 5), and the analyses based on the combined data from three studies presented in this chapter, was to quantify the relationship between ODR and response to treatment. To increase accuracy for the estimate, the individual cow was the unit of concern. On average, if you treat any cow with an anthelmintic at calving (at any given time during the year, provided she is at least in her second lactation) you would expect to increase her milk production throughout her current lactation if her previous late lactation ODR value was greater than ~0.2. The estimated amount of milk loss has been plotted in Fig. 7.5. However, producers would rather use the herd as the unit of concern for economic and practical reasons.

To provide guidelines for BT samples, several analyses had to be performed; the serial accumulation of these steps, each with their strengths and weaknesses, provided the final BT guidelines. Each step will be discussed individually.

7.4.1. Estimating Annual BT ODR (Process 'A')

This process converts two BT ODR values taken within one season, to an estimate of the 'Annual BT ODR' for the herd. This value was used as an overall estimate of the parasite burden in the herd that was independent of the season of testing.

An important component to this analysis was to determine the number of samples needed within one season. The results showed that the largest improvement in correlations (as shown in Table 7.1) occurred between sampling once and twice within a season (average increase of 7.7%); there was an improvement between sampling twice and thrice (average increase of 2.5%), however, given the large improvement in correlations between one and two samples, compared to two and three samples, it was deemed that two samples would be sufficient to represent the herd BT within one season, while making it practical enough for producers to comply with the sampling scheme. The 'parasite' study (Chapter 5) had a downward trend to the ODR values as the study progressed. This was likely due to the continuous treatment of every 2nd calving cow throughout the study, thus reducing the overall parasite burden in the herd. The final estimates for analysis 'A' had factored in a time-dependent correction factor. The pattern for the final estimate of seasonal effects was very similar to what has been reported in several studies. Sanchez & Dohoo (2002), in Prince Edward Island, Canada, had the smallest ODR values in January and progressively increased until October, and Charlier *et al.* (2007b), in Belgium, had the peak BT ODR in July-August (~0.7) with the trough being in January-February (~0.4). The extremes in the values were much larger in Belgium (~0.30 ODR difference) compared to the Canadian ones (~0.02 ODR difference in this study and ~0.15 ODR difference in the study conducted by Sanchez & Dohoo). This large difference in extreme

values could be partially attributed to the climatic differences, and perhaps, to a lesser extent to the analytical time-dependence factor applied to this study.

7.4.2. Estimating the Representative Cow ODR (Process 'B')

This process converts the estimate of the 'Annual BT ODR' to an estimate of the 'Representative Cow ODR' for the herd. This value was used as an estimate of a hypothetical 'average' cow in the herd being evaluated. The data collected from the 'parasite' study were too few (10 herds) to estimate the 'Representative Cow ODR', and therefore data from another study in Belgium were included into the analysis (24 herds). The results from analysis 'B', based on data from these two studies (50% increase in 'Annual BT ODR' compared to 'Representative Cow ODR'), were similar to those reported by an earlier small study by Charlier *et al.* (2007a), where there was a 53% increase in BT ODRs compared to the average of the individual ODRs in two herds.

There are many factors that contribute to the BT ODRs, including, but not limited to, overall level of infection (high or low), individual level of infection (what proportion of the individuals carry the majority of the parasites), milk yield (dilution factor), number of cows contributing to the BT, etc.

The purpose of this analysis was not to identify or quantify the effect of important factors contributing to a BT ODR, but rather to develop a simple estimate for the overall difference (and its *SE*) between the BT ODR and an expected cow level ODR from the same herd.

7.4.3. Estimating the Representative Cow Milk Loss (Process 'C')

This process converts the 'Representative Cow ODR' to an estimate for the 'Representative Cow Milk Loss' (kg/cow/day) for the herd. The fractional polynomial, estimated from the model in section 7.3.3, is a statistically significant predictor of milk production losses (kg/cow/day) based on individual ODR values taken from a milk sample from the cow's previous lactation. There is no simple function for the fractional polynomial, and it was easier to generate a prediction (with its relevant *SE*) for each ODR unit than to mathematically convert values.

For simplicity, a hyperbolic function that closely matched the fractional polynomial was generated. The major difference between the two functions was that the hyperbolic function assumed that milk loss continued to increase (although at a very slow rate) as ODRs increased past 0.6 (Fig. 7.5); this assumption was considered to be biologically plausible. There is no reason to expect any reduction in the effects of parasites at ODR values >0.6. The observed decline (based on the fractional polynomial) was probably a function of very few observations in this range in the data. The predicted production loss at ODR=0.5 from this combined analysis was ~0.6 kg/cow/day. For all the studies predicting milk production responses to anthelmintic treatments (as found in the literature and summarized in Table 5.1), losses could be as high as ~2.5 to 6 kg/cow/day for ODR values greater than 0.5 (Charlier *et al.*, 2007b; Charlier *et al.*, 2010; Ploeger *et al.*, 1989; Sanchez *et al.*, 2005; Sanchez *et al.*, 2002a). Only one study has reported more conservative

losses at 0.385 (Sithole *et al.*, 2005a), however, only cattle with limited outdoor exposure were included in that study. Consequently, the losses predicted by the nomogram developed in this study are likely conservative relative to those that might be based on other literature estimates.

7.4.4. Average Individual Milk Loss (Process 'D')

This process converts the estimate for the 'Representative Cow Milk Loss' (kg/cow/day) for the herd to the 'Average Individual Milk Loss' (kg/cow/day). This final analysis ('D'), to the knowledge of the authors, has never been reported in literature. It was a necessary analysis since milk loss from one 'Representative Cow Milk Loss' value for the herd was not likely going to be the same as averaging the milk losses from each individual cow in the herd. In a way, this process also served as a validation method to compare real BT ODR values that were converted into 'Representative Cow Milk Loss', using processes 'A', 'B', and 'C', with those from individual cow ODR values that were converted into 'Average Individual Milk Loss', using process 'C' (as shown in Fig. 7.1)

There were only ten herds with the necessary data for this analysis, so the results of the analysis have limited precision. However, the scatterplot from the CCC analysis (Fig. 7.6) shows that 'Representative Cow Milk Loss' values less than 0.4 kg/cow/day (as calculated from BT ODRs within one season and using the four processes ('A', 'B', 'C', and 'D') as illustrated in the nomogram) will likely underestimate the 'true' amount of milk loss for the herd, had each

cow been individually sampled and their predicted individual cow milk loss values averaged for the entire herd. The opposite is also true, when 'Representative Cow Milk Loss' values were greater than 0.4 they generally overestimated the true 'Average Individual Milk Loss' for a herd.

7.4.5. Stochastic Prediction for 'Average Individual Milk Loss'

The median values from the stochastic predictions were very similar to those found in the deterministic nomogram. This was anticipated because the only mathematical difference between the two is found in process 'C', where the stochastic method used the fractional polynomial equation (with relative standard errors), while the deterministic method used the hyperbolic function. The cumulative probability plot (Fig. 7.8) highlights some of the differences between the seasons, where this difference proportionally increases as averaged BT ODR values increase. Averaged BT ODR values less than the 5th percentile cutpoint for treatment effect (Housed<=0.12, Shoulder<=0.13, and Grazed<=0.13) are deemed to produce a production response to treatment only one time out of 20. Similarly, averaged BT ODR values greater than the 95th percentile for treatment effect (Housed>0.52, Shoulder>0.56, and Grazed>0.57) are deemed to be large enough to produce a treatment effect 19 times out 20. Averaged BT ODR values above the 50th percentile (Housed>0.27, Shoulder>0.28, Grazed>0.29) would be expected to result in a response to treatment more often than not (better than a coin toss). This is consistent with the deterministic nomogram which indicates that response to

treatment is expected to start in the 0.26-0.29 range (depending on the sampling season). Averaged BT ODR values, greater than the 0.52-0.57 range, will consistently predict a beneficial treatment effect.

The results from the sensitivity analysis showed that the random variable for the milk loss (process 'C') was the most influential on the outcome ('Average Individual Milk Loss'), followed by the random variable for process 'D', converting the 'Representative Cow Milk Loss' to the 'Average Individual Milk Loss'. Interestingly, both processes 'A' and 'B' had a very small influence on the outcome ('Average Individual Milk Loss').

The most influential parameter ('Process C') was derived from the model predicting milk loss. The second most influential parameter (process 'D') was derived from the analysis with the smallest sample size (n=10).

The easiest study to repeat in the future (by increasing the sample size), with the ability to reduce uncertainty in the stochastic prediction, would be the study that was included for analysis 'D'; having more herds with both a BT ODR and ODRs from each individual cow simultaneously would improve the estimates for analysis 'D' and thus improve the stochastic model. Reducing the standard errors within process 'C' would be very difficult since the combined clinical trials to obtain those standard errors (derived from the fractional polynomial model) included more than 2,000 cows.

7.4.6. Simplified Deterministic Nomogram

The nomogram was developed as a tool for diagnosticians, veterinarians, and

producers to interpret ELISA results, such as Svanovir®, in terms of milk yields (kg/cow/day) in dairy cattle for North American herds. Though some of the data for this study were derived from European studies, the majority of the influence on the final outcome to predict milk loss relied on the estimates from three Canadian studies investigating the prediction ability of an ELISA test from individual cow samples.

Canadian ODRs are usually smaller than European ODRs (based on observations from studies summarized in Table 5.1), which may be due to different climates. The grazing seasons in Europe are longer than those in Canada, and winters are milder than the Canadian winters; these two factors would probably increase infection levels in Europe (Yazwinski and Tucker, 2006). However, a difference in ODR values is not the same as a difference in the effect of intestinal parasites on milk production.

Some factors which may influence the effect of parasites on a cow's milk production could be related to the differences in nematode populations between regions (e.g. genetic diversity), or the differences in dairy cattle populations (e.g. maximum production capacity, immune status, genetics, etc.). It is still reasonable to assume that while there are some differences between the populations of nematodes and dairy cattle around the world, those similar populations will probably share similarities in how nematodes affect dairy cattle milk production. As such, estimates from these guidelines may be applicable in other regions of the world, even if there are climatic

differences.

Forbes *et al.* (2008) had developed a chart for BT samples taken in Europe, as explained in the introduction, and seen in Figure 7.11. Some of the differences between the two guidelines (nomogram vs. Forbes *et al.* (2008) chart) are that the European predicted milk losses and BT ODR values are much larger since they are based on another study by Charlier *et al.* (2005a) and they estimated the milk loss to increase linearly from 0 to 3.2kg/cow/day when BT ODR values increase from 0.5 to 1.5. In contrast, the nomogram has a maximum BT ODR value of 1.0 with little increase in milk loss above 0.6 kg/cow/day. Another difference is that Forbes *et al.* (2008) assume that there is no milk loss when ODR values are less than 0.5. The nomogram from this study shows that some milk loss can be expected from ODR values greater than ~0.25. Forbes *et al.* (2008) do not explain how their *SE* were derived, in contrast, the uncertainties are shown and explained in this study. The last minor difference is the y-axis in the nomogram is inverted compared to the European guidelines.

Although uncertainties have been included in the study, care is still needed when interpreting values from this study, particularly when using the nomogram. It is difficult to appreciate the amount of uncertainty that is present with the deterministic methods used for this model, which is why a second y-axis, with an approximate cumulative probability of having a positive treatment effect, was added to the nomogram. Even this cumulative

probability of positive treatment effect is an approximation from the observed data that comprised this study.

The final estimates from the overall model, which include processes 'A' through 'D', were derived specifically from the studies described in this chapter, and it will be important to update this overall model as new data is collected from other studies. It is also probable that new data will yield results that may be slightly different to what has been estimated and graphed in this chapter. Therefore, it is important to understand that these estimates should be used as a rough guide for interpretation.

