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Abstract

Gastrointestinal (GIT) nematodes have been shown to have a detrimental effect on milk production and reproduction in adult dairy cows. Fecal egg counts (FEC) have been shown to be unreliable measures of worm burden in adult cows. An indirect crude adult worm Ostertagia ostertagi enzyme-linked immunosorbent assay (ELISA) has shown promise in its ability to measure worm burden. ELISA optical density ratio (ODR) values are associated with milk production and pasture exposure in a predictable way. Eprinomectin treatment of adult dairy cows at is associated with increased in milk production in pastured herds. The ELISA has a lot of promise in its ability to predict a milk production or reproduction response to anthelmintic treatment. The ELISA could be used as a herd health management tool to predict cows/herds that will benefit from deworming. The main goals of this study were 1) to establish the benefit of deworming cows with eprinomectin at calving on milk production and reproduction in 64 dairy herds with limited outdoor exposure in the summer, 2) to evaluate the ability of a bulk milk ELISA in identifying dairy herds whose milk production and reproduction would benefit from eprinomectin treatment 3) to evaluate the accuracy of DHI reproduction records, 4) to evaluate the association between GIT nematodes and energy status of cows at calving, and 5) to further develop the ELISA by evaluating a selection of blocking agents, conjugates and antigens.

Eprinomectin treatment did not improve milk production and reproduction in herds with limited pasture exposure. However, it did provide some evidence that the ELISA may be more effective than FEC at identifying herds in which a positive treatment effect might be expected. The bulk milk ELISA was not able to identify herds whose calving to conception intervals would benefit from eprinomectin treatment. A large-scale study involving herds with some exposure to pasture will be required to adequately quantify the relationship between late lactation ODR values and production response to anthelmintic treatment.

Reproduction records from veterinary clinics (farm data) that provided a herd health service were used in the reproduction analysis, because reproduction records are provided optionally to the dairy herd improvement (DHI) organization by producers. DHI records were validated using farm records. Only 21% of cows that had farm reproduction data had comparable DHI records. There was very good agreement in calving dates, calving to last insemination and calving to conception intervals, between both data sources. However, poor agreement was observed in the calving to first insemination interval and number of inseminations per conception.

Because earlier studies found a significant treatment effect in cows that were dewormed around calving, a study to establish the impact of deworming on the peripartum energy status, was conducted. Parasite burden in the previous lactation, had a negative impact on the energy status of the cows, at ODR values greater than 0.4. However, the lack of an anthelmintic treatment effect may have been due to the low parasite burdens in the study herds.

The stability of ELISA plates prepared with one of 3 blocking agents and used with one of 2 conjugates (monoclonal and polyclonal) at various time intervals after
preparation of the plates, was assessed. ODR values of all the blocking agent/conjugate combinations were consistent over a year. Two of the blocking agents enabled plates to be stored dry, at room temperature, making them convenient to use and transport. ELISA results suggested that the larval stage 4 and adult excretory/secretory antigens elicit a stronger humoral immune response than the crude adult antigen. Further studies to compare the abilities of the antigens in identifying cows/herds that would benefit from anthelmintic treatment need to be conducted.
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First and foremost, I am very grateful to Dr. Ian Dohoo for not only giving me the opportunity to pursue my PhD studies at this great university, but also being a fantastic supervisor. Having been under his supervision for over two and a half years, I cannot recall (no exaggeration) a time when our relationship was strained. Not that I have not made mistakes. I made mistakes and Ian helped me overcome them without diminishing my confidence. After working with Ian I have one thing to say: What a good man. Thank you Dr. Dohoo!

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<td>ANOVA</td>
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<td>BHB</td>
<td>Beta-hydroxybuterate</td>
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<td>CI</td>
<td>Confidence interval</td>
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<td>CCC</td>
<td>Concordance correlation coefficient</td>
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<td>CDHMS</td>
<td>Canadian dairy herd management system</td>
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<td>DHI</td>
<td>Dairy herd improvement</td>
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<td>FEC</td>
<td>Fecal egg counts</td>
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<td>GIT</td>
<td>Gastro-intestinal tract</td>
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<td>HR</td>
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<td>Incidence rate ratio</td>
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<td>Ig</td>
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<td>ln</td>
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<td>NEFA</td>
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1 General introduction

1.1 Gastrointestinal tract (GIT) nematodes in temperate climatic regions

*Ostertagia ostertagi* and *Cooperia* species are the most common GIT nematodes in adult dairy cattle of the northern temperate regions (1-3). Other GIT nematodes, found in cattle are *Trichostrongylus axei*, *Oesophagostomum radiatum*, *Haemonchus placei* and *Nematodirus helvetianus*. *O. ostertagi* has been shown to be the most prevalent and economically significance nematode in temperate regions (4;5). As a result, it is the most studied GIT nematode in cattle. GIT nematodes have been shown to exert a negative impact on production in lactating animals, as seen by improved production following elimination of the worms using anthelmintics (6;7)

1.2 *Ostertagia ostertagi*

1.2.1 Life cycle

*O. ostertagi* closely resembles *Teladorsagia* which is a parasite of sheep and goats. It is usually less than 14 mm long and brownish in colour. Nematodes of the super-family trichostrongyloidea have a life cycle that is short and direct (do not need an intermediate host). Mature adult *O. ostertagi* worms located in the abomasum of cows produce eggs that are shed in the feces onto pasture. Under optimal conditions of moisture and temperature, eggs hatch into first larval stage (L1) in as little as 24 hours.
L1 then molts into L2 larvae and finally into the infective L3 larvae. The process of maturation from L1 to L3 larvae normally takes about 21 days. L3 larvae develop a thick sheath that enables them to survive the cold winter temperatures in northern temperate climates in a process known as overwintering or arrested development. Cows grazing on pasture ingest the L3 larvae, which molt into L4 in the abomasum and ultimately into the adult worm parasite. During cold winter temperatures, L4 larvae within the host may not molt into adult worms, a process called hypobiosis. This is a period when the L4 remain in the abomasal mucosa and their metabolism is reduced. In spring, when the weather gets warmer, the L4 hypobiotic larvae resume development and molt into adult worms.

1.2.2 Epidemiology in temperate climates

There are 3 distinct worm populations; a free-living population on pasture (eggs and L1 – L2 larvae), infective L3 larvae on pasture available to the host, and the worm burden (mainly L4 and adult worms) present in cows (8). Numerically, the free-living population in fecal material on pasture is the largest followed by the L3 infective stage that is available to the host and least is the worm burden in cattle. Seasonal climatic differences and management practices have an effect on all the populations.
1.2.2.1 Free-living stages (eggs, LI and L2)

Hatching of eggs is regulated primarily by temperature and moisture (9). The moisture present in fecal material is normally sufficient for the development of the infective stage (L3). Cold temperatures have a detrimental effect on the hatching of the eggs as well larval survival. Exposure to direct sunlight kills eggs and larvae rather quickly. During autumn, in temperate climates, the weather may be wetter than in the summer and the temperatures are mild. These conditions favour hatching and rapid development of larvae into the infective stage.

1.2.2.2 Infective stage

The infective stage larvae must be freed from the fecal pat in order to enable their transmission to grazing cattle. The migrational powers of larvae are generally limited, therefore in the absence of rainfall/moisture the larvae cannot leave the fecal pat. Rain is believed to contribute significantly towards breaking up the fecal pat, aiding the dispersal of larvae throughout the pasture (10). Other factors such as hooves of grazing animals and farm equipment also aid the dispersal of L3 larvae, making it available to cows. Highest numbers of infective L3 larvae in temperate climates occur in the fall. Therefore, nematode transmission to grazing cattle is highest during the fall. L3 larvae die during the hot and dry summers. As mentioned previously, L3 larvae on pasture go into arrested development during the cold winter months and resume development during the spring, mainly as a response to increasing temperatures.
1.2.2.3 Abomasal worm burden

The number of L4 in the abomasums is higher than that of adult worms during the cold winter periods (11;12). However, the number of adult worms rises (with a reduction in L4 numbers) during warmer months, reaching a peak in summer.

1.2.3 Immune response

Immune response mechanisms to GI nematodes are still not completely understood, however, both specific and non-specific responses are believed to play a role. The immune response is directed at different stages of the parasite life cycle. Both humoral and cellular mechanisms are involved in the specific response (13). Calves repeatedly infected with *O. ostertagia* show IgG1, IgG2, IgA, IgM and IgE responses in their serum (14). Many studies have used levels of serum or milk IgG against *Ostertagia* as a measure of parasite worm burden in cattle (15-17). IgA antibodies are barely detected in serum and their presence is a spill-over as result of their secretion into the abomasal lumen as a response to infection (18).

Older cows are generally more immune to GIT nematodes than younger ones. Calves that are exposed to GI nematodes during their first-grazing season develop a long lasting immunity to them (19). Development of a long-lasting response is affected by the magnitude and duration of exposure to the parasite. A study showed that repeated exposures to small doses of parasites are more important in ensuring longer lasting immunity than one large dose (20), while another reported the opposite (19). Cows
develop resistance to parasites like *Nematodirus* and *Cooperia* faster (age of about 1 year) than they do to *Ostertagia* and *Trichostrongylus*. A possible reason for the slow development of protective immunity against *O. ostertagia* may be the presence of antigens that suppress the host immune response to *O. ostertagi* (21). *O. ostertagia* excretory-secretory (ES) antigens (especially the L4 ES) are more immunogenic than the somatic antigens.

Immunity to GIT nematodes in cattle manifest in a sequence of events: 1) decreased fecundity, 2) stunning of growth, 3) increased retardation and arrested development, 4) adult worm expulsion and finally, and 5) resistance to the establishment of new infections.