7.5. Conclusion

The best scenario to predict milk loss in cattle due to gastrointestinal parasitism is to test every individual cow in the herd and derive individual milk loss values for each cow; treatments would then be considered individually. For economic and logistical reasons, producers would rather test BT milk samples to decide if a herd-level intervention against intestinal parasites should be undertaken (i.e. pasture rotation, introduce an anthelmintic treatment programme, etc.).

The nomogram developed in this study is currently the most informative option for North American dairy herds planning to use an ELISA test (e.g. Svanovir®) to predict milk loss associated with gastrointestinal parasites from BT milk samples. Like all guidelines, these should remain dynamic and flexible, and should also be updated on a regular basis, as more information

is made available. More specifically, as additional information between 'Representative Individual Milk Loss' and the 'Average Individual Milk Loss' is made available, the better the estimates for the guidelines will be.

The nomogram was developed deterministically, as a guide for decision makers, however, stochastic processes give better understanding of overall uncertainties, but at a cost of complicating the final outcome and decision. Some of the findings from the stochastic process can be applied to the nomogram. Specifically, when ODR values fall below the nomogram scale (<~0.26), the probability of an effect from treatment falls below 50%. However, when ODR values are greater than 0.57, there will be a benefit from treatment 19 times out of 20. Treating a herd with BT ODR values (2 samples averaged within one season) between 0.26 and 0.56 will likely yield beneficial results (greater than 50% and less than 95% chance of success), and the predicted milk yield values from the nomogram will show what would be expected in the long run (asymptotically). There is some evidence, albeit from a small sample size of 10 herds, that estimated milk loss values less than 0.4 kg/cow/day will likely underestimate the true value for that herd, while estimated milk loss values greater than 0.4 may overestimate the true value.

Nevertheless, the nomogram, intended to inform decision makers, will help with the ultimate decision to control (or not control) for gastrointestinal parasites, however, the choice of methods to control remains with the veterinarians and the producers, and should be considered on a farm-by-farm

basis.

7.6. References

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Table 7.1. Summary results from 'Process A' analysis, using a simulation with 1,000 iterations for each combination of seasons and sampling protocols.

Combinations	Correlations (R)		Coefficients (no constant)	
	Mean	St. Dev.	Mean	St. Dev.
Grazed, 1 Sample	0.8181	0.0582	0.8942	0.0559
Grazed, 2 Sample	0.8815	0.0274	0.9281	0.0309
Grazed, 3 Sample	0.9077	0.0156	0.9360	0.0199
Shoulder, 1 Sample	0.8687	0.0504	0.9520	0.0424
Shoulder, 2 Sample	0.9001	0.0220	0.9500	0.0256
Shoulder, 3 Sample	0.9027	0.0200	0.9448	0.0243
Housed, 1 Sample	0.7778	0.0531	0.9607	0.0742
Housed, 2 Sample	0.8698	0.0241	1.0005	0.0363
Housed, 3 Sample	0.9070	0.0111	1.0139	0.0129

Table 7.2. Regression analysis between BT ODRs and averaged individual ODRs, as part of 'Process B' analysis. 'Days Apart' refers to the number of days between the BT ODR and the individual ODRs for each herd.

Days Apart	(n) herds	Coefficient (no constant)	Standard Error (no constant)	Correlation (R)
<= 7	19	0.6830	0.0270	0.8168
<= 10	26	0.6727	0.0241	0.8481
<= 15	34	0.6561	0.0224	0.8185

Table 7.3. Results from the multilevel mixed-effects model to predict milk production, as part of 'Process C' analysis. The model contains 87 herds, 2,018 cows, and 12,524 observations for milk yield (kg/cow/day).

Fixed effects

Variable	β	SE	95% CI	P
Intercept	150.813	2.946	145.040, 156.586	<0.001
Time Period				<0.001
Shoulder 1999	Baseline			
Housed 2000	-1.781	1.254	-4.240, 0.678	0.156
Grazed 2000	-2.292	1.308	-4.854, 0.271	0.080
Shoulder 2000	-1.951	1.374	-4.645, 0.743	0.156
Housed 2001	-1.830	1.607	-4.981, 1.320	0.255
Grazed 2002	0.293	1.619	-2.881, 3.466	0.857
Shoulder 2002	-0.752	1.592	-3.872, 2.368	0.637
Housed 2003	-0.277	1.595	-3.403, 2.848	0.862
Grazed 2003	-0.742	1.643	-3.963, 2.479	0.652
Grazed 2007	1.112	1.468	-1.766, 3.989	0.449
Shoulder 2007	0.575	1.439	-2.247, 3.396	0.690
Housed 2008	1.987	1.439	-0.833, 4.806	0.167
Grazed 2008	2.238	1.450	-0.604, 5.080	0.123
Shoulder 2008	1.402	1.474	-1.486, 4.290	0.341
Calving Season				<0.001
Grazed	Baseline			
Shoulder	1.177	0.368	0.455, 1.899	0.001
Housed	1.286	0.296	0.706, 1.866	<0.001
Lactation Group				<0.001
2nd	Baseline			
3rd	2.360	0.304	1.764, 2.957	<0.001
4th and greater	2.238	0.291	1.668, 2.808	<0.001
Days in milk				<0.001
DIM centered	-0.123	0.002	-0.126,-0.119	<0.001
DIM $^{0.05}$	-145.072	3.153	-151.253,-138.891	<0.001
Log Somatic Cell Count	-1.016	0.044	-1.103,-0.929	<0.001
ODR and Treatment Interaction§				0.009*

Random Effects

Level	Variance	Standard Error	Rho (p)
Herd	11.811	2.226	
Cow	17.055	1.037	
Residual (AR(1))	29.733	0.719	0.443

§ FP structure of treatment and ODR are described in text and displayed graphically

* Likelihood Ratio Test between the full (displayed in this table) and the reduced model (not shown)

Table 7.4. Summary results for each process (A, B, C, and D), as illustrated in Figure 7.1.

	Process	Est.	*SE*	Cumulative Percentile 1	5	25	50	75	95	99	Data Required	Data Used
A	Grazed	0.928	0.031	0.856	0.877	0.907	0.928	0.949	0.979	1.000	Multiple BT samples Throughout the year	Clinical Trial (n=38 herds)
	Shoulder	0.950	0.026	0.890	0.908	0.933	0.950	0.967	0.992	1.010		
	Housed	1.005	0.036	0.921	0.945	0.981	1.005	1.029	1.065	1.090		
B		0.667	0.027	0.604	0.623	0.649	0.667	0.685	0.711	0.730	BT ODR and average of individual cow ODR	Subpopulation of CT and Belgian Data (n=34 herds)
C	ODR=0.2	0.105	0.313	-0.623	-0.410	-0.106	0.105	0.316	0.619	0.832	Individual cow data from clinical trials	Clinical Trials from Chapter 5, Sanchez et al. (2002a), and Sithole et al. (2005a) (n=87 herds)
	ODR=0.5	0.657	0.326	-0.101	0.121	0.437	0.657	0.877	1.193	1.414		
	ODR=0.7	0.644	0.463	-0.434	-0.118	0.331	0.644	0.956	1.406	1.722		
D		0.875	0.120	0.596	0.677	0.794	0.875	0.956	1.072	1.154	BT ODR and individual cow ODR from at least 75% of the cows in a herd	Subpopulation of CT (n=10 herds)

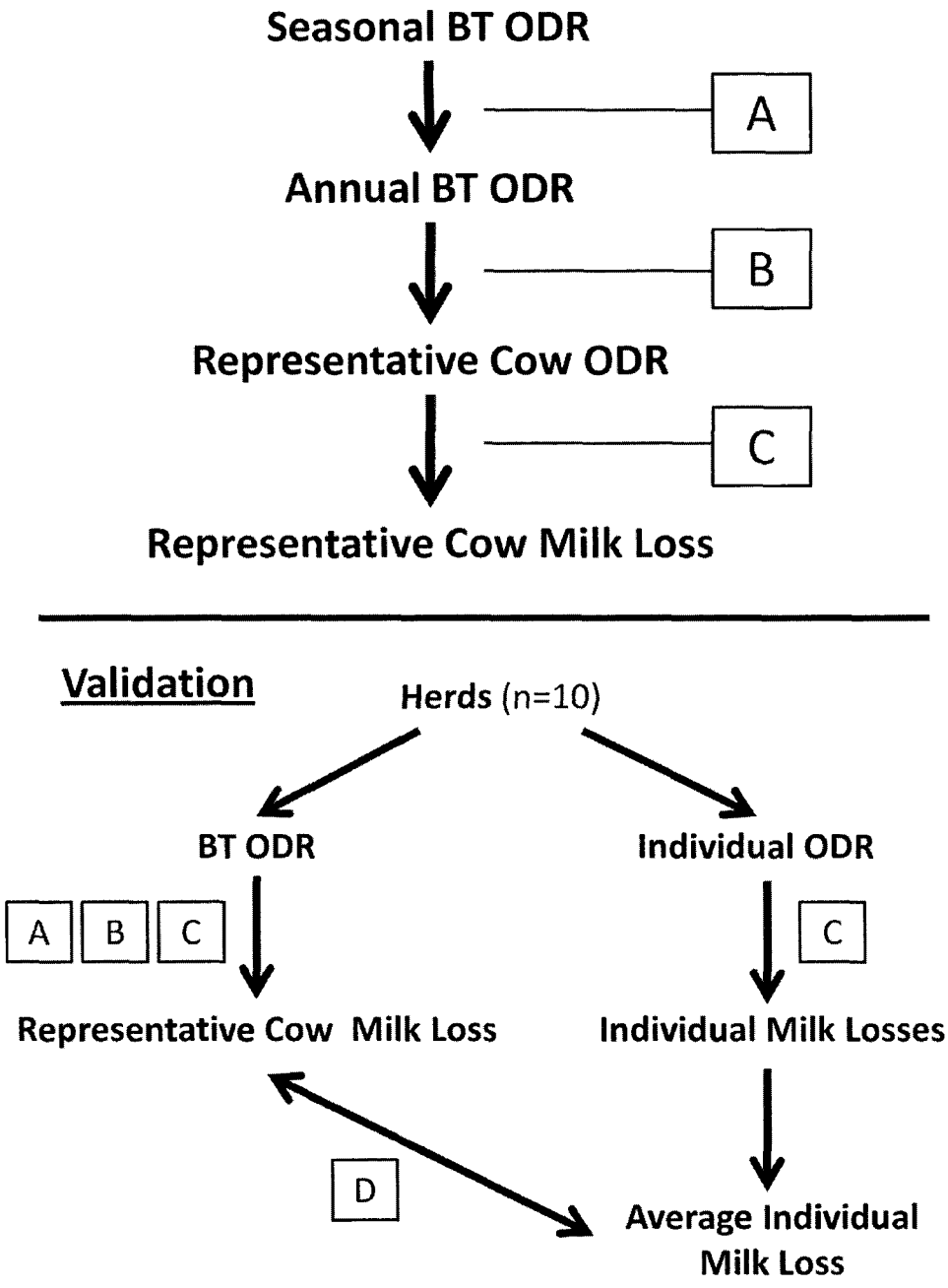

Figure 7.1. Flow chart for the sequence of analyses required to develop guidelines. The four boxes containing letters (A, B, C, or D) are the analyses and indicate their location in the sequence. The final outcome for the guidelines is to predict the amount of milk loss that is associated with intestinal parasitism of dairy cattle ('Average Individual Milk Loss').

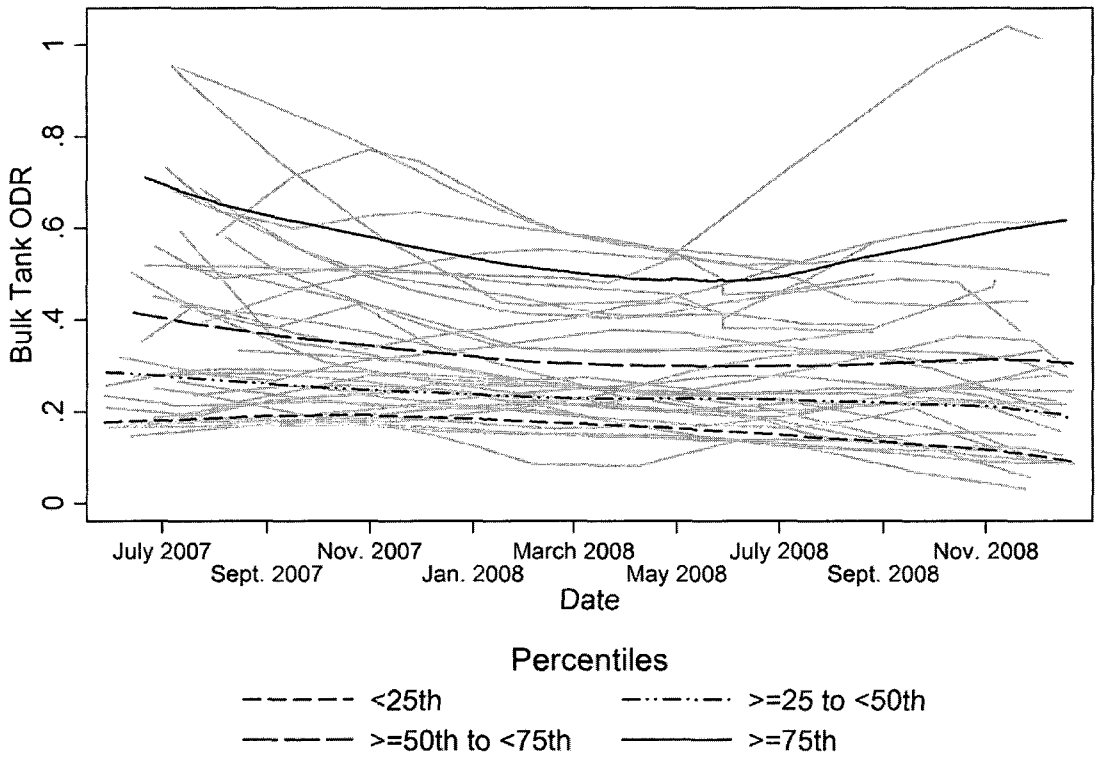

Figure 7.2. Locally weighted smoothed scatterplot curves for BT ODRs. Specifically, grey lines are lowess curves for BT ODRs, from 38 herds, over the course of the study. Black lines are averaged BT ODRs for herds falling within one of four percentile groups (<25th, 25th to <50th, 50th to <75th, and greater than and equal to 75th). Notice the downward trend to the values over time.

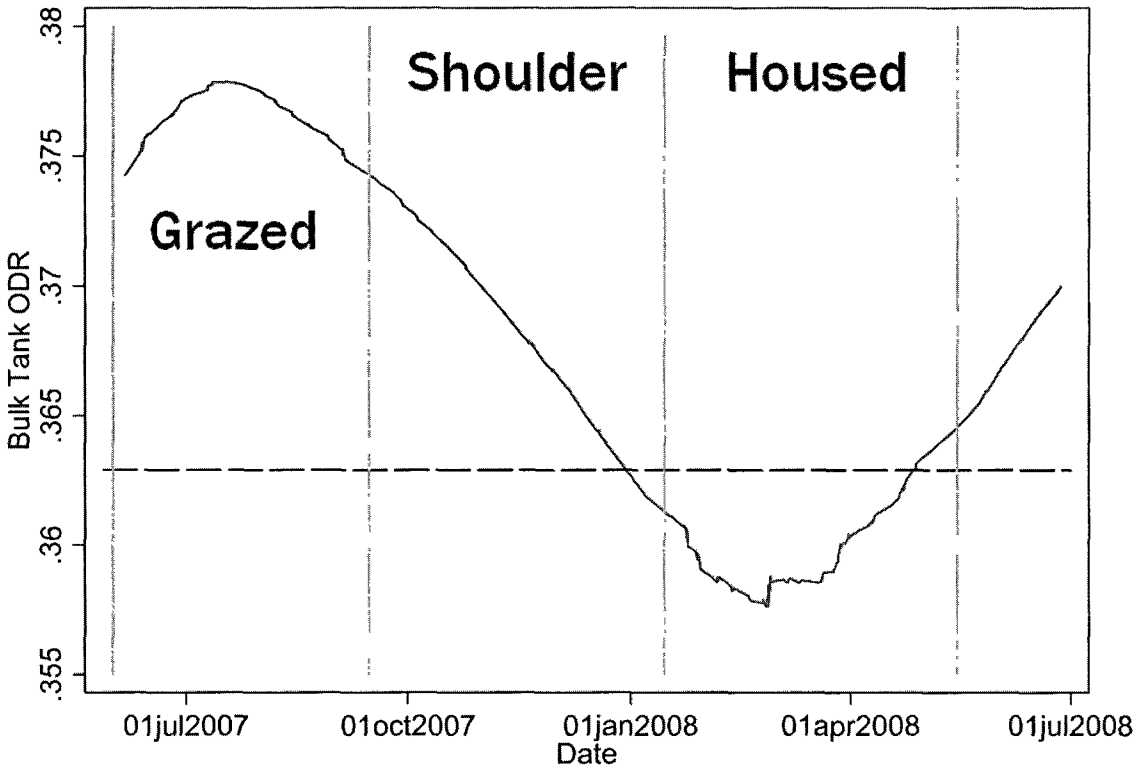

Figure 7.3. Seasonal variation of BT ODR values using time-adjusted ODR. Vertical lines demarcate the seasons (Grazed, Shoulder, Housed). Horizontal dash-line is the average annual BT ODR for all the herds.

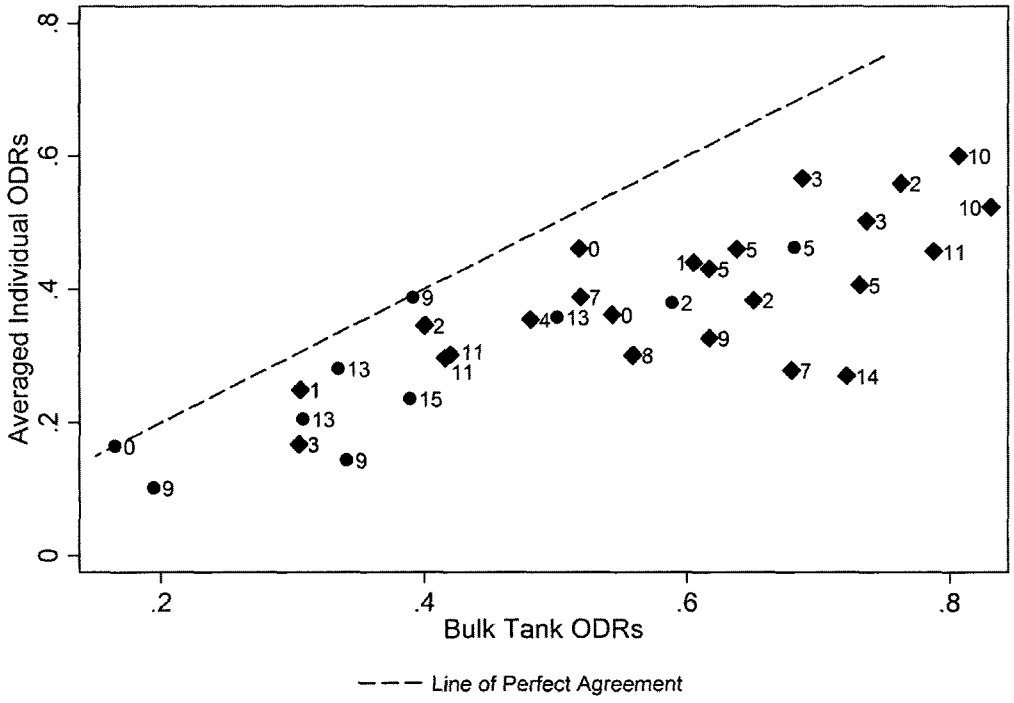

Figure 7.4. Individual versus BT ODR values. Diamonds are herds from Charlier *et al.* (2010) and circles are herds from the 'parasite' study (Chapter 5). Numbers indicate the differences in days between sampling the cows and the BT milk sample. The dashed line represents perfect agreement between the two variables.

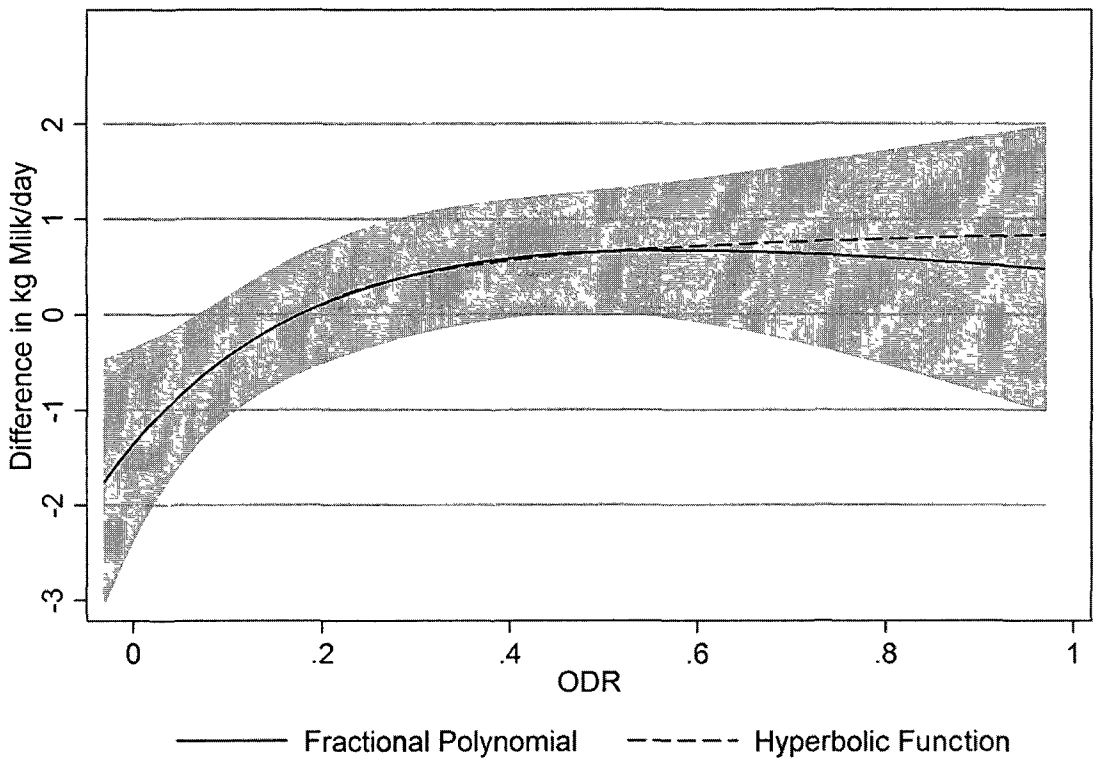

Figure 7.5. Predicted milk loss associated with intestinal parasitism from individual cow ODR values, using combined data from three Canadian studies. Shaded area represents the 95%CI for the fractional polynomial estimates. The fractional polynomial predictions are based on a model for the effects of ODR, and the hyperbolic function is based on the fractional polynomial predictions.