1.2.4 Pathophysiology

1.2.4.1 Local structural and functional changes

Initial cellular changes that occur as a result of infection with L4 larvae begin within several hours after infection. As L4 larvae enter the mucosal gastric glands, mucosal cells become hyperplastic. During L4 larvae development into adults, mucosal cell hyperplasia persists resulting in the appearance of nodules on the abomasal mucosal surface. Prior to the emergence of young adult worms, the gastric glands become distended resulting in the flattening of glandular epithelium. Inflammatory cell infiltration and oedema are not extensive until about 18 days after infection when the worms begin emerging from the gastric glands. Glands that are distended by larvae lack
differentiated acid-producing parietal cells and pepsinogen-producing chief cells. During emergence of worms from the gastric glands, junctions between post-capillary venule endothelial cells are separated. Functional changes in the abomasum are directly related to the following structural changes: 1) decrease in acid secretion as a result of the loss of parietal cells, 2) lack of conversion of pepsinogen to pepsin due to the absence of an acidic environment, 3) increase in gastrin production due to decreased acid production, and 4) blood serum proteins such as albumin leak into the abomasum through mucosal cell intercellular junctions that are widened by the emergence of worms from the glands.

1.2.4.2 Systemic changes

Studies on the pathophysiology of infection with GIT worm parasites have focused on 3 topics: mechanisms of appetite depression, changes in gastrointestinal function and alterations in protein metabolism.

Voluntary feed intake depression is widely reported as the major impact in the pathogenesis of worm infestation. Lack of appetite in cattle may account for over 73% of the difference in weight gain between Ostertagia-infected and control animals (22). Elevated blood gastrin levels are associated with reduced feed intake. Alternative explanations for reduced feed intake in situations where blood gastrin concentrations are not elevated, have been studied. Work done in Trichostrongylus-infected sheep suggested that central satiety signals may be associated with inappetance (23).
Worm infestation is also accompanied by alterations in GIT motility, gastrointestinal secretions, digestion and absorption. Studies by Fox et al. (1989) demonstrated a reduction in the gut motility of calves infected by *Ostertagia ostertagi*. Substances of parasite origin such as cholinesterases may also influence GIT motility. Destruction of parietal cells in the abomasum results in impaired abomasal digestion. Elevations in blood pepsinogen levels in parasitised animals have been linked to a steep gradient in pepsinogen concentration between the abomasal mucosa and local capillaries as well as an increase in capillary permeability (24;25). However, more recent work suggests that the mechanisms surrounding increased pepsinogen blood levels may be more complicated and multi-factorial. Hypergastrinaemia seen in *Ostertagia*-infected calves seems to be due to an increase in the secretion of gastrin rather than a change in the number of gastrin-secreting G-cells.

Impaired protein digestion in parasitised animals can be overcome by increases in the dietary protein content, which can also improve innate resistance and accelerate the development of acquired immunity.

1.2.5 Clinical signs

Clinical signs are normally observed in young animals. Two disease patterns are observed in cattle, Type 1 and Type 2 ostertagiosis. Both are characterized by diarrhoea, weight loss and growth failure. Type 1 ostertagiosis results from a rapid acquisition of large numbers of L3 larvae that complete development to the adult stage about 3 weeks after infection. It is commonly seen in young cattle near weaning to the age of 18

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months primarily during late summer and autumn in northern temperate climates. Most animals in a herd may be affected and mortalities can be reduced by prompt treatment with endectocide. Type 2 ostertagiosis results when large numbers of inhibited L4 larvae resume development and mature to adults simultaneously. It occurs during late winter and spring in northern temperate climates, commonly in yearling steers and 2-year old heifers in late gestation. Prevalence of Type 2 clinical disease is lower than with Type 1 disease, however, the prognosis is graver than in Type 1.

1.3 Cooperia

1.3.1 Life cycle, epidemiology and immunology

Cooperia are about 9 mm in length and are less pathogenic than Ostertagia. The most commonly found species in the northern temperate regions is Cooperia oncophora. The life cycle of Cooperia is similar to that of Ostertagia except that it completes its life cycle in the small intestine. They also undergo hypobiosis at the L4 stage. Results of the study by Nodvedt et al. (1) suggest that Cooperia may have a higher fecundity than O. ostertagi.

Epidemiology of Cooperia is very similar to that of Ostertagia. A study showed that marked inhibition of development occurred during late fall, with a lower level of inhibition observed for Ostertagia (26). Resumption of development of inhibited Cooperia L4 larvae to adults resumed earlier during the stabling winter period, than that for Ostertagia.
Both humoral and cellular mechanisms are involved in the immunity against *Cooperia*. Humoral response is mainly characterized by IgG1 and IgA (27).

Experimental infection of calves with *C. oncophora* has demonstrated the existence of 3 responder types: high, intermediate and low (27). High responders were characterized by low FEC (and low worm burdens) after infection while low responders had high FEC. This study demonstrated that there are individual differences in the immune response and that worm treatment strategies that minimise the use of anthelmintics could be adopted by treating only those animals that are low responders.

1.3.2 Pathogenesis and clinical signs

L4 larvae usually coil around the tips of duodenal villi or lie deep within the crypts between the villi. *C. punctata* and *C. pectinata* have been reported to penetrate into the intestinal mucosa and submucosa (hence are more severe pathogens than *C. oncophora*). Close contact of adult worms to villi results in compression or distortion of villi and villous atrophy and destruction may follow leading to impaired digestion and absorption. The adult worms penetrate the mucosa of the small intestine and suck blood and heavy infections in calves may cause serious disease. Clinical signs are rarely observed (especially in *C. oncophora* infections). They include diarrhoea, inappetance and weight loss.
1.4 Diagnostic tests

Diagnostic tests for GIT nematodes include: fecal egg counts (FEC), fecal larval cultures, blood pepsinogen values, blood gastrin values and serology (e.g. \textit{O. ostertagia} ELISA). FEC (eggs/gram) have been traditionally used as measures of worm burden, however, they are inaccurate in adult cattle. Absence of eggs in adult cows does not mean that they are uninfected. By virtue of larger volumes of feces that are produced by adult animals, FEC are bound to be lower than in younger animals. However, when initial infection levels are low at the beginning of the first grazing season in calves, a gradual build up of FEC occurs, which has a high correlation with worm burden approximately 2 months after turnout (28). Fecal larval cultures are normally done to differentiate between the GIT nematode species especially in trichostrongylids since their eggs show considerable similarity.

Blood pepsinogen levels have been used as a diagnostic tool for \textit{O. ostertagi}. The problem with using pepsinogen is that no standardised method is used and between-laboratory comparisons are lacking. The method is labour-intensive and thus too expensive. Gastrin has been shown to be far less sensitive in detecting levels of \textit{O. ostertagi} than FEC, pepsinogen and serology (29).

An indirect \textit{O. ostertagi} ELISA (30) using crude worm antigens has been used as an indicator of worm burden. The problem with using crude antigen is that epitopes are shared by many nematodes and cross-reactions occur (14). Another problem is that it is difficult to standardise a crude worm ELISA, considering that each batch of worms may differ. However, a study has found that there is a high repeatability between ELISA tests.
that used 2 batches of crude adult worm antigen (31). The ability to do larval cultures has made it possible to produce purer ES antigens that may have a higher analytic sensitivity and specificity (32). An ELISA was developed at Utrecht University that was based on a specific recombinant low molecular weight protein of *C. oncophora* (33). The test did not show any cross-reactions with other nematodes under experimental conditions. However, it was not able to perform well when field samples were used to validate it, most likely because differences between experimental and field strains.

1.5 Anthelmintic treatment

Classes of anthelmintics that are commonly used in cattle include, benzimidazoles (e.g. albendazole and fenbendazole), imidazothiazoles (e.g. levamisol), tetrahydropyrimidines (e.g. pyrantel and morantel), piperazines, and the macrocyclic lactones. Macrocyclic lactones (avermectins) are the most recent ones on the market. They include, ivermectin, doramectin, eprinomectin and moxidectin all of which are antibiotic in nature since they are produced by streptomycete microorganisms. They produce their effects by binding to glutamate-gated chloride channels, triggering chloride influx, which hyperpolarizes the parasite neuron and prevents initiation or propagation of normal action potentials. This results in the paralysis and death of the parasite. Macrocyclic lactones have revolutionized treatment of parasitic disease. They are generally highly effective and provide a true broad spectrum of activity against GIT nematodes and ectoparasites (hence they are also called endectocides). The major disadvantage of macrocyclic lactones is that they are expensive. Resistance to
Avermectins has been reported in sheep (34) where anthelmintic use is generally more than in cattle. Therefore judicious use of anthelmintics should be emphasized to reduce the risk of development of resistance.

1.5.1 Eprinomectin

Eprinomectin (4'-epiacetylamino-4'deoxyavermectin B1) is a second generation macrocytic lactone. It is suitable for use in lactating dairy cows because its low milk partitioning coefficient enables a zero milk withdrawal period (35;36). It is presently available for dairy cows as a pour-on formulation (for topical use), making it convenient to apply. Even when it is applied under rainfall conditions, it is efficacious. It also has a wide safety margin (35). Topically applied eprinomectin has been shown to be effective against the adult and immature stages of Haemonchus placei, O. ostertagi, T. axei, C. oncophora, C. punctata, Nematodirus helvetianus, Oesophagostomum radiatum and Dictyocaulus viviparous. The efficacy of eprinomectin (against GIT nematodes) is persistent with an efficacy ≥ 90 % for 3 to 4 weeks after topical application (37). Its been shown to exert a positive milk production treatment effect for 180 days after treatment at calving in dairy cows (38). It is also effective against mange mites of cattle.

1.6 Research at the Atlantic Veterinary College (AVC) leading to present study

1.6.1 The "summer-fall slump" project
The "summer-fall slump" project was conducted on dairy herds in Prince Edward Island (PEI) to investigate seasonal milk production trends and to identify factors that were associated with these trends (39). One of the studies in the project evaluated the association between parasite burden (as measured with the indirect ELISA) and other predictors such as milk production. This study found a negative association between milk production and parasite burden. This was the first time the ELISA had been used at the AVC. This study paved the way for more investigation on the relationship between ELISA test and milk production and herd management practices.