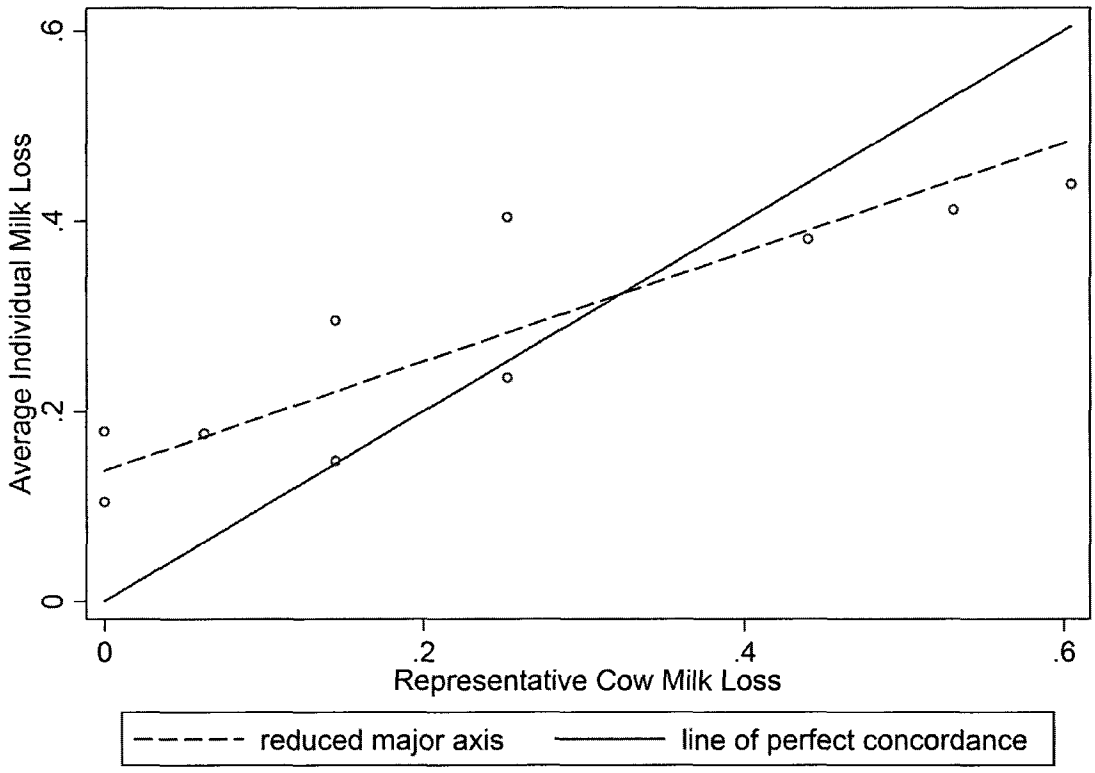

Figure 7.6. Scatterplot, from the concordance correlation coefficient in process 'D', **for 'Average Individual Milk Loss' versus 'Representative Cow Milk Loss'.**

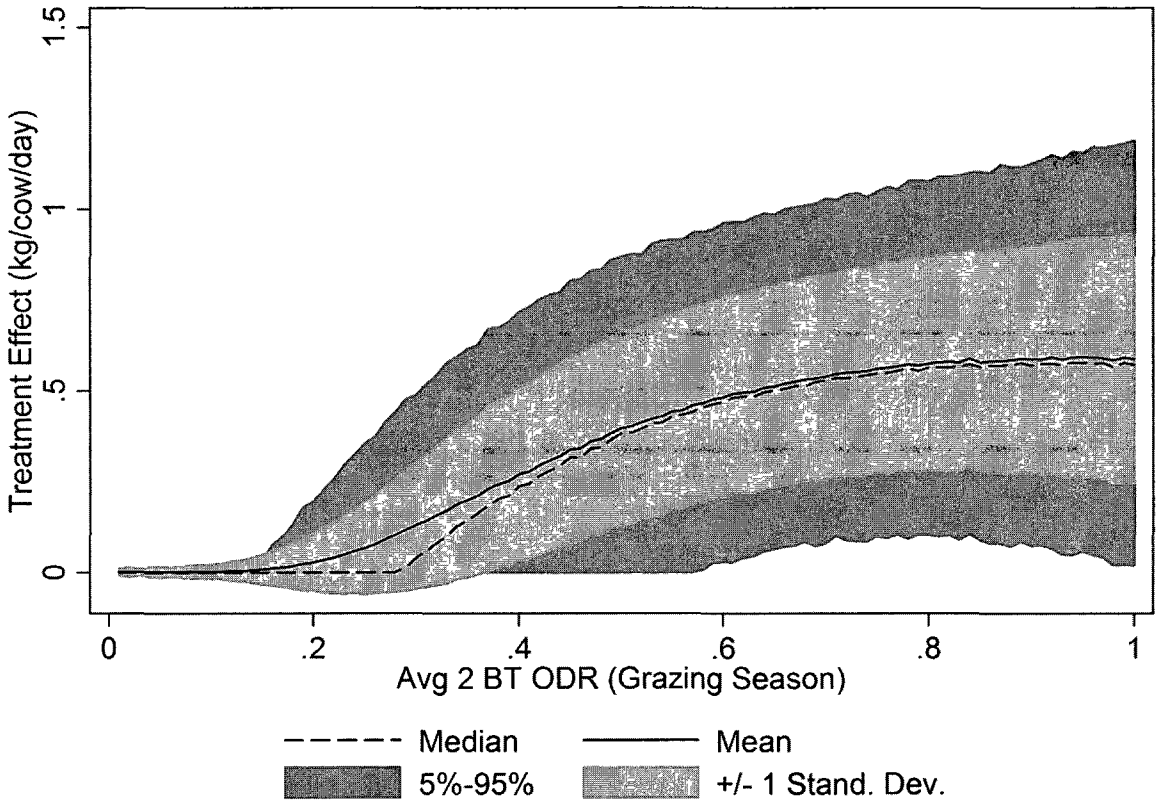

Figure 7.7. Stochastic prediction for the averaged individuai milk loss. The 5th, 50th (median), and 95th percentiles, as well as the mean and standard deviation for the averaged individual milk loss (kg/cow/day) are shown for the average of two BT ODR values taken during the grazing season.

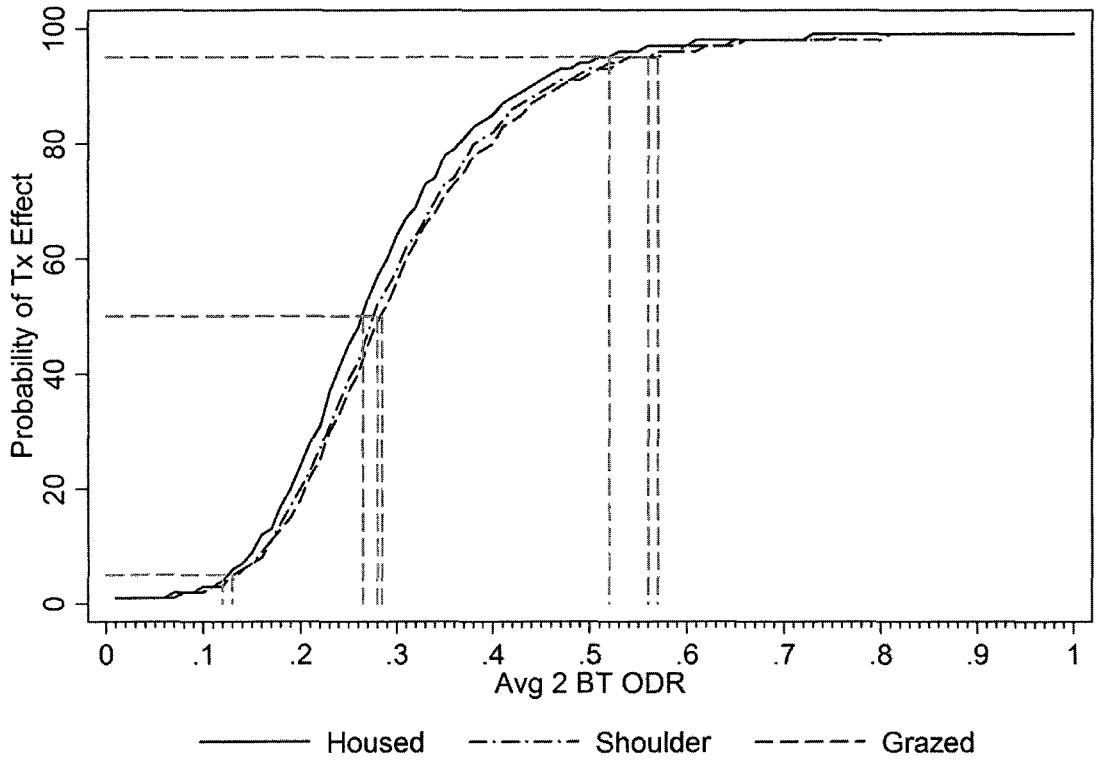

Figure 7.8. Cumulative probability of treatment effect. *Horizontal dashed lines are present at the 5th, 50th and 95th percentiles for treatment effect.*

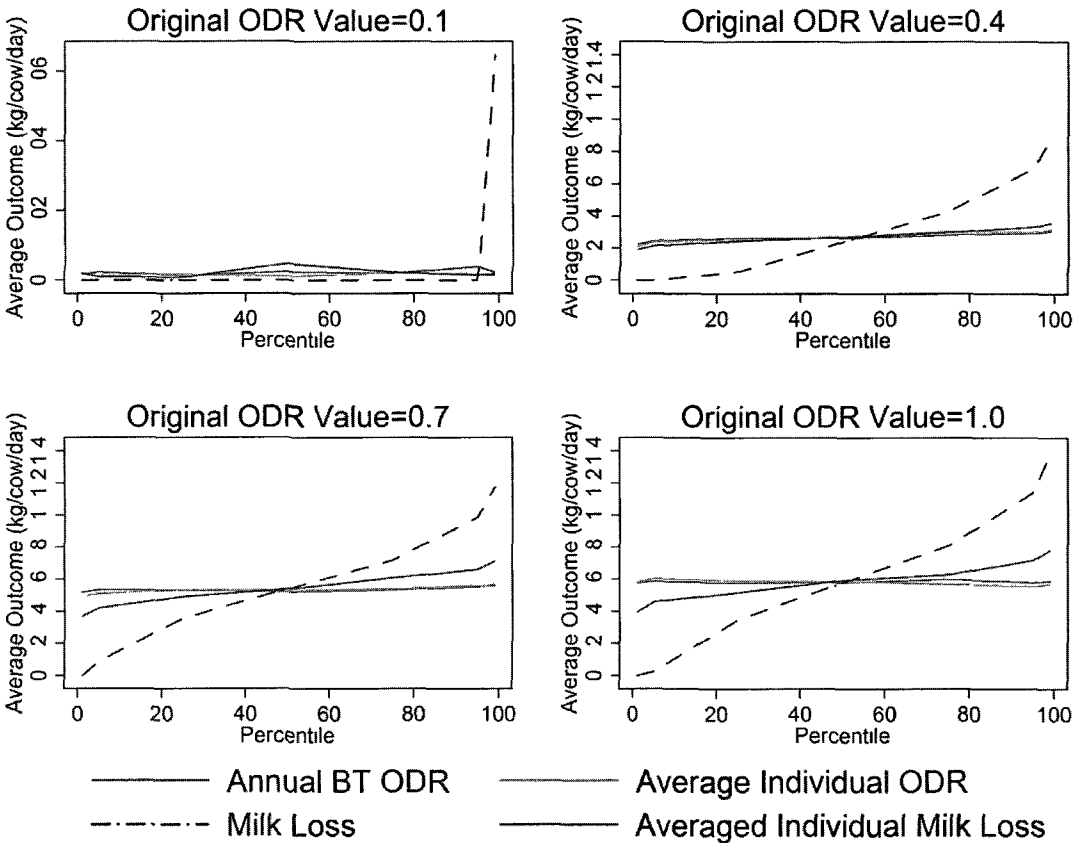

Figure 7.9. Sensitivity analysis, showing 'spider' plots for ODR values at 0.1, 0.4, 0.7, and 1.0. Each spider plot contains the four parameters from the stochastic model. The vertical range produced by each parameter shows the range of expected outcomes if the values within the parameter were fixed somewhere between the 1st and 99th percentiles.

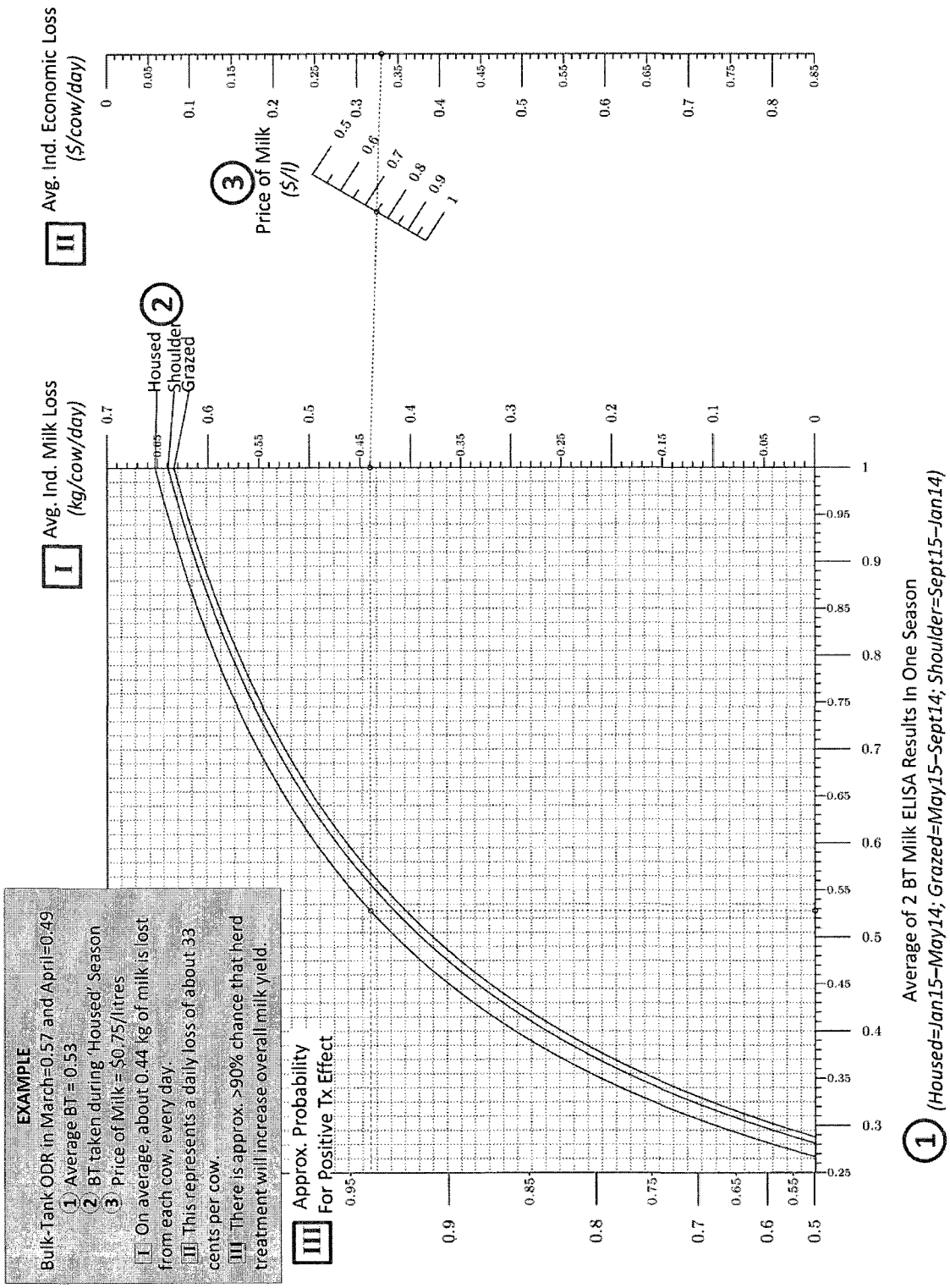

Figure 7.10. Nomogram to calculate the averaged individual milk loss (and associated economic loss) **from two averaged BT ELISA readings within one season.** The interpreter should input values for steps 1 to 3, and observe the outcomes for I, II, and III.

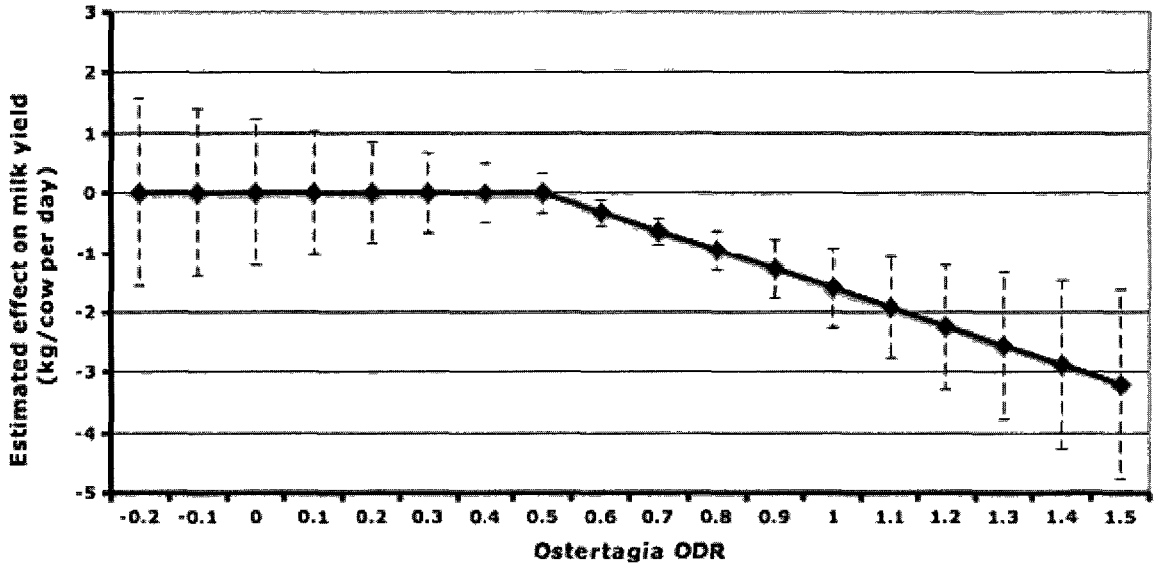

Figure 7.11. European guidelines, as seen in Forbes *et al.* (2008). This plot is also included in the ELISA kits supplied by Svanova.

Chapter 8

Summary

Gastrointestinal parasites, such as *Ostertagia ostertagi*, adversely affect milk production in dairy cattle (Sanchez *et al.*, 2004). These gastrointestinal nematodes are ubiquitous in temperate climates, however, even with their associated production losses, clinical signs of infection are rarely seen in adult cattle (Gibbs, 1988; Louw, 1999; Williams *et al.*, 1993).

There are many management options to reduce the amount of gastrointestinal parasites (e.g. limiting pasture exposure, 4-year pasture/crop rotation, not mixing heifers, dry cows, and milking cows on the same pastures, etc.), and several treatments (anthelmintics) to eliminate them (e.g. ivermectin, eprinomectin, fenbendazole, etc.). Current gastrointestinal nematode chemical control programs (using anthelmintics), as summarized by Stromberg and Gasbarre (2006), are 1) therapeutic, 2) preventive, or 3) suppressive. Therapeutic deworming occurs when cattle are treated with anthelmintics when they are affected by parasites, while preventive deworming is a program of three or more treatments at regular intervals (e.g. 6 weeks or less), and an increased number of treatments may lead to suppressive deworming. Suppressive deworming is used to prevent adult parasite poluations from developing to maturity and contaminating the pasture.

Preventive deworming is a popular method for controlling nematode infections, and unfortunately, has the potential for selecting anthelmintic resistance. Other management practices contributing to anthelmintic

resistance are to underdose the animals (e.g. estimating animal weights, using the dose for another animal species, or using a different route to administer the drug), and regularly switching drugs instead of using one drug until it is no longer effective (Craig, 2006).

Therapeutic deworming, on the other hand, preserves the efficacy of anthelmintics (Craig, 2006) by targeting individual animals that have the largest numbers of worms and treat them, and/or evaluate the entire herd for parasite levels (Stromberg and Gasbarre, 2006).

As mentioned above, dairy cattle do not show clinical signs of infection, but their milk production is negatively affected by nematode infections. Diagnostic tests are, therefore, necessary to measure levels of nematode infections in subclinical cattle and may help with therapeutic deworming programmes. As will be discussed in further details below, an enzyme-linked immunosorbent assay (ELISA) can be used to quantify levels of intestinal parasites in dairy cattle from milk samples.

The theme to this thesis was to investigate how an ELISA test can be used to quantify parasite levels and production losses in dairy cattle. Additionally, some technical questions regarding the ELISA test were also investigated.

The important question for producers is "When should we treat or change management practices to reduce the effects that gastrointestinal parasites have on our dairy production?". To answer this question we need to better understand the diagnostic tools available to quantify parasite burden and how

to relate these test values with production losses.

8.1. Introduction

Diagnostic techniques to identify and/or quantify intestinal parasites have improved over the years. Fecal egg counts have traditionally been utilized to identify and quantify infections, however, this test is plagued with false negatives, high variability between consecutive tests (low repeatability), and overall they underestimate the level of infection (Agneessens *et al.*, 2000; Borgsteede *et al.*, 2000; Eysker and Ploeger, 2000; Gross *et al.*, 1999). Serum pepsinogen tests have had some success with quantifying the levels of parasitism in first-season grazers, but were less reliable when used to quantify infections in adult cattle. Unfortunately, the quality of the pepsinogen test depends on the current life cycle stages of the nematodes in the host (Gross *et al.*, 1999).

The first enzyme-linked immunosorbent assay (ELISA) test to quantify nematode infections in cattle was developed in 1981 (Keus *et al.*), and since then, many in-house ELISA tests have been developed for studies to quantify the infections and their related production losses. More recently, Svanova (Svanova Veterinary Diagnostics, Uppsala, Sweden) developed a commercial ELISA test (Svanovir®) available in Europe. This test is designed to be used on milk samples, making the collection process very simple, and bulk-tank (BT) or cow milk samples can be used.

One major advantage of having a commercial ELISA test is the standardization

that is now available for the test controls. Results are normalized, using the controls, and reported as optical density ratios (ODRs), thus permitting the ELISA results to be compared between plates, kits and, to a certain extent, studies and regions (Charlier *et al.*, 2005; Sanchez *et al.*, 2001; Sanchez *et al.*, 2002a; Sithole *et al.*, 2005; Vanderstichel *et al.*, 2010). ODR values represent a percent positivity between the negative and the positive controls supplied by the company and range approximately between -0.2 and 1.2; an ODR of 0.5 would indicate that the test sample is halfway between a cow with almost no parasites (negative) and one that is heavily infected (positive).