1.6.2 Relationship between the ELISA and milk production and pasture exposure

Bulk tank milk surveys (40;41) of *O. ostertagi* antibodies in PEI and Nova Scotia (NS) showed that high ELISA optical density ratio (ODR) values were predictably related to pasture exposure. The PEI and NS studies showed that an increase in ODR from the observed 25th percentile to the 75th percentile in each study was associated with a drop in milk production of 1.2 kg/cow/day and 1.25 kg/cow/day, respectively. These studies showed that the ELISA had a lot a promise in being used as a tool to identify herds or cows whose production would benefit from endectocide treatment.
1.6.3 Treatment effects and predictive ability of the ELISA

A randomized, double blind clinical trial was performed in 28 dairy herds in Canada. The objective of the study was to evaluate the effect of treatment with eprinomectin pour-on solution (Eprinex®) at calving on milk production, in cattle that had exposure to pasture (38). Cows treated with eprinomectin produced an additional 0.94 kg of milk per day when compared to controls over a period of 200 days. Another study showed that cow-level ODR values determined late in the previous lactation had a marginally significant (most likely because of a small sample size) effect on treatment response, suggesting that high parasite burden cows responded better to anthelmintic treatment (15). One study evaluated the effect of eprinomectin on reproductive performance (42). The study found a marginally significant effect of treatment on the calving to conception interval but not on calving to first service interval. A significant reduction in the number of breedings to conception for eprinomectin-treated cows was also observed. These studies provided further evidence that the ELISA may be useful in determining the cows to treat.

1.6.4 ELISA test validation

The crude worm indirect ELISA was evaluated using serum and milk samples from adult dairy cows. The repeatability of the test between and within plates, antigen batches, milk preservation methods and sample storage conditions was generally good (31). This provided a step towards the use of the ELISA on a larger scale.
1.6.5 Parasite burden of non-pastured herds

The 2 bulk tank milk ELISA ODR surveys conducted in Atlantic Canada showed unexpectedly high ODR values in some herds in which cows were not exposed to pasture during summer. There have also been claims in the past that transmission of gastrointestinal nematodes between cows occurs during housing but there has also been strong evidence refuting these claims (43). This led to the question whether production in cows that had limited or no exposure to pasture, would be increased by anthelmintic treatment. This question was addressed by studies described in this thesis.

1.7 Objectives of the present study

1.7.1 Overall goals

The broad objectives of the present study were: 1) to determine if treatment of dairy cows with eprinomectin at the time of calving had any beneficial effect on milk production and reproduction in herds that kept their adult cows in total confinement or provided limited outdoor exposure, 2) to determine if the ELISA was able to identify herds in which a treatment effect would be expected, and 3) to further develop the ELISA and improve its accuracy and efficiency by evaluating several possible antigens, blocking agents and conjugates.
1.7.2 Specific objectives of 6 research chapters

1.7.2.1 Effect of eprinomectin on milk production

Goal: To evaluate if there are milk production benefits of deworming cows with limited pasture exposure as well as to determine if the bulk milk ELISA can identify herds that would benefit from eprinomectin treatment.

Specific objectives: 1) To determine if treatment of dairy cows with eprinomectin at the time of calving had any beneficial effect on milk production in herds that kept their adult cows in total confinement or provided limited outdoor exposure, and 2) to determine if either FEC or the ELISA was able to identify herds in which a treatment effect would be expected.

1.7.2.2 Effect of eprinomectin on reproduction

Goal: To evaluate if the reproduction in cows with limited pasture exposure would benefit from deworming as well as to determine the predictive ability of the ELISA.

Specific objectives: 1) To investigate if eprinomectin treatment of adult dairy cows around calving had any beneficial effects on the calving to first artificial insemination interval, calving to conception interval, and number of inseminations per conception in herds with no or limited pasture exposure, 2) to investigate whether the ELISA could be used to identify herds whose calving to conception interval could benefit from eprinomectin treatment.
1.7.2.3  **DHI reproduction data evaluation**

Goal: To evaluate if DHI reproduction parameters extracted from DHI records can be used in place of on-farm records.

Specific objectives: 1) To determine the proportion of herds and cows within herds, whose reproduction records were submitted to DHI, and 2) to determine the agreement in the calving dates and reproduction indices (calving to first artificial insemination interval, calving to last insemination interval, calving to conception interval and number or inseminations per conception) between DHI data and data obtained from clinics in Ontario and Quebec that provided a herd health service to these farms (farm data).

1.7.2.4  **Effect of GIT nematodes on energy status of dairy cows**

Goal: To evaluate if deworming dairy cows around calving improves their energy status.

Specific objective: To investigate if treatment of cows with eprinomectin before calving had any effect on energy balance pre- and post-calving. The relationship between parasite burden (as determined by milk antibody levels) at the end of one lactation and energy balance in the subsequent peri-parturient period was also evaluated.

1.7.2.5  **Stability of ELISA plates over time**

Goal: To evaluate if ELISA plates can be stored dry at room temperature.

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Specific objective: To assess the stability of plates prepared with one of 3 blocking agents, and used with one of 2 conjugates, at various time intervals after preparation of the plates.

1.7.2.6 Evaluation of 3 antigens in the ELISA

Goal: To compare the performance of ES antigens with crude antigen in the ELISA

Specific objective: 1) to determine the agreement between ELISA tests conducted using 3 O. ostertagia antigens: crude adult worm, L4 ES and adult ES.
1.8 References


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Effect of eprinomectin treatment at calving on milk production in dairy herds with limited outdoor exposure

2.1 Abstract

The objective of this study was to determine the effect of endectocide treatment at calving on milk production in herds that were totally or semi-confined during the summer. In totally confined herds lactating and dry cows were housed throughout the summer and had no access to pasture. In semi-confined, herds lactating and dry cows had limited outdoor exposure to a small pasture or paddock but were still fed a ration that met all their nutritional requirements. The study was carried out between February 2002 and February 2003 in 64 herds enrolled with DHI and distributed in 4 regions in Canada and 1 state in the United States. Cows were randomly allocated to receive eprinomectin or a placebo, with treatment being administered on or close to the day of calving. In May/June 2002, 8 fecal samples were collected from each farm and fecal egg counts (FEC) were determined. Monthly bulk milk samples from each farm were tested with an indirect ELISA using a crude Ostertagia ostertagi antigen. Monthly test-day milk production data were recorded for 200 days after calving. In general, FEC were very low (mean = 1 EPG, range = 0 to 27). Mean herd bulk milk ELISA optical density ratio (ODR) values for the whole year ranged between 0.22 and 0.80. The ODR values were dichotomized into high and low using a cut-point of 0.5. Treatment effects were analyzed using a linear mixed model with herd and cow as random effects. The analysis was restricted to 4789 cows (23956 test-day records) treated between 21 days before and
7 days after calving. Overall, there was no significant effect of treatment. However, there was a marginally significant (P = 0.15) interaction between treatment and ODR, which suggested a larger treatment effect in high-ODR herds than in low-ODR herds. The confidence intervals for the treatment effects (kg/day per cow of milk) in both high-ODR herds (-0.33 to 1.10) and in low-ODR herds (-0.53 to 0.14) were both wide and included zero. Consequently, this study failed to show a beneficial effect of eprinomectin treatment in totally- and semi-confined herds.
2.2 Introduction

Many studies have shown that de-worming adult dairy cows has beneficial effects on milk production. Sanchez et al. (1) reported a mean increase of 0.46 kg/cow per day in 75 clinical trials, although an adjustment to eliminate publication bias reduced this value to 0.35 kg/cow per day.

Eprinomectin is a third generation macrocytic lactone de-wormer that is suitable for use in dairy cows because its low milk partitioning coefficient (2) alleviates the need for a milk withdrawal period. A recent study has identified a 0.94 kg/day per cow increase in milk production when cows are treated at calving with eprinomectin pour-on especially in dairy herds exposed to pasture (3). However, the effect of eprinomectin on milk production in herds with little or no exposure to pasture has not been investigated.

Fecal egg counts (FEC) have been used for a long time to measure parasite burden but are an inaccurate measure of worm burden in adult animals (4;5). An ELISA test using crude *Ostertagia ostertagi* antigen (6-8) has been used to evaluate worm burden in several studies (7;9) and has been found to be a better measure of worm burden than FEC.

There have been claims in the past that cow-to-cow contamination of gastrointestinal nematode larvae occurs during housing (10) but there has also been strong evidence refuting these claims (11). Two bulk tank milk ELISA ODR surveys conducted in Atlantic Canada have shown that the ODR values found in some herds that did not expose cows to pasture during summer were unexpectedly high (12;13). This led
to the question whether milk production in cows that had limited or no exposure to pasture would be increased by endectocide treatment.

The primary objective of this study was to determine if treatment of dairy cows with eprinomectin at the time of calving has any beneficial effect on milk production in herds that keep their adult cows in total confinement or provide limited outdoor exposure. The secondary objective was to determine if either FEC or the ELISA is able to identify herds in which a treatment effect would be expected.

2.3 Materials and methods

2.3.1 Herd selection

The study was a randomized clinical trial that was performed in 65 herds between February 2002 and February 2003. There were 5, 9, 16, 12 and 11 dairy herds enrolled in the Canadian provinces of Prince Edward Island (PEI), Nova Scotia (NS), Ontario, Quebec and Saskatchewan, respectively. An additional 12 dairy herds were enrolled in Minnesota, USA. Originally, 68 herds were recruited into the study but 3 dropped out, one in each of the provinces of Ontario, Quebec and Saskatchewan. The 2 herds in Ontario and Quebec dropped out at the beginning of the study. The Saskatchewan herd dropped out in the middle of the study after we had collected some bulk tank milk and fecal samples. Therefore, those data were included only in the assessment of parasite burden and not in the analysis of milk production.
Cows in this study were Holstein Fresian and classified as either totally or semi-confined. Totally confined herds were defined as herds in which lactating and dry cows were housed throughout the summer and had no outdoor exposure at all. Outdoor exposure for the lactating and dry cows in the semi-confined herds was limited to a yard or paddock; nevertheless, the cows were still fed a ration that met all of their nutritional requirements, i.e. the composition and quantity of stored feeds (components or total mixed ration) fed to lactating or dry cows was not changed when the cows went outside. The herds had to be enrolled with the Dairy Herd Improvement (DHI) organization and willing to provide access to those data. They were also required to provide monthly bulk tank milk samples either through the dairy laboratory or through on-farm collection. No endectocide could have been administered to lactating cows during the summer of 2001. There were no restrictions on housing type for heifers in selected herds.