It is important to understand that this ELISA test does not count the number of parasites present in a cow, but rather quantifies the amount of immunological response, against intestinal parasites, that is present in the cow. Consequently, it acts as a surrogate count for parasites, much like a California Mastitis Test is used to indicate the severity of mastitis, without actually detecting bacterial infections.

8.2. Chapter 2 - Farm Management and Environmental Factors on BT ODRs

As was mentioned earlier, management practices can have an influence on parasite levels, and theoretically, environmental factors also play a role in these levels. Both environment and management practices were investigated to understand how important they were in contributing to the herd's overall parasite load.

This study was part of a larger mastitis research project (Olde Riekerink *et al.*,

2006) which started in late 2003 and continued until the middle of 2005. A total of 115 herds from 9 provinces successfully completed this study, which contributed over 400 bulk tank samples. Each producer filled out a questionnaire, and environmental data (e.g. weather station information, remote sensing data from satellites, digital elevation maps, etc.) were downloaded online from various governmental databases (e.g. Natural Resources Canada, Statistics Canada, Environment Canada, etc.). Statistical models, accounting for repeated measures (multiple bulk tank ODRs for each farm) and for clustering of farms within a region (province or ecoregion), were used to analyze environmental and farm management data. Overall, the greater the exposure that heifers and milking cows had to pasture, the higher the levels of anti-parasite antibodies were in bulk tank samples. Sharing pastures between heifers, dry cows and milking cows was also associated with higher BT ODRs. Treating the entire herd or treating milking cows at calving, with anthelmintics, reduced BT ODR values. Farms in areas with higher number of rainy days, higher vegetation index values (more greenery), and lower land surface temperatures (mostly near the eastern and western coastline), were also likely to have higher BT ODRs. Seasonal variation was such that late summer and early fall, when parasite load was at its highest, yielded larger BT ODRs. Despite these environmental factors having some statistically significant influence, factors at the herd level (e.g. pasturing methods, anthelmintic administration) had a higher potential

impact on bulk-tank measurements than the herd's surrounding environmental factors.

8.3. Chapter 3 - Milk Handling on ELISA Test Results

Before we can start running an ELISA test, we need to make sure that the milk samples that are submitted to the laboratory, from the farm or through a Dairy Herd Improvement (DHI) programme (e.g. Valacta or CanWest), have not been handled in such a way that the test results could be compromised.

The manufacturers of Svanovir® recommend using a fresh milk sample centrifuged to remove the lipid layer (referred to as defatting). However, routine collection of such samples will be expensive, and samples available for routine screening (e.g. DHI) will likely have been subjected to one or more transportation, processing, and storage stressors (e.g. heating, freezing).

A study was undertaken to determine if heating, freezing, re-freezing or defatting milk samples had an effect on ODR values from a milk ELISA test (Svanovir®) measuring *O. ostertagi* antibodies. Two herds agreed to give approximately 140ml of fresh milk from each cow, during one milking session, and from those milk samples, 40 were selected to undergo a series of 'storage stressors'.

The variation in the ODR values was different between the frozen and the fresh samples. The difference in variation between treatment groups meant that one of the statistical assumptions for the more traditional model (split plot design) was violated. As such, the mixed model was extended to allow for

differences in variation between fresh and frozen samples.

Findings showed that, although the outcome of the test is slightly influenced by the storage method, length of storage and defattening process, the differences were minimal and would have little effect on the interpretation of the results. Fresh, whole, heated milk, the most likely sample to be used in DHI based surveillance programs, will yield reliable results. So too will frozen, whole milk, the most likely to be used in large scale research projects.

8.4. Chapter 4 – Applying Kinetic Methods to an Indirect ELISA

Another important technical aspect of the ELISA test is whether we could improve the ELISA test by applying kinetic methods to an indirect ELISA.

The final process of an indirect ELISA is to incubate the samples for 30 minutes after enzymes have been added. The enzymatic reaction is finally stopped after the incubation by adding a 'stop' solution (an acid), and the amount of color change is read (quantified) with a specialized machine, called a spectrophotometer. An indirect ELISA can become a kinetic ELISA if the sample is read and recorded at regular short intervals (e.g. 45 seconds) during the incubation period. Therefore, the kinetic ELISA does not require stop solutions, and measurements are taken in real-time.

The manufacturer's instructions describe using the test as an endpoint ELISA (e-ELISA); however, kinetic ELISAs (k-ELISA) have certain advantages over e-ELISAs. These advantages motivated this study to understand the relationship between e-ELISA and k-ELISA results from Svanovir®. More

specific objectives were to determine whether it is possible to run both k-ELISA and e-ELISA methods on the same plate, and to establish an appropriate time interval for k-ELISA measurements. A normalization method for the k-ELISA slopes (slope ratio) was proposed to compare ELISA methods. A concordance correlation coefficient (CCC) is used to evaluate the agreement between two series of continuous measurements, where, values close to 1 indicate very good agreement while values approaching zero reflect very poor agreement; CCCs were calculated and used to compare results between the two ELISA methods.

The study found that running a k-ELISA has no effect on ODR results of an e-ELISA on the same plate, and that agreement was very strong at both 15 and 28 minutes, indicating that a 15-minute slope is sufficient for a k-ELISA method using Svanovir®.

8.5. Chapter 5 – Use of ELISA Test Results to Predict Individual Cow Milk Yields

So far, we have discussed the influence that producers have on the herd's overall parasite load, and also discussed technical aspects of the ELISA test which will quantify the amount of parasite burden present in the herd or the cow, depending on which milk sample is tested (either BT or Individual cows). The next important task is to link the ODR values with milk production losses. To make this link, a large clinical trial was undertaken across Canada involving over 3,000 cows from nearly 40 herds in 9 provinces.

Milk samples were collected from cows in their late lactation (>200 days in milk) to measure individual ODRs. Producers would then apply a treatment (randomly allocated to either an anthelmintic or placebo) to cows as they calved throughout the study period. Milk production records were acquired from Dairy Herd Improvement programmes for individual cows on a monthly basis for the duration of the study.

Statistical analyses had to account for many complicated factors present in this study. The structure of the data was such that there were repeated measures (multiple milk-production records for each cow), and clustering of cows within herds.

Since the treatment effect (anthelmintic) in the clinical trial was expected to depend on the level of parasitism in the cow, where low ODR values from the ELISA test indicated low levels of parasitism, the estimates from the interaction between ODR and treatment on milk production would be of particular interest. However, the relationship between ODR and milk yield was not linear (Sanchez *et al.*, 2005), and therefore a more complex functional form for the ODR values was required. A 2-degree fractional polynomial (FP), using two terms, was the most parsimonious method to obtain a good fit to the data (Royston and Sauerbrei, 2008), and was applied to the ODR term, which transcended to the interaction terms. To accurately predict the treatment effect on milk production, other important factors that contribute to milk production had to be included in the model (Dohoo *et al.*, 2009).

Specifically, test season, calving season, parity (2nd, 3rd, and >=4th), somatic cell counts (log transformed), and days in milk (following Wilmink's function (Schaeffer *et al.*, 2000)) were accounted for in the model.

The findings from this study were consistent with other similar, yet smaller studies. Despite having a large number of cows involved in this study the statistical estimates of the effect were only borderline significant. To increase the statistical power of the study, datasets from two previous smaller Canadian studies were included with this dataset (analysis and results in Chapter 7). The final statistical model was nearly identical to the original clinical trial model predicting milk losses, except for two variables, (1) which accounted for the differences between years, seasons, and studies, and (2) was derived from the questionnaire in Chapter 5. The large combined dataset was able to predict the amount of individual milk loss (kg/cow/day), based on the ELISA test results (ODRs) from individual cows in a herd (see Figure 7.5).

8.6. Chapter 6 – Use of ELISA Test Results to Predict Reproduction Parameters

In the literature, the effect of parasitism on fertility in dairy cattle has been reported inconsistently between studies (Sanchez *et al.*, 2002b; Sithole *et al.*, 2006; Walsh *et al.*, 1995). In fact, studies investigating the effect of anthemintics on fertility in beef cattle, as well, have had conflicting results (Hawkins, 1993). Using the data collected from the clinical trial, the effect of parasitism on fertility was investigated.

Specifically, four fertility measurements (outcomes) were investigated separately; 1) Number of services per conception (NSC), 2) First-service conception risk (FSCR), 3) Days to first-service (DFS), and 4) Days to conception (DC). The number of services per conception was analyzed using multilevel negative binomial regressions, and the first-service conception risk was analyzed using multilevel logistic regressions; multilevel analyses allow for clustering effects of cows within herds. Both the days to first-service and the days to conception outcomes were analyzed using Cox proportional hazards models extended with a random effect (frailty) for herd. (Dohoo *et al.*, 2009)

Overall, there was no significant effect of anthelmintic treatments on reproduction, even when accounting for the level of parasite exposure. The lack of significance may be due to a deficiency in statistical power of the study, or simply to a weak or non-existent effect.

Both milk yield (within the lactation) and days to first-service were variables that remained in most of the reproduction models. Briefly, milk yield was consistently significant for the reproduction parameters, and generally, higher milk yields were associated with detrimental reproductive results. Higher yielding cows had a greater NSC, lower risk of conceiving on the first service, and a decreased hazard for both first-service and conception; the milk yield hazard for both first-service and conception was, however, not decreasing as much as the lactation period progressed (linear time-varying effect).

The predicting variable of days to first-service was significant for the NSC, and FSCR. It was estimated that the longer it took a producer to commence breeding for a cow, the greater the odds of conception on the first-service, and generally, the fewer the number of services were required for that conception.

8.7. Chapter 7 - Guidelines for Interpretation of ELISA Test Results

In general, study designs and investigation methods to predict the effect of intestinal parasitism on dairy production have varied between studies and regions of the world (Table 5.1). The major differences between the studies were the 'unit of concern' for the analysis. When selecting the herd as the unit of concern, bulk tank (BT) milk samples were used to measure the level of antibodies against parasites, and production losses were measured at the herd-level (averaged milk production). The treatment intervention, however, could be administered either individually (cow-level), as cows calved, or to the entire herd. When selecting the cow as the unit of concern, individual milk samples were taken for the ELISA test, and the individual cow milk yield was recorded. Once again, the intervention could be either at the individual or herd level.

These differences in sampling and treatments have made the development of general guidelines more difficult. Individual predictions, such as those performed in the clinical trial, are more consistent and accurate than using pooled milk samples, such as BT samples. However, bulk tank samples are

much easier to collect from farms, compared to collecting a milk sample from each cow. Running one test for the herd is financially more appealing than having to run, potentially, dozens of cow samples, especially if the producer plans to treat the entire herd.

To develop the final guidelines, a series of four analyses ('A', 'B', 'C', and 'D', as shown in Fig. 7.1) were performed to bridge the gap between BT ODR values and the prediction of milk loss from individual cows. Briefly, the first process, 'A', was to create a protocol for averaging several BT values collected within one season ('Seasonal BT ODR') and using this average to estimate an annual herd BT ODR value 'Annual BT ODR'. Since BT samples report higher ELISA values than the average of the individual ELISA values in the herd, a second analysis, 'B', converted the 'Annual BT ODR' into a representative individual ODR for the herd 'Representative Cow ODR'. Using the coefficients from the model to predict milk loss from parasitism at the individual cow level (based on Chapter 5) as the third step, 'C', a single value to estimate milk loss (kg/cow/day) could be calculated ('Representative Cow Milk Loss'). Calculating milk loss for one representative ODR value for the herd ('Representative Cow Milk Loss') is not the same as averaging the calculated milk loss from each individual cow within that herd ('Average Individual Milk Loss'), therefore, the fourth step, 'D', was necessary to account for this difference.