Selection of herds into the study was based on their proximity to a veterinary school and/or collaborating veterinary clinic. Five collaborating clinics were identified in the Atlantic provinces (PEI and NS), 2 in Saskatchewan and 1 in each of the 3 other study sites. To detect a difference of 0.5 kg/cow per day of milk between eprinomectin- and placebo-treated cows in a study with 80 % power, a total sample size of 4688 cows was needed (half from each confinement group). Another goal was to balance the number of totally-confined and semi-confined herds. The parameters (std. dev. = 6.17 kg/day, within-cow correlation = 0.32) used in the sample size calculation were based on the results of the clinical trial by Nodvedt et al. (3). Since some attrition from the study was anticipated given the low level of study technician supervision, a starting sample size of 6000 cows was planned. Based on the assumption that the average herd
size was 100 cows, selecting 12 herds per study site ensured that the attrition-corrected sample size of 6000 cows was met. A minimum of 40 and a maximum of 300 milking cows per herd was set for participating herds. Within each study site, no more than 4 herds were to be over 150 cows so as to avoid the influence of a small number of large herds on the study. During the study period, 4 follow-up visits and monthly phone calls were made to ensure that the study protocol was being followed. Data related to herd size, cow and heifer housing and other management practices were recorded.

2.3.2 Treatment protocol

The dose of eprinomectin pour-on (5 mg/ml) per cow was calculated on the basis of a 725 kg cow applied at 500 \( \mu g/kg \) (1 ml/10 kg) resulting in a dose per cow of 72.5 ml. Eprinomectin was dispensed as individual doses into brown colored plastic bottles and an equal quantity of placebo (mineral oil) was dispensed in a similar way. The similarity in the bottles containing eprinomectin and placebo ensured that the producer would be unaware of the contents of each bottle. The bottles were uniquely labeled with sequential numbers (alternating placebo and eprinomectin) and packed into compartmentalized cardboard boxes containing 18 bottles each. The boxes were then distributed to all the participating herds at the beginning of the trial and additional bottles provided if a herd ran low. Treatment of cows or heifers was to be done between 3 weeks before the expected calving date and up to 1 week after calving. There was a label on the box containing treatment bottles informing the producer to take the bottles from the left to the right of each row whenever treatment was due. Treatment was done
by pouring the entire contents of the bottle along the dorsal midline of the animal. Cow name or number, treatment date, calving date and bottle number were then recorded on a form that was given to the producer.

2.3.3  Fecal samples, bulk tank milk and mange scores

On the second farm visit (May/June, 2002), fecal samples were collected from 4 first-parity and 4 second- and above-parity milking cows that had not been treated at calving. FEC in eggs per gram (EPG) were determined using the modified Wisconsin sugar flotation technique (14).

Monthly bulk tank milk samples from each herd were collected between March 2002 and February 2003. All milk samples were sent to the Atlantic Veterinary College where an indirect ELISA using a crude *O. ostertagi* antigen was performed (6).

A mange score (0 to 3) for each cow was determined and recorded at the time of treatment by the producer according to a mange scoring chart (15). Producers were trained in the use of the scoring system at the beginning of the study. Mange status was evaluated to determine if the milk production of cows with mange would benefit from eprinomectin treatment.

2.3.4  Milk production data

Individual test-day milk yield records for the Canadian sites were obtained from the Canadian Dairy Herd Management System database in Montreal. The records for the
Minnesota herds were obtained from United States National Dairy Herd Improvement. This process of data collection ensured that the study was blinded since the technicians that measured milk yield were unaware of the treatment status of the cows. Test-day records were collected from February 2002 to August 2003 in order to ensure that cows that calved at the end of the treatment period (February 2003) had completed 200 days of lactation before data collection was stopped. Information on parity, days in milk (DIM), somatic cell count (SCC) and calving date were obtained from the same sources. Two herds in Quebec dropped out of DHI but were retained in the study and farm-recorded milk production data were used in the analysis.

2.3.5 **Descriptive statistics**

The FEC distribution across housing types and sites was summarized. In each herd, the percentage of cows with an egg count greater than 0 was determined and herds were classified into high and low FEC herds based on a cut-point of 50 %. The temporal distribution of bulk tank milk ELISA ODR values was evaluated graphically. A mean 12-month ODR was computed for each herd and dichotomized into high and low ODR using a cut-point of 0.5. Mange scores were dichotomized into absent (score 0) or present (greater or equal to 1). The study site prevalence of cows with any signs of mange was determined based on this dichotomized variable.

Equality in the distribution of eprinomectin and placebo treated cows was verified within herds, lactation groups and calving seasons. Four categorical variables based on the housing of dry cows, lactating cows, bred heifers and young heifers were
generated with each variable having 3 categories (pasture/paddock, gravel yard and confined). The distribution of herds according to dry- and lactating-cow housing and ODR was summarized. The study period was divided into 4 calving seasons: winter (January to March), spring (April to June), summer (July to September) and fall (October to December). Parity was classified into 3 classes: first lactation, second lactation or third lactation and above.

2.3.6 Multi-variable methods

The analysis of FEC and their association with the predictors (parity, housing type and study site) used generalized estimating equations with an exchangeable correlation structure to account for the correlation between cows in the same herd (16). A negative binomial distribution was assumed as it showed a significantly better fit than a Poisson distribution, and no improvements were obtained by adding a zero-inflation component (16).

The monthly bulk milk ELISA ODR and their association with the predictors (testing season, housing type and study site) were analyzed by linear mixed models to account for the repeated measurements on herds. Different within-herd correlation structures were examined and in order of increasing complexity these are: compound symmetry, first order auto-regressive, auto-regressive moving average (arma) and stationary (or Toeplitz). The best model was selected based on Akaike's Information Criterion (AIC) and likelihood-ratio tests.
Individual cow milk production for the first 200 days in milk was analyzed by linear mixed models with herd random effects and correlated errors of observations on the same cow to account for the repeated measurements. The highest level in the hierarchy, study site, was included in the model as a fixed effect (predictor). In a linear mixed model it is assumed that all random effects follow a normal distribution, and with only 5 study sites it was difficult to make this assumption. Other predictors included were eprinomectin treatment, days in milk, parity, ln SCC, calving season, calendar month of test, housing type, mange status and gastrointestinal tract nematode parasite burden indicators. As the housing type of milking and dry cows could be expected to have a more direct effect on milk production than the housing type for heifers, both dry and lactating cow housing variables were included in the model. The associations of the two parasite burden indicators, FEC and herd-level ODR status, with milk production and/or treatment response were examined by including interaction terms with treatment in separate models. Calendar month of test was included in the model to account for seasonal variation. DIM was included as Wilmink’s function (17) consisting of a linear term (DIM) and a power term ($\text{DIM}^{0.05}$). Main effects of interest were kept regardless of their $P$-value and so was the interaction between treatment and ODR. The within-cow correlation structure was explored in a similar fashion as in the linear mixed model for bulk milk ELISA ODR. Pair-wise correlation coefficients of all parameter estimates were determined to investigate any problems of collinearity between predictors.

SAS, Proc Mixed, version 8.2 (18) was used to fit the linear mixed models and Stata version 8 (19) was used for data editing and all other statistical procedures. For all
analyses, the significance level was set at $P < 0.05$. Linear mixed model analyses used the Satterthwaite method to approximate the degrees of freedom for significance tests.

2.4 Results

2.4.1 Parasite burden

In general, FEC (Table 2.1) were very low (mean = 1, range = 0 to 27, standard deviation = 2). In the study by Borgsteede et al. (5) that was conducted on adult dairy cattle, 30% of the cows had FEC higher than 25 EPG while in this study only 2 out of 556 cows had FEC higher than 25. A total of 677 monthly bulk tank milk samples (Figure 2.1) were tested from the 66 herds. The herd average bulk milk ODR values had a mean of 0.41, a standard deviation of 0.13 and a range of 0.22 to 0.80.

2.4.2 Mange score prevalence

The prevalence of mange was generally low. No cases of mange were recorded in 19 herds and only 4 herds had a mange prevalence greater than 0.4. The cow-level prevalences of mange in the regions were 0.06 (Atlantic study site), 0.08 (Quebec), 0.16 (Ontario), 0.12 (Saskatchewan) and 0.13 (Minnesota).

2.4.3 Test animals
Effort was made to link records in the treatment file with those in the production data file. Out of a total of 6162 cows that were treated in the trial, 5477 (89 %) had production records. Treated cows without production records consisted of cows with inconsistent identification or cows that were removed from the herd before any production data were recorded for the lactation. Restriction of the analysis to cows treated between 21 days before and 7 days after the day of calving was the main reason for the drop from 5477 to 4789 cows (78 % of treated cows). One herd with 75 cows did not have SCC records, so it was excluded from the statistical models where SCC was a predictor.

Out of the 4789 cows with production records and treated within the allotted time period, 50.4 % were treated with eprinomectin and the rest with placebo. The equality in the distribution of eprinomectin and placebo treated cows within the lactation groups provided evidence that random allocation of treatment to cows had been achieved. Equality of treatment groups within regions and calving seasons provided a good indication that the exclusion of cows did not introduce any selection bias.

The parity distribution of the third- and above-parity cow group was as follows: 44% third parity, 27% fourth parity, 15% fifth parity, 7% sixth parity and the rest was composed of parities 7 to 13.

2.4.4 Cow housing and herd parasite burden

Nine of the 13 (69%) high-ODR herds had lactating cows which had some degree of outdoor exposure compared to 12 of the 51 (24%) low-ODR herds (Table 2.2).
None of the high-ODR herds and 20 (39%) of the low-ODR herds confined both their lactating and dry cows indoors.