Overall, the best scenario to predict milk loss in cattle due to gastrointestinal

parasitism is to test every individual cow in the herd and derive individual milk loss estimates for each cow; treatments would then be considered individually.

For economic and logistical reasons, producers would rather test BT milk samples, and therefore, a nomogram (Figure 7.10) was developed to summarize the series of anlayses to go from BT ODRs to estimated averaged individual milk loss (kg/cow/day). It was also found that if ODR values, from bulk tank samples (average of 2 samples from one season), were greater than 0.57, anthelmintic treatment would result in a positive production effect 19 times out of 20, and if ODR values were less than 0.26, the treatment was not likely to be beneficial (<50% chance of beneficial effect on production).

Comparing the results from 'Representative Cow Milk Loss' to those from 'Average Individual Milk Loss' (assumed to be the 'true' estimate) showed that the 'Representative Cow Milk Loss' estimates with values less than 0.4 kg/cow/day were likely to underestimate the 'true' value, while values greater than 0.4 were likely to overestimate the 'true' value.

Care is still needed when interpreting values from this study, particularly when using the nomogram. It is difficult to appreciate the amount of uncertainty that is present with the deterministic methods used for this model, which is why a second y-axis, with an approximate cumulative probability of having a positive treatment effect, was added to the nomogram (Fig. 7.10). Even this cumulative probability of positive treatment effect is an approximation from

the observed data that comprised this study.

The final estimates from the overall model, which include processes 'A' through 'D', were derived specifically from the studies described in Chapter 7, and it will be important to update this overall model as new data is collected from other studies. It is also probable that new data will yield results that may be different to what has been estimated and graphed in Chapter 7. Therefore, it is important to understand that estimates from this study should be used as a rough guide for interpretation.

Nevertheless, this nomogram is the most informative option for North American dairy herds planning to use an ELISA test (e.g. Svanovir®) to predict milk loss associated with gastrointestinal parasites from BT milk samples. Like all guidelines, these should remain dynamic and flexible, and should also be updated on a regular basis, as more information is made available. More specifically, as additional information is available linking milk yield loss (kg/cow/day) with the average of all combined individual milk yield, the better the estimates for the guidelines will be.

The nomogram, intended to inform decision makers, will help with the ultimate decision as to whether or not to increase the control of gastrointestinal parasites. However, the choice of methods to control the parasites remains with the producers and veterinarians, and should be considered on a farm-by-farm basis.

8.8. Closing Remarks

While statistical model building is important to highlight useful information, suggest conclusions, and support decision making, these models can only reflect the observed data that was collected from field studies. Also, there are inherent assumptions within a study design or statistical analyses, as discussed individually in each chapter, which mustn't be forgotten when using the information presented in this thesis.

As with every research, there are unknowns which should be further investigated and may influence some of the results presented in this research. An example being topical absorption time for eprinomectin (Eprinex®) which would influence the risk of mechanical transfer of anthelmintics between cattle; this could alter the conclusion of a slight bias towards the null, as described in Chapter 5.

Another unknown, and an assumption throughout this research, is the link between ODR values and actual numbers of parasites in the animal. This assumption was indirectly justified by associating ODR values with production losses, and quantifying this production loss with a clinical trial for an anthelmintic treatment; it was also assumed that the anthelmintic treatment reduced the parasite load. However, without slaughtering the animals and counting the number of adult nematodes in the abomasums, we cannot make that explicit association between ODR and nematode count.

Some of the potential future research was described as it pertained to each

chapter. However, more general future research could focus on 1) the cow's immune system and how that relates to ELISA results, worm counts, and/or milk production, 2) association between ODR values and total worm counts (on post-mortem examinations), or 3) run a similar large clinical trial in other regions of the world (e.g. Europe, South America, etc.) to see if the effect that nematodes have on milk production is similar between populations and regions.

One suggestion for any future investigators would be to either use Svanova's *Ostertagia ostertagi* sample controls supplied with the ELISA kits (both negative and positive controls) or to calibrate their in-house controls with those supplied by Svanova. The ODR values of ELISA tests depend on the sample controls, and therefore, if the controls are all calibrated equally, the ODR results can be compared between studies and regions. There are many published studies in Europe using Svanovir® and most of the ODR values used in this thesis were derived from controls supplied by Svanova. Also, Svanova holds ISO accreditation (SS-EN ISO 9001:2008) and maintains a standardized stock of control solutions.

Overall, the research and guidelines presented in this thesis are timely and relevant. The guidelines and testing methods are useful for producers now and it could prove even more useful in the future. As mentioned above, the current trends in chemical control against gastrointestinal nematodes in ruminants favour anthelmintic resistance. There is little development for new

drugs (or classes of drugs) against nematode infections. Feed prices are volatile and using pastures to supplement the nutritional requirements of cattle may become more popular as the cost of energy increases. Using pasture management techniques (e.g. crop rotation) to reduce parasite load may prove more difficult when higher agricultural production output will become financially necessary. All of these potential factors could lead to more dairy cattle on pastures. In the future, general trends in chemical control against intestinal parasites will likely shift from a preventive to a therapeutic approach. To achieve adequate therapeutic control, diagnostic tools capable of quantifying production losses and/or nematode infections will become indispensable.

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Appendix A

Survey # «survey_id»

Dear Dairy Producer:

Researches at the Atlantic Veterinary College are evaluating a new test for measuring parasite (worm) burdens in dairy herds using bulk tank milk samples.

To assist in the evaluation of the test, we are collecting some basic management data about each dairy herd participating in the mastitis survey. Would you please take a few minutes to fill in this very brief survey and return it along with the mastitis survey in the self-addressed, postage paid envelope.

All information will be used only for research purposes and will be kept strictly confidential.

If you have any questions, please do not hesitate to contact us by phone or email:

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ALL QUESTIONS RELATED TO THE SUMMER OF 2004

Heifers (Breeding Age or Pregnant)

1 In the Summer of 2004, **heifers** were (check one):

a. totally confined (in the barn) 24 hrs./day ☐
b. given access to a concrete or gravel surface exercise yard (outdoors) some time each day. ☐
c. given access to a small field for the purpose of exercise(not primarily for grazing). ☐
d. spent some time grazing and met some of their nutritional requirements from pasture ☐

	Yes	No
2 If **heifers** had access to pasture for grazing (not just for exercise), did they graze on pastures that had also been grazed by **dry cows** during **2004**?	☐	☐

3 Which of the following treatments were used for worm control in **heifers**? (Check all that apply)

a. pour on or injectable deworming in **Fall 2003** ☐
b. pour on or injectable deworming in **Spring or Summer 2004** ☐
c. Ivomec sustained release bolus in **Summer 2004** ☐
d. pour on or injectable deworming in **Fall 2004** (before October 1st) ☐
e. no treatments between **Fall 2003 and Fall 2004** ☐

Milking cows

4 In the Summer of 2004, **milking cows** were (check one):

a. totally confined (in the barn) 24 hrs./day ☐
b. given access to a concrete or gravel surface exercise yard (outdoors) some time each day. ☐
c. given access to a small field for the purpose of exercise (not primarily for grazing). ☐
d. spent some time grazing and met some of their nutritional requirements form pasture. ☐

	Yes	No
5 If **milking cows** had access to pasture for grazing (not just for exercise), did they graze on pastures that had also been grazed by **heifers** during **2004**?	☐	☐

6 Which of the following worm control treatments were used in **milking cows** in **2004**? (Check all that apply)

a. no treatment ☐
b. oral dewormers (in feed or by mouth) ☐
c. pour on or injectable treatment at dry off ☐
d. pour on or injectable treatment at calving ☐
e. pour on or injectable treatment of whole herd ☐

- Your milk samples will be tested to evaluate the level of worms in your herd. If you would like this information sent to you please check this box .

- I give permission for researches at the Atlantic Veterinary College to send the results from my bulk tank samples to me and to use my herd's production data from the mastitis project for this research. I understand that this information is to be used for research purposes only and all information from my farm will be kept strictly confidential.

_____ _____

Signature Date

Sondage # «survey_id»

Cher/ère producteur/trice :

Des recherches en cours au *Atlantic Veterinary College* de l'Ile-du-Prince Édouard évaluent actuellement une nouvelle technique pour mesurer la charge de parasites (vers) dans les troupeaux laitiers en utilisant les échantillons de lait en vrac.

Pour appuyer l'évaluation de cette technique, nous aimerions obtenir quelques informations sur la régie de base de chacun des troupeaux laitiers qui participent à l'étude sur la situation de la mammite au Canada. Nous vous saurions gré de prendre quelques minutes pour remplir ce court sondage et de nous le retourner avec le questionnaire sur la mammite dans l'enveloppe-réponse préaffranchie.

Toutes les informations seront utilisées pour des fins de recherche seulement et seront gardées strictement confidentielles.

Si vous avez des questions, svp n'hésitez pas à nous contacter par téléphone ou par courriel:

Dr. Sanchez: (902) 566 0803 – jsanchez@upei.ca
Dr. Dohoo: (902) 566 0640 – dohoo@upei.ca

Javier Sanchez
Associé de recherche
Régie de la santé
Atlantic Veterinary College

Ian Dohoo
Professeur - Epidémiologie
Régie de la santé
Atlantic Veterinary College

TOUTES LES QUESTIONS SE RAPPORTENT À L'ÉTÉ 2004

Taures (à l'âge de la reproduction ou gestantes)

1 À l'été 2004, **les taures** : (cochez un choix):

a. étaient en confinement complet (dans l'étable) 24 h/jour. ☐
b. avaient accès à un parc d'exercice avec une surface en ciment ou en gravier (à l'extérieur) durant une certaine période à chaque jour. ☐
c. avaient accès au pâturage pour l'exercice (le but principal n'étant pas de brouter). ☐
d. ont passé du temps au pâturage et ont rencontré la majorité de leurs besoins nutritifs en broutant dans les champs. ☐

	Oui	Non
2 Si les **taures** avaient accès au pâturage pour brouter (pas seulement pour l'exercice), ont-elles été dans des champs où des vaches taries avaient séjourné au	☐	☐

cours de l'année **2004**?

3 Lequel ou lesquels des traitements suivants ont été administrés pour le contrôle des vers chez les **taures**? (Cochez toutes les réponses qui s'appliquent)

a. traitement vermifuge par injection ou versable à **l'automne 2003.** ☐
b. traitement vermifuge par injection ou versable au **printemps ou à l'été 2004.** ☐
c. capsule d'Ivomec à action prolongée à **l'été 2004.** ☐
d. traitement par injection ou versable à **l'automne 2004** (avant le 1er octobre). ☐
e. aucun traitement entre **l'automne 2003 et l'automne 2004.** ☐

Vaches en lactation

4 À l'été 2004, **les vaches en lactation...** (cochez un choix):

a. étaient en confinement complet (dans l'étable) 24 h/jour. ☐
b. avaient accès à un parc d'exercice avec une surface en ciment ou en gravier (à l'extérieur) durant une certaine période à chaque jour. ☐
c. avaient accès au pâturage pour l'exercice (le but principal n'étant pas de brouter). ☐
d. ont passé du temps au pâturage et ont rencontré la majorité de leurs besoins nutritifs en broutant dans les champs. ☐

	Oui	Non
5 Si les **vaches en lactation** avaient accès au pâturage pour brouter (pas seulement pour l'exercice), ont-elles été dans des champs où des **taures** avaient séjourné au cours de l'année **2004**?	☐	☐

6 Lequel ou lesquels des traitements vermifuges suivants ont été utilisés chez les **vaches en lactation** en **2004**? (Cochez toutes les réponses qui s'appliquent)

a. aucun traitement ☐
b. vermifuge oral (dans les aliments ou dans la gueule) ☐
c. traitement versable ou en injectable au tarissement ☐
d. traitement versable ou en injectable au vêlage ☐
e. traitement versable ou en injectable pour tout le troupeau ☐

- Vos échantillons de lait seront analysés pour évaluer le niveau des vers dans votre troupeau. Si vous voulez que nous vous fassions parvenir les résultats, veuillez cocher cette case. ☐

- Je permets aux chercheurs du *Atlantic Veterinary College* de m'envoyer les résultats d'analyse de mes échantillons de lait en vrac et d'utiliser les données de production de mon troupeau déjà fournies dans le cadre du projet sur la situation de la mammite au Canada. Je comprends que ces informations seront utilisées pour des fins de recherche seulement et que toutes les informations de ma ferme seront gardées strictement confidentielles. ☐

_____ _____

Signature Date

Appendix B

CBMRN Question used for validation:
(Circulated between September and December 2007)

Did your milking cows have access to pasture in the last 12 months?