2.4.5 Multi-variable models

2.4.5.1 Parasite burden

The bulk milk ELISA ODR model that assumed a Toeplitz correlation structure fit much better than the rest of the models that assumed other structures. This model showed that ODR was significantly related to season \((P < 0.001)\) and study site \((P < 0.001)\). Bulk milk ODR values in the winter were 0.03 (SE = 0.02) less than in the fall while those in the summer were 0.04 (SE = 0.01) higher than those in the fall. Figure 2.2 shows that ODR values peaked during the warmer months. There was no significant relationship between ODR and housing type \((P = 0.16)\) although there was a trend towards higher ODR values in semi-confined herds than confined ones (Figure 2.2). A total of 672 observations from 66 herds were used in this analysis.

Out of 556 cows (from 66 herds) whose fecal samples were collected, 411 were used in the analysis because the parity status of the rest was not recorded. The negative binomial regression model showed that parity \((P = 0.047)\) and housing type \((P < 0.001)\) were significantly related to ln FEC. Mean ln FEC in cows that were in their second parity and above was 0.72 (SE = 0.36) less than that from first-parity cows. The mean ln FEC of semi-confined cows was 2.24 (SE = 0.52) higher than that in totally confined cows. The effect of study site on ln FEC was marginally significant \((P = 0.063)\). Cows
in Ontario had the highest ln FEC while cows in Quebec and Saskatchewan had the lowest.

2.4.5.2 Milk production

The arma correlation structure was chosen because it fit the data better than the auto-regressive structure which has been used in the past for such test-day models. The Toeplitz correlation structure produced a slightly better fit (as determined by both the AIC and likelihood-ratio test) than the arma, but it is a more complicated model which requires the estimation of many more correlation parameters.

Results of the final model are shown in Table 2.3. Overall, there was no significant effect of treatment ($P = 0.291$). However, there was a marginally significant ($P = 0.149$) interaction between treatment and ODR, with a larger treatment effect (kg/day per cow of milk) in high-ODR herds (0.38 kg) than in low-ODR herds (-0.20 kg). The confidence intervals for the treatment effects in high-ODR herds (-0.33 to 1.1) and in low-ODR herds (-0.53 to 0.14) were both wide and included zero. Cows in low-ODR herds that received the placebo produced 2.8 kg of milk more than cows in high-ODR herds that received the placebo.

In a model in which ODR was replaced by FEC as an indicator of herd parasite burden, the interaction of FEC and treatment ($P = 0.37$) was not significant. This model was not considered any further and details have not been presented.

Lactating-cow housing and dry-cow housing were not significantly associated with milk production. Study site was statistically insignificant ($P = 0.342$) and was
removed from the model. Parity, DIM, month of test, calving season and ln SCC were all statistically significant (Table 2.3). Interaction terms between treatment and all the other variables were tested and found to be non-significant. There was no indication of collinearity because the pair-wise correlations between the parameter estimates of all predictors were low.

The effect of mange at the time of treatment was evaluated in a model together with the other predictors and was found not to have a significant effect on milk production nor was there any treatment by mange interaction effect.

2.5 Discussion

Consistent with other studies (4;5), the ODR values reached a peak in the warmer months and declined in colder months. Consistent with a study done in Canada (3), FEC were higher in younger cows than in older ones. The older cows are more likely to have developed adequate immunity to keep the FEC low. The FEC of cows in semi-confined herds were higher than those in totally-confined herds most likely because of some pasture exposure in the former group.

Nine of the 13 (69%) high-ODR herds had lactating cows which had some degree of outdoor exposure compared to 12 of the 51 (24%) low-ODR herds. None of the high-ODR herds and 20 (39%) of the low-ODR herds confined both their lactating and dry cows indoors. This distribution is consistent with expectations since cows with outdoor exposure have a higher probability of consuming parasite larvae and getting infected than cows kept indoors.
Overall, there was no significant effect of treatment. These results are consistent with a study conducted in the USA that also evaluated the effect of endectocide treatment on milk production in confined cows (20). However, there was some evidence that the difference between treated and non-treated cows was 0.58 kg/cow per day higher in high-ODR than in low-ODR herds. On the other hand, there was no evidence that the effect of treatment depended on whether the herd was low-FEC or high-FEC. Therefore, this study did provide some indication that the ELISA test was better than FEC at being able to identify herds in which a positive treatment effect might be expected. Sanchez et al. (7) found that ODR values of late-lactation-cow milk samples had a marginally significant effect ($P = 0.07$) on treatment response in the subsequent lactation, suggesting that high-ODR cows responded better to the anthelmintic treatment.

Cows in low-ODR herds that received the placebo produced 2.8 kg of milk more than cows in high-ODR herds that received the placebo. This provides evidence that being a high-ODR herd was associated with lower production. Despite controlling for the type of housing (confinement, yard or paddock) that lactating and dry cows had, it was impossible to tell if the effect was totally due to parasites or partially due to outdoor exposure. If it was entirely due to parasites, we would have expected a larger treatment effect.

There was no evidence that mange status at calving had any influence on milk production nor was there evidence that the effect of eprinomectin depended on the mange status. This may be attributed to the generally low mange prevalence observed in the study herds. Mange scoring was done by the producers. In order to standardize the
reporting of mange lesions, a mange scoring system was used (15). Despite the fact that the system was evaluated and found to be useful, it does have some shortcomings. It is subject to mis-classification bias especially when the lesions were mild (level 1) as was the case in the majority of the mange cases in the study. Cows that have few lesions are likely to be missed and classified as non-cases. On the other hand, non-cases could be wrongly classified as mange cases especially in thin cows as they tend to lose hair around the bony prominences of the lumbo-sacral area. Dry dung and dirt around the lumbo-sacral area may appear as crusts that may be mistaken for mange lesions. Given the large number of herds in the study, and their wide geographic distribution, it was impossible to have study personnel visit the farms on a sufficiently frequent basis to do the mange scoring.

Interpretation of all the significant predictors of milk production (DIM, parity, month of test, ln SCC and calving season) was consistent with the findings of other studies. First- and second-parity cows produced 8 kg and 1.4 kg less milk respectively, than the third- and above-parity cows. The linear mixed effects model showed that test-day milk production was generally highest in the winter and spring and lowest in the summer and fall. The results of a study by Ray et al. (21) on the effect of season and parity on milk production are consistent with the results of the present study. A unit increase in ln SCC resulted in a decrease of 1 kg of milk. Many previous studies have shown that increased SCC were associated with reduced milk production. Cows calving in winter produced 0.4 kg/day more milk than ones calving in the fall. Milk production of cows calving in spring and summer was 0.07 kg/cow per day and 1 kg/cow per day less respectively, than ones calving in the fall. The study by Nodvedt et al. (3) that
evaluated the effect of eprinomectin in pastured herds did not have enough power to
detect a significant association between calving season and milk production.

2.6 Conclusions

Overall, this study failed to show a beneficial effect of eprinomectin treatment in
totally- or semi-confined herds. However, this study did provide some evidence that the
ELISA may be more effective than FEC at being able to identify herds in which a
positive treatment effect might be expected.
2.7 References


(18) SAS. Cary, NC, USA: 1999.


Table 2.1: Mean, range and standard deviation values (std.dev.) of fecal egg counts (FEC) and bulk tank milk ELISA optical density ratio (ODR) values from 66 herds according to study site, housing type and season (for ODR) in a clinical trial on the effect of eprinomectin on milk production in confined and semi-confined dairy herds.

<table>
<thead>
<tr>
<th>Site (n herds)</th>
<th>Eggs per gram</th>
<th>Bulk Milk ODR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (range)</td>
<td>std. dev.</td>
</tr>
<tr>
<td>Atlantic (14)</td>
<td>1 (0-26)</td>
<td>4</td>
</tr>
<tr>
<td>Minnesota (12)</td>
<td>0 (0-10)</td>
<td>1</td>
</tr>
<tr>
<td>Ontario (16)</td>
<td>1 (0-27)</td>
<td>3</td>
</tr>
<tr>
<td>Quebec (12)</td>
<td>0 (0-6)</td>
<td>1</td>
</tr>
<tr>
<td>Saskatchewan (12)</td>
<td>0 (0-6)</td>
<td>1</td>
</tr>
<tr>
<td>Housing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Confined (34)</td>
<td>0 (0-6)</td>
<td>1</td>
</tr>
<tr>
<td>semi-confined (32)</td>
<td>1 (0-27)</td>
<td>3</td>
</tr>
<tr>
<td>Season</td>
<td></td>
<td></td>
</tr>
<tr>
<td>winter</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Season</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>summer</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>fall</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1FEC distribution was right skewed and all median values were zero.
Table 2.2: Distribution of 64 herds by parasite burden (high/low ODR), dry and lactating cow housing in a clinical trial on the effect of eprinomectin on milk production in confined and semi-confined dairy herds.

<table>
<thead>
<tr>
<th>Lactation cow housing</th>
<th>Paddock/pasture</th>
<th>Yard</th>
<th>Indoors</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low ODR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paddock/pasture</td>
<td>6</td>
<td>0</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Yard</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Indoors</td>
<td>14</td>
<td>5</td>
<td>20</td>
<td>39</td>
</tr>
<tr>
<td>TOTAL</td>
<td>22</td>
<td>6</td>
<td>23</td>
<td>51</td>
</tr>
</tbody>
</table>

| High ODR              |                 |      |         |       |
| Paddock/pasture       | 4               | 0    | 1       | 5     |
| Yard                  | 1               | 3    | 0       | 4     |
| Indoors               | 2               | 2    | 0       | 4     |
| TOTAL                 | 7               | 5    | 1       | 13    |
Table 2.3: Coefficients, standard errors (S.E.) and \( P \)-values from a linear mixed effects model (with a herd random effect and an arma within-cow correlation structure) of the effect of eprinomectin on test-day milk production (kg/day per cow) within the first 200 days after calving (4789 cows from 64 herds).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>Coefficient</th>
<th>S.E.</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td></td>
<td>167.830</td>
<td>2.472</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Eprinomectin</td>
<td>yes</td>
<td>0.385</td>
<td>0.366</td>
<td>0.291(^a)</td>
</tr>
<tr>
<td></td>
<td>no</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ODR</td>
<td>low</td>
<td>2.819</td>
<td>1.234</td>
<td>0.037(^a)</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eprino*ODR</td>
<td>yes,low</td>
<td>-0.584</td>
<td>0.405</td>
<td>0.149</td>
</tr>
<tr>
<td>Lactating cow</td>
<td>pasture</td>
<td>-0.908</td>
<td>1.197</td>
<td>0.719(^b)</td>
</tr>
<tr>
<td></td>
<td>yard</td>
<td>0.177</td>
<td>1.420</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indoor</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry cow</td>
<td>pasture</td>
<td>0.426</td>
<td>0.988</td>
<td>0.398(^b)</td>
</tr>
<tr>
<td></td>
<td>yard</td>
<td>1.887</td>
<td>1.386</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indoor</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parity</td>
<td>1</td>
<td>-8.207</td>
<td>0.187</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-1.421</td>
<td>0.196</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3+</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ln SCC</td>
<td></td>
<td>-1.075</td>
<td>0.033</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DIM</td>
<td></td>
<td>-0.110</td>
<td>0.002</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DIM(^{0.05})</td>
<td></td>
<td>-145.290</td>
<td>2.344</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Calving season</td>
<td>winter</td>
<td>0.380</td>
<td>0.223</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Season</td>
<td>Coefficient</td>
<td>p-value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>---------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>spring</td>
<td>-0.071</td>
<td>0.235</td>
<td></td>
<td></td>
</tr>
<tr>
<td>summer</td>
<td>-0.947</td>
<td>0.217</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fall</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Test month, \(^1\) <0.001\(^b\)

\(^a\) Test includes both the main effect and interaction terms.

\(^b\) For categorical variables, \(P\)-value reflects test of overall significance, not individual levels of the variable.

\(^1\) Dummy variables for each of the 12 months not shown.
Figure 2.1: Frequency distribution of 677 monthly bulk tank milk ODR values obtained from 66 confined and semi-confined dairy herds that participated in a clinical trial on the effect of eprinomectin on milk production.
Figure 2.2: Temporal distribution of the monthly average bulk milk ELISA ODR values according to housing type (March 2002 - August 2002) in 66 confined and semi-confined dairy herds that participated in a clinical trial on the effect of eprinomectin on milk production.
3 Effect of eprinomectin pour-on treatment around calving on reproduction parameters in adult dairy cows with limited outdoor exposure

3.1 Abstract

The objective of this study was to investigate if treatment of cows with eprinomectin around calving had any beneficial effects on reproduction as measured by the calving to first artificial insemination interval, calving to conception interval, and number of inseminations per conception in totally and semi-confined dairy herds. In totally confined herds lactating and dry cows were housed throughout the summer and had no access to pasture. In semi-confined herds lactating and dry cows had limited outdoor exposure to a small pasture or paddock but were still fed a ration that met all their nutritional requirements. The study was carried out between February 2002 and February 2003 in 35 herds (2381 cows) located in Quebec, Ontario and Minnesota (USA) participating in a larger clinical trial. The herds kept electronic reproduction records. Cows were randomly allocated to receive eprinomectin or a placebo, with treatment being administered on or close to the day of calving. Monthly bulk tank milk samples from each farm were tested with an indirect ELISA using a crude Ostertagia ostertagi antigen and these data were averaged over the study year. The optical density ratio (ODR) values were then dichotomized into high and low using a cut-point of 0.50. Treatment effects were analyzed using Cox proportional hazards survival models with herd frailty effects for calving to conception and calving to first service intervals. Aalen’s linear hazards model was used to investigate time-varying effects in the Cox
models. A random effects poisson regression model was used to model the number of services per conception. Other predictor variables tested in the models were parity, calving season, study site, peak milk production, ODR and the lactating and dry cow housing variables. Overall, there was no significant effect of treatment on the 3 indices of reproductive performance. The effect of season of calving depended on how much time had passed since calving. Presumably this effect reflected a seasonal effect at the time of breeding. Hazard of conception in younger cows was higher than in older cows. Cows bred early tended to have a higher number of inseminations per conception than those bred late.
3.2 Introduction

Anthelmintic treatment of adult dairy cows has a beneficial effect on reproductive performance indices (1). In a study conducted in Canada, cows from pastured herds were treated with eprinomectin (Eprinex®) at calving in order to evaluate if treatment had any beneficial effects on reproduction. The study found a marginally significant effect of treatment on the calving to conception interval as well as a significant reduction in the number of artificial inseminations per conception in eprinomectin treated cows (2). The same study evaluated the effect of treatment on milk production and found that cows treated with eprinomectin produced 0.94 kg/day of milk more than placebo-treated cows.

Eprinomectin is a third generation macrocytic lactone endectocide that is suitable for use in dairy cows. It has low milk partitioning coefficient (3) that alleviates the need for a milk withdrawal period. However, determining which cows/herds would benefit from treatment has been difficult. Fecal egg counts are an unreliable measure of parasite worm burden in adult dairy cattle (4;5). An indirect microtitre ELISA that uses *Ostertagia ostertagi* antigen (6) determined parasite burden better than fecal egg counts. Studies have been conducted to investigate the ability of the ELISA to identify cows that would benefit from anthelmintic treatment. Sanchez et al. (2) found that among untreated cows, the rate of conception was lower for cows with a high ELISA optical density ratio (ODR) compared to low ODR cows, suggesting that higher parasite burdens had an adverse effect on reproduction.
There have been claims that transmission of gastrointestinal nematodes between cows occurs during housing (7). However, there has also been strong evidence refuting these claims (8). Two bulk tank milk ELISA ODR surveys conducted in Atlantic Canada showed unexpectedly high ODR values in some herds in which lactating cows were not exposed to pasture during summer (9;10). This led to the question as to whether reproductive performance in cows that had limited or no exposure to pasture would be increased by endectocide treatment.

The primary objective of this study was to investigate if eprinomectin treatment of adult dairy cows around calving had any beneficial effects on the calving to first insemination interval, calving to conception interval, and number of inseminations per conception in herds with no or limited pasture exposure. The secondary objective was to investigate whether bulk milk ODR could be used to identify herds whose calving to conception interval could benefit from eprinomectin treatment.

3.3 Methods and materials

3.3.1 Study animals

The study was performed in 35 herds from 2 provinces of Canada (Ontario and Quebec) and Minnesota (USA). The study herds were part of a larger clinical trial that was carried out to investigate if eprinomectin treatment at calving had any benefits on milk production in totally- and semi-confined dairy herds (11). A total of 65 herds from
5 provinces in Canada and Minnesota (USA) were enrolled in that study, between February 2002 and February 2003.

Participating herds had to be Holstein Fresian and be totally or semi-confined. Totally confined herds were defined as herds in which lactating and dry cows were housed throughout the summer, and had no outdoor exposure at all. Semi-confined herds were defined as herds that had limited outdoor exposure of lactating and/or dry cows to a yard or paddock, but which still fed a ration that met all their nutritional requirements. In other words, the composition and quantity of stored feeds (components or total mixed ration) fed to lactating or dry cows was not changed when the cows went outside. The herds also had to be enrolled with the Dairy Herd Improvement (DHI) organization, and be willing to provide access to those data. Herds used in this study were those with computerized reproductive records. They were also required to provide monthly bulk tank milk samples either through the dairy laboratory or through on-farm collection. Participating herds ranged in size from 40 to 300 milking cows. Within each study site, no more than 4 herds were to be over 150 cows so as to avoid the influence of a small number of large herds on the study. No anthelmintics could have been administered to lactating cows during the summer of 2001. There were no restrictions on housing type for heifers in selected herds.

Selection of herds into the study was based on their proximity to the respective veterinary school and/or collaborating veterinary clinics (11). Information related to herd size, cow and heifer housing and other management practices were recorded during the first 3 herd visits.
3.3.2 Treatment protocol

The dose of eprinomectin pour-on (5 mg/ml) per cow was calculated on the basis of a 725 kg cow applied at 500 μg/kg (1 ml/10 kg) resulting in a dose per cow of 72.5 ml. Eprinomectin was dispensed as individual doses into brown colored plastic bottles and an equal quantity of placebo (mineral oil) was dispensed in a similar way. The bottles were uniquely labelled with sequential numbers (alternating placebo and eprinomectin) and packed into compartmentalized cardboard boxes containing 18 bottles of each. The boxes were then distributed to all the participating herds at the beginning of the trial. Additional bottles were provided if a herd ran low. Treatment of cows or heifers was to be done between 3 weeks before the expected calving date and up to 1 week after calving. There was a label on the box containing treatment bottles informing the producer to take the bottles from the left to the right of each row whenever treatment was due. Treatment was done by pouring the entire contents of the bottle along the back midline of the animal. Cow name or number, treatment date, calving date and bottle number were then recorded on a form that was given to the producer. Cows that calved between February 2002 and February 2003 were enrolled in the study.

3.3.3 Bulk tank milk

Monthly bulk tank milk samples from each herd were collected between March 2002 and February 2003. All milk samples were frozen and sent to the Atlantic
Veterinary College, where an indirect ELISA using a crude adult *Ostertagia ostertagi* antigen was performed (12). Results were recorded as the optical density ratio (ODR).

3.3.4 Reproduction data

Information on milk production, lactation number and calving date for the Canadian provinces was obtained from the Canadian Dairy Herd Management System database in Montreal. First insemination date, number of inseminations and conception date were obtained from computerized records kept at the Farm Service Ambulatory unit at the University of Montreal for the Quebec site and from a collaborating veterinary clinic in Ontario. All records for the Minnesota herds were obtained from United States National Dairy Herd Improvement.

3.3.5 Descriptive statistics

A mean 12-month ODR was computed for each herd and dichotomized into high and low ODR using a cut-point of 0.50 (11). Equality in the distribution of eprinomectin and placebo treated cows was verified within study sites, lactation groups and calving seasons. The number of herds in each study site, ODR group, lactating-cow housing category and dry-cow housing category was summarized. Calving to first insemination intervals, calving to conception intervals and the number of inseminations per conception were summarized.
Pasture exposure variables were generated from the data obtained from the producers during the second and third farm visits. Two categorical variables based on the housing of lactating and dry cows were generated with each variable having 3 categories (pasture/paddock, gravel yard and confined). The study period was divided into 4 calving seasons: winter (January - March), spring (April - June), summer (July - September) and fall (October - December). Parity was classified into 3 classes: first lactation, second lactation or third lactation and above.