[ ] No, they are kept inside year-round
[ ] No, but they had access to a grassed exercise yard (less than 5 acres per 100 cows)
[ ] No, but they had access to a non-grassed (paved or dirt) exercise yard (less than 5 acres per 100 cows)
[ ] Yes, they were on pasture from the month of _____ to the month of _____

Parasite Questionnaire circulated to all producers involved with the study between May and November of 2008.

Parasite Control **BEFORE** the study:

Between May 2006 and April 2007, did you use any medications for deworming and/or external parasite control?

Yes [ ] (proceed to next table)
No [ ] (skip to "Parasite Control DURING the study")

		Anthelmintics (Dewormers)								External Parasiticides (to remove lice/mites)										OTHER
		Oral			Pour-On or Injectable					Ear Tags					Pour-On (Topical)					
		Safe-Guard®	Panacur™	Valbazen®	Eprinex®	Cydectin®	Dectomax	Noromectin®	Ivomec®	Megamectin®	Bovatd®	Protector®	Eclogard™	Atroban®	Eliminator	Saber™	CyLence®	DeLice®	Dri-Kill™	Vetolice®	Boss	Lause Kill®	
2006 Milking Cows	Tick if Yes																					
	Whole herd in Spring	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	_____
	Whole herd in Summer	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	_____
	Whole herd in Fall	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	_____
	Whole herd in Winter	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	_____
	Individual cows near the time of calving	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	_____
2006 Heifers (before first calving)																							
	All heifers in Spring	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	_____
	All heifers in Summer	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	_____
	All heifers in Fall	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	_____
	All heifers in Winter	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	_____
	Individual heifers near the time of calving	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	_____

Parasite Control **DURING** the study:

After applying the content of the bottle, most of the treated cows were (check one):

Kept separated from other cows for approximately 1 hour [ ]
Kept separated from other cows for approximately 6 hours [ ]
Kept separated from other cows for approximately 12 hours [ ]
Kept separated from other cows for approximately 24 hours [ ]
Kept separated from other cows for more than 24 hours [ ]
Not able to keep them apart [ ]

Were any external parasiticides used **during** the study (between May 2007 and May 2008)? (It is important for us to know if such products were used - e.g.CyLence®, Vetolice®, etc., even if these products had no effect on our study)

Yes [ ] No [ ]

Pasture Exposure:

In the Summer of 2007, **milking cows** were (check one):

Totally confined (in the barn) 24hr/day [ ]
Given access to a concrete or gravel surface exercise yard (outdoors) some time each day [ ]
Given access to a small field (for the purpose of exercise and not primarily for grazing) [ ]
Spent some time grazing and met some of their nutritional requirements from pasture [ ]

In the Summer of 2007, **dry cows** were (check one):

Totally confined (in the barn) 24hr/day [ ]
Given access to a concrete or gravel surface exercise yard (outdoors) some time each day [ ]
Given access to a small field (for the purpose of exercise and not primarily for grazing) [ ]
Spent some time grazing and met some of their nutritional requirements from pasture [ ]

In the Summer of 2007, **heifers** (before first calving) were (check one):

Totally confined (in the barn) 24hr/day [ ]
Given access to a concrete or gravel surface exercise yard (outdoors) some time each day [ ]
Given access to a small field (for the purpose of exercise and not primarily for grazing) [ ]
Spent some time grazing and met some of their nutritional requirements from pasture [ ]

Were any pastures shared between groups of cows (milking cows, heifers and/or dry cows)?

Yes [ ] No [ ]

...If yes, which groups were shared (check one):

Milking cows with heifers [ ]
Milking cows with dry cows [ ]
Heifers with dry cows [ ]
Milking cows, dry cows and heifers [ ]

Contrôle des parasites **AVANT** l'étude

Avez-vous utilisé des vermifuges et/ou des médicaments de contrôle des parasites externes entre mai 2006 et avril 2007?

Oui [ ] (passez au tableau suivant)
Non [ ] (allez à « Contrôle des parasites PENDANT l'étude »)

Produits de contrôle des parasites utilisés entre mai 2006 et avril 2007

Vaches en lactation en 2006	Cochez si traie	Anthelmintiques (vermifuges)								Antiparasitaires externes (contre les poux/acariens)										AUTRE
		Oral			Cutané ou injectable					Etiquettes d'oreille						Cutané (topique)				
		Safe-Guard ®	Panacur ®	Valbazen ®	Eprinex TM	Cydectin ®	Dectomax ®	Noromectin ®	Ivomec ®	Megamectin ®	Bovaid ®	Protector ®	Ectogard TM	Atroban ®	Eliminator ®	Saber TM	CyLence ®	DeLice ®	Dri-Kil TM	Vetolice ®	Boss ®	Louse Kill ®	
Troupeau entier au printemps	☐																					
Troupeau entier à l'été	☐																					
Troupeau entier à l'automne	☐																					
Troupeau entier en hiver	☐																					
Vaches individuelles à l'approche du vêlage	☐																					
Génisses en 2006 (avant le premier vêlage)																						
Toutes les genisses au printemps	☐																					
Toutes les genisses à l'éte	☐																					
Toutes les génisses à l'automne	☐																					
Toutes les génisses en hiver	☐																					
Génisses individuelles à l'approche du vêlage	☐																					

Contrôle des parasites **PENDANT** l'étude

Après l'application du contenu d'une bouteille, la plupart des vaches ont été (cochez une seule réponse) :

Séparées des autres vaches pendant environ 1 heure [ ]
Séparées des autres vaches pendant environ 6 heures [ ]
Séparées des autres vaches pendant environ 12 heures[ ]
Séparées des autres vaches pendant environ 24 heures[ ]
Séparées des autres vaches pendant plus de 24 heures[ ]
Incapable de les séparer [ ]

Des antiparasitaires externes ont-ils été utilisés **pendant** l'étude (soit entre mai 2007 et mai 2008)? (Il est important pour nous de savoir si de tels produits ont été utilisés - p. ex. CyLence®, Vetolice®, etc., même s'ils n'ont aucun effet sur notre étude)

Oui [ ] Non [ ]

Mise au pâturage

À l'été 2007, les **vaches en lactation** ont (cochez une seule réponse) :

Été complètement confinées (à l'étable) 24 heures sur 24 [ ]
Eu accès à une aire d'exercice en béton ou en gravier (à l'extérieur) pendant un certain temps chaque jour [ ]
Eu accès à un petit champ (aux fins d'exercice et pas uniquement de pâturage) [ ]
Passé du temps au pâturage et ont répondu à certains de leurs besoins nutritionnels au pâturage [ ]

À l'été 2007, les **vaches en tarissement** ont (cochez une seule réponse) :

Été complètement confinées (à l'étable) 24 heures sur 24 [ ]
Eu accès à une aire d'exercice en béton ou en gravier (à l'extérieur) pendant un certain temps chaque jour [ ]
Eu accès à un petit champ (aux fins d'exercice et pas uniquement de pâturage) [ ]
Passé du temps au pâturage et ont répondu à certains de leurs besoins nutritionnels au pâturage [ ]

À l'été 2007, les **génisses** (avant le premier vêlage) ont (cochez une seule réponse) :

Été complètement confinées (à l'étable) 24 heures sur 24 [ ]
Eu accès à une aire d'exercice en béton ou en gravier (à l'extérieur) pendant un certain temps chaque jour [ ]
Eu accès à un petit champ (aux fins d'exercice et pas uniquement de pâturage) [ ]
Passé du temps au pâturage et ont répondu à certains de leurs besoins nutritionnels au pâturage [ ]

Est-ce que les groupes de vaches (vaches en lactation, génisses et/ou vaches en tarissement) ont utilisé les mêmes pâturages?

Oui [ ] Non [ ]

...Si oui, quels groupes ont utilisé les mêmes pâturages? (cochez une seule réponse)

Vaches en lactation et génisses [ ]
Vaches en lactation et vaches en tarissement [ ]
Génisses et vaches en tarissement [ ]
Vaches en lactation, vaches en tarissement et génisses [ ]

Appendix C

Python script to run the nomogram in PyNomo:

```
import sys
sys.path.insert(0, "..")
#sys.path[:0] = [".."]
from pynomo.nomographer import *


### Second Block (Multiplicative)

N_params_gain={
        'u_min':0.00,
        'u_max':0.85,
        'function':lambda u:(u),
'tick_side':'left',
'tick_levels':3, # Duplicate labels
'tick_text_levels':2, # Duplicate labels
                }
N_params_mlkprice={
        'u_min':0.50,
        'u_max':1.00,
        'function':lambda u:(u),
        'tick_levels':2,
        'tick_text_levels':1,
                }
N_params_indmlkloss={
      'tag':'mlk',
        'u_min':0.00,
        'u_max':0.70,
        'function':lambda u:(u),
        'tick_levels':3,
        'tick_text_levels':2,
        'tick_side':'right',
                }

block_2_params={
             'block_type':'type_2',
             'width':15.0,
             'height':15.0,
             'f1_params':N_params_gain,
             'f2_params':N_params_mlkprice,
             'f3_params':N_params_indmlkloss,
'isopleth_values':[['x',0.75,0.44]],
             }
```

```
### First Block (Graph)

block_BTODR_params={
'u_tag':'mlk',
   'block_type':'type_5',
   'width':25,
   'height':15,
   'u_func':lambda u:u,
   'v_func':lambda x,v:((-0.181/((x*v)*0.6667))+1.017)*0.8747,
   'v_values':[1.0005,0.9500,0.9281],
   'v_manual_axis_data':
      {
  1.0005:['Housed', {'draw_line':True, 'x_corr':1.5, 'y_corr':-0.1}],
  0.9500:['Shoulder', {'draw_line':True, 'x_corr':1.5, 'y_corr':-0.2}],
  0.9281:['Grazed', {'draw_line':True, 'x_corr':1.5, 'y_corr':-0.4}],
      },
# X-axis Description:
   'wd_tick_levels':3,
   'wd_tick_text_levels':2,
   'wd_tick_side':'right',
   'manual_x_scale':True,
       'x_min':0.25,
       'x_max':1.00,
# Y-axis Description:
   'u_scale_opposite':True,
   'horizontal_guides':True,
'u_values':[0.0,0.7],

'isopleth_values':[[0.44,1.0005,'x']],
 }



### Putting it all together:
main_params={
              'filename':'Nomogram_FINAL.pdf',
              'paper_height':15.0,
              'paper_width':25.0,
              'block_params':[block_BTODR_params,block_2_params],
              'transformations':[('rotate',0.01),('scale paper',)],
'isopleth_params':[
          {'color':'black',
           'linewidth':'thin',
           'linestyle':'dashed',
          },
                  ],
              }
Nomographer(main_params)
```