3.3.6 Multivariable methods

In this study, time to conception and time to first insemination were both modeled using maximum likelihood Cox-proportional hazard survival models with herd frailty effects (13;14). A gamma distribution of herd frailty was assumed. The gamma distribution was checked using a chi-square probability distribution. The predictor variables tested in all models were eprinomectin treatment, parity, calving season, study site, peak milk production, ODR (high/low) and the lactating and dry cow housing variables. The significance of categorical variables and two-way interactions were tested using a likelihood-ratio test and they were kept in the model if \( P < 0.1 \) with parameters having \( P \)-values between 0.05 and 0.1 being considered to be marginally significant. Eprinomectin treatment was forced to remain in all the models. Cows that were not bred during the study period were excluded from the analyses.

Follow-up time for all the cows was set at 200 days, post-calving and cows that had not conceived by that time were considered censored. Cows with calving to first
insemination intervals of less than 40 days were excluded from the analyses as these were considered to be recording errors. Two separate Cox models were fit. In the first model, the effect of eprinomectin treatment on calving to first insemination interval was studied. The second model evaluated the effect of eprinomectin treatment on calving to conception interval. The effect of eprinomectin treatment on calving to conception interval in high and low ODR herds was evaluated in this model by testing the interaction between eprinomectin treatment and ODR. In both models, peak milk production values were divided by 10 so as to obtain coefficients and confidence intervals that represented the effect of a 10 kg change in peak production. Evaluations of the proportional hazards assumption and residuals were done for each model. The Aalen's linear hazards model was fit in order to investigate time varying effects of variables that had not satisfied the proportional hazards assumption (15). Based on the time points where there seemed to be a change in the effects of the variables, the variables that incorporated time varying effects were generated and included in a Cox model. Goodness of fit of each model was evaluated by incorporating design variables (based on the ranked values of the deciles of the estimated risk) into the fitted proportional hazards model. A partial likelihood-ratio test was subsequently applied to compare the models, with a non-significant result suggesting that the model fit the data (16). For each analysis, the quantiles of the cumulative observed versus the cumulative estimated expected number of events, was plotted (Arjas plot). The model was deemed correct when the points followed a 45° line commencing at the origin.

The number of inseminations per conception in placebo and eprinomectin treated cows was modeled using poisson regression. Herd was included in the model as a
random effect so as to account for the within-herd correlation in the number of services per conception. Only cows that conceived were included in this analysis. In addition to the predictor variables mentioned earlier, days to first insemination and an interaction effect with treatment were included in the analysis. A gamma distribution was assumed for the random effects. Since it was not possible to retrieve the random effects, a poisson model that assumed normally distributed random effects was run. The parameter estimates of this model were the same as the ones obtained using gamma-distributed random effects. The residuals of these random effects were normally distributed. Stata 8 (17) statistical software package was used for all the analyses.

3.4 Results

3.4.1 Study animals

A total of 2688 cows that were treated also had electronic reproduction records. Out of these, 2381 cows remained after the exclusion of cows that were not inseminated as well as cows with a calving to first insemination interval less than 40 days.

There was an equal distribution of eprinomectin and placebo treated cows in the study (Table 3.1). The uniform distribution of treatment groups at all levels of pregnancy status, calving season, province was an indication that restricting the analysis of reproduction data to only those herds with electronic records did not introduce a selection bias. The equality of treatment groups within all parity groups was an indication that random allocation of treatment was achieved and that the exclusion of
herds from the milk production study, which did not have electronic records did not affect this balance. Most herds in the study kept their lactating-cows indoors and that only 4 out of 35 herds (11% of herds with a total of 125 cows) were high-ODR herds (Table 3.2). In the overall milk production study (11) 13 of 65 (20%) herds were high ODR.

The mean calving to conception intervals of cows that were treated with eprinomectin and placebo were 131 days and 130 days, respectively. Figures 3.1 and 3.2 show that the distributions of calving to conception intervals in cows treated with eprinomectin and placebo, respectively, were very similar. Eprinomectin-treated cows had a mean calving to first insemination interval of 83 days while that of placebo-treated cows was 82 days. The mean number of services for cows treated with eprinomectin and placebo was 2.6 and 2.5, respectively.

3.4.2 Effect of treatment on calving to first insemination interval

Figure 3.3 shows a Kaplan Meier cumulative survival plot for the effect of treatment on calving to first insemination interval. A visual inspection of the plot shows that the cumulative probability of being bred at any time during the follow up period was the same in eprinomectin and placebo treated cows.

The gamma distribution of the frailty effects was checked, and this assumption was not violated. One herd seemed to be an outlier, therefore another model was run without it. Minor changes to the parameter, implied this outlier was not influential. The frailty effects in this model (with 3 herds dropped) followed a gamma distribution. The
test for the proportional hazards assumption revealed that lactating-cow housing (yard level) violated this assumption (this resulted in the overall model violating the assumption). Aalen's linear hazards model indicated that the two time-points that the effects of lactating-cow housing (yard only) changed were at 60 and 80 days after calving. Consequently, the effects of lactating-cow housing (yard level) and milk production were each split into 3 time intervals, 41-60 days (early), 61-80 days (mid) and 81-200 days (late). The results of a Cox proportional hazards model that took into account the time varying effects of the 3 variables are shown in Table 3.3. Results of the Cox-proportional hazards model showed that treatment was not significantly related to the hazard of being inseminated. There was an indication of a very slight increase in the first insemination rate of cows calving in the summer and fall. A very small lactation effect was observed. The effect of housing type on the hazard of a cow being inseminated was marginally significant (P = 0.09). The results suggested that the hazard of insemination in cows housed indoors was about twice as high as that of cows that had access to a paddock. There was evidence in this study that peak milk production had an effect on the hazard of a cow being inseminated (P < 0.01). During the early period, the hazard of inseminating a cow decreased as peak milk production. However, in the mid and late periods, the hazard of inseminating a cow slightly increased as peak milk production increased.

3.4.3 Effect of treatment on calving to conception interval
Six cows did not have milk production records, reducing the number of cows used in the analysis from 2381 to 2375. Figure 3.4 shows a Kaplan Meier cumulative survival plot for the effect of treatment on calving to conception interval. A visual inspection of the plot shows that the cumulative probability of not conceiving at any time during the follow up period was the same in eprinomectin and placebo treated cows.

The gamma distribution of the frailty effects was checked, and this assumption was found to be slightly violated due to 3 herds that were outliers. Another model was run without these 3 herds and there were minor changes in the coefficients of this model, implying that the outliers were not influential. The frailty effects in this model (with 3 herds dropped) followed a gamma distribution. The proportional hazards assumption test revealed that ODR and calving season violated this assumption. Aalen’s linear hazards model indicated that the two time-points at which the effects of ODR or calving season changed were 80 days and 120 days after calving. The effect of ODR was constant until approximately day 80, when it increased (Figure 3.5) (the change at 120 days was due to the changing effects of calving season). Consequently, the effects of ODR, calving season (all levels) and peak milk production were each split into 3 time intervals, 40-80 days (early), 81-100 days (mid) and 121-200 days (late). The results of a Cox proportional hazards model that took into account the time varying effects of the 3 variables are shown in Table 3.4. There was no evidence that this final model violated the proportional hazards assumption. The effect of treatment on the hazard of conception was not significant, nor was its interaction with ODR. ODR was not a significant (P = 0.26) predictor on the hazard of conception. The hazard of conception in
cows calving in the spring (relative to that of cows calving in the winter) depended on the number of days after calving. During the early period (i.e. summer inseminations for spring-calving cows), the hazard of conception was lower than in cows that calved in the winter. However, during the mid and late follow-up periods (which would correspond to fall inseminations for spring-calving cows) the hazard ratio increased. A similar pattern was observed in cows calving in the summer and fall. The ability to conceive decreased as the cows got older. The hazard of conception in cows kept indoors was twice as high as that of cows that had access to a paddock. There was evidence in this study that peak milk production had an effect on the hazard of conception (P < 0.01). During the early period, there was a slight decrease in the hazard of conception as peak milk production increased. However, in the mid and late periods, the hazard of conception increased as peak milk production increased.

3.4.4 Effect of treatment on the number of artificial inseminations per conception

A total of 1794 cows that conceived during the study period were used in this analysis. There was no significant association between treatment (P = 0.60) and the number of inseminations per conception (Table 3.5). Calving season (P < 0.01) and calving to first insemination interval (P < 0.01) were significantly associated with number of inseminations per conception. The number of services per conception in cows calving in spring and summer were higher than those for winter-calving cows. There was also some evidence that as the calving to first insemination interval increased, the number of services per conception decreased. The herd ODR (P = 0.05) and peak milk
production \( (P = 0.09) \) were significantly and marginally significantly associated with the number of inseminations per conception. Cows in high ODR herds had 20\% fewer inseminations than those in low ODR herds. The number of services required increased as peak milk production increased. A 10 kg increase in peak milk production was associated with an increase of one extra insemination.

3.5 Discussion

This study showed that there was no association between eprinomectin treatment and the three reproduction parameters: calving to first insemination interval, calving to conception interval, and number of inseminations per conception. The study by Sanchez et al. (2002), that was conducted on pastured dairy herds revealed a marginally significant treatment effect on calving to conception interval (hazard ratio = 1.24, \( P = 0.06 \)), but not on calving to first insemination interval. The same study observed a significant reduction in the number of services per conception for cows treated with eprinomectin. Among untreated cows, the hazard of conception was lower for high-ODR cows relative to low-ODR cows suggesting that high gastro-intestinal parasite burdens had a negative impact on reproductive performance. The hazard of conception in eprinomectin-treated high-ODR cows was similar to that in low-ODR cows indicating that treatment prevented the negative effects due to higher parasite burdens. A study investigating whether eprinomectin treatment at calving had any beneficial effects on milk production in confined and semi-confined dairy herds did not show any treatment effect (11). Unlike in the milk production study, where there was an indication that the
bulk milk ODR was able to identify herds that would benefit from treatment, there was no such indication in this study. The fact that the study had only 4 high-ODR herds (11% of total herds) may have reduced the power to observe this effect. These studies conducted in herds with limited pasture exposure showed that endectocide treatment of adult dairy cows did not have any beneficial effects on either reproduction or milk production. The low parasite burden of cows in these herds did not have a negative impact on production as was observed in the studies conducted on pastured herds (2,18).

In spring-calving cows, the hazard of conception during the early period (40 – 80 days after calving) was lower than in cows that calved in the winter. However, during the mid and late follow-up periods the hazard ratio increased. The middle and late periods of cows calving in the spring coincided with the cooler months of the fall. This explains the results, since the conception rates were higher in cooler months than in warmer ones where heat stress may have a negative impact on the cows’ physiology and production (19). The current study showed that the ability to conceive decreased as the age of the cows increased which is consistent with other studies (20). Calving season and lactation number (age) were not significantly associated with conception rate in the study by Sanchez et al. (2). This may have been due to the smaller sample size (549 cows) that reduced the power of the study. There was an indication that cows kept indoors were bred earlier than cows that had access to a paddock. These results are not consistent with expectations since heat detection in cows kept indoors is more difficult than those kept outdoors. However, the results may be explained by the fact that the majority of the herds (6 out of 9) whose lactating cows had access to a paddock during the summer, kept lactating cows in tie stall barns during the colder months. The
advantage of better heat detection in the paddocks during summer was overshadowed by being kept in the more restrictive tie stall barns during colder months. On the other hand, a larger proportion of the lactating cows that did not have exposure to paddocks in the summer, were kept in free-stall barns (all year round) where heat detection is easier than in tie stall barns.

The evidence that the hazard of conception decreased with increasing peak milk production during the early period may be due to increased susceptibility of high-producing cows to get into negative energy balance after calving due to the high energy demands imposed by lactation. Negative energy balance has a negative impact on the return to normal reproductive function during early lactation (21). The same explanation can be given for the effect of peak milk production on the hazard of servicing a cow for the first time after calving.

There was some indication that the number of inseminations per conception was higher in cows calving in the spring and summer despite their hazard of conception being better than those for cows calving in the winter. This may have been due to the presence of conditions (like access to an exercise yard or paddock) that enabled the cows to show heat better during the warmer months than in cooler months. As a result these cows were bred more often since they showed signs of heat more readily than during the cooler months.

Contrary to expectations, there was an indication that the number of inseminations per conception in high ODR herds was lower than in low ODR herds. This may have been due to the scarcity of high ODR herds in the study. A study with more high ODR herds may be better able to evaluate the interaction between treatment
and ODR. There was evidence that as the calving to first insemination interval shortened, the number of inseminations per conception increased. This may be partly explained by the fact that the uterus of cows that develop post-parturient uterine diseases like endometritis, needs more time to sustain the next pregnancy (22).

3.6 Conclusions

This study did not show a beneficial effect of eprinomectin treatment on reproduction parameters in totally- or semi-confined dairy herds. Increasing the number of herds may improve the power of the study to detect whether bulk milk ODR is able to identify herds that would benefit from eprinomectin treatment.

3.7 References


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Table 3.1: Distribution of treatment groups by study site, lactation groups, calving season and pregnancy status for 2381 cows that took part in a multi-site clinical trial to evaluate the effect of eprinomectin treatment on reproduction parameters.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Level</th>
<th>Treatment</th>
<th>Eprinomectin</th>
<th>Placebo</th>
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</thead>
<tbody>
<tr>
<td>Study site</td>
<td>Quebec</td>
<td>182</td>
<td>185</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ontario</td>
<td>510</td>
<td>498</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Minnesota</td>
<td>494</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>Lactation</td>
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<td>419</td>
<td>407</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>323</td>
<td>314</td>
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<tr>
<td></td>
<td>3+</td>
<td>444</td>
<td>474</td>
<td></td>
</tr>
<tr>
<td>Calving season</td>
<td>Winter</td>
<td>236</td>
<td>246</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>250</td>
<td>251</td>
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<tr>
<td></td>
<td>Summer</td>
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<td></td>
<td>Fall</td>
<td>339</td>
<td>337</td>
<td></td>
</tr>
<tr>
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<td>745</td>
<td>765</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Not Pregnant</td>
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<td>430</td>
<td></td>
</tr>
<tr>
<td>Total Treatment</td>
<td></td>
<td>1186</td>
<td>1195</td>
<td></td>
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Table 3.2: Distribution of 35 herds by study site, lactating-cow housing, dry-cow housing and bulk milk ODR groups in a multi-site clinical trial to evaluate the effect of eprinomectin treatment on reproduction parameters.

<table>
<thead>
<tr>
<th>Variable</th>
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</thead>
<tbody>
<tr>
<td>Study site</td>
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<td>7 (20%)</td>
</tr>
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<td></td>
<td>Ontario</td>
<td>16 (46%)</td>
</tr>
<tr>
<td></td>
<td>Minnesota</td>
<td>12 (34%)</td>
</tr>
<tr>
<td>Lactating-cow housing</td>
<td>paddock</td>
<td>9 (26%)</td>
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<tr>
<td></td>
<td>yard</td>
<td>2 (6%)</td>
</tr>
<tr>
<td></td>
<td>confined</td>
<td>24 (68%)</td>
</tr>
<tr>
<td>Dry-cow housing</td>
<td>paddock</td>
<td>12 (34%)</td>
</tr>
<tr>
<td></td>
<td>yard</td>
<td>6 (17%)</td>
</tr>
<tr>
<td></td>
<td>confined</td>
<td>17 (49%)</td>
</tr>
<tr>
<td>ODR</td>
<td>high</td>
<td>4 (11%)</td>
</tr>
<tr>
<td></td>
<td>low</td>
<td>31 (89%)</td>
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Table 3.3: Results of a Cox proportional hazards survival model on the effect of eprinomectin treatment on calving to first insemination interval in 2375 cows from confined and semi-confined dairy herds.

<table>
<thead>
<tr>
<th>Variable</th>
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<th>Estimate</th>
<th>Std. error</th>
<th>P-value</th>
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<th>CI</th>
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<td>Treatment</td>
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<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eprinomectin</td>
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<td>0.04</td>
<td>0.74</td>
<td>0.99</td>
<td>0.91;1.07</td>
</tr>
<tr>
<td>Calving</td>
<td>Winter</td>
<td>Baseline</td>
<td></td>
<td>0.02°</td>
<td></td>
<td></td>
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<td></td>
<td>Spring</td>
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<td>0.97</td>
<td>0.85</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>0.10</td>
<td>0.06</td>
<td>1.11</td>
<td>0.98</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>Fall</td>
<td>0.13</td>
<td>0.06</td>
<td>1.13</td>
<td>1.00</td>
<td>1.28</td>
</tr>
<tr>
<td>Lactation</td>
<td>1</td>
<td>Baseline</td>
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<td>&lt;0.05°</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.04</td>
<td>0.06</td>
<td>1.04</td>
<td>0.92</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td>3+</td>
<td>-0.09</td>
<td>0.06</td>
<td>0.91</td>
<td>0.81</td>
<td>1.03</td>
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<tr>
<td>Lactating-cow</td>
<td>Yard_early</td>
<td>0.04</td>
<td>0.46</td>
<td>1.04</td>
<td>0.42</td>
<td>2.57</td>
</tr>
<tr>
<td></td>
<td>Yard_mid</td>
<td>-0.75</td>
<td>0.46</td>
<td>0.47</td>
<td>0.19</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>Yard_late</td>
<td>-0.57</td>
<td>0.42</td>
<td>0.57</td>
<td>0.25</td>
<td>1.30</td>
</tr>
<tr>
<td></td>
<td>Indoor</td>
<td>0.53</td>
<td>0.21</td>
<td>1.70</td>
<td>1.12</td>
<td>2.58</td>
</tr>
<tr>
<td>Peak milk</td>
<td>Early</td>
<td>-0.10</td>
<td>0.06</td>
<td>&lt;0.01°</td>
<td>0.90</td>
<td>0.81;1.00</td>
</tr>
<tr>
<td></td>
<td>Mid</td>
<td>0.07</td>
<td>0.04</td>
<td>1.07</td>
<td>0.98</td>
<td>1.17</td>
</tr>
<tr>
<td></td>
<td>Late</td>
<td>0.06</td>
<td>0.04</td>
<td>1.06</td>
<td>0.98</td>
<td>1.16</td>
</tr>
</tbody>
</table>

° Overall P-value for all levels of the variable/time-periods of follow-up
Table 3.4: Results of a Cox proportional hazards survival model on the effect of eprinomectin treatment on calving to conception interval in 2375 cows from confined and semi-confined dairy herds.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Level</th>
<th>Estimate</th>
<th>Std. Error</th>
<th>P-value</th>
<th>HR</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Placebo</td>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eprinomectin</td>
<td></td>
<td>-0.03</td>
<td>0.05</td>
<td>0.55</td>
<td>0.97</td>
<td>0.88;1.07</td>
</tr>
<tr>
<td>ODR</td>
<td>Low</td>
<td>Baseline</td>
<td></td>
<td>0.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High_early</td>
<td></td>
<td>0.18</td>
<td>0.32</td>
<td>1.20</td>
<td>6.33</td>
<td>2.25</td>
</tr>
<tr>
<td>High_mid</td>
<td></td>
<td>0.65</td>
<td>0.30</td>
<td>1.91</td>
<td>1.07</td>
<td>3.41</td>
</tr>
<tr>
<td>High_late</td>
<td></td>
<td>0.58</td>
<td>0.31</td>
<td>1.78</td>
<td>0.97</td>
<td>3.25</td>
</tr>
<tr>
<td>Calving</td>
<td>Winter</td>
<td></td>
<td></td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring_early</td>
<td></td>
<td>-0.30</td>
<td>0.15</td>
<td>0.74</td>
<td>5.55</td>
<td>0.99</td>
</tr>
<tr>
<td>Spring_mid</td>
<td></td>
<td>0.02</td>
<td>0.14</td>
<td>1.02</td>
<td>0.77</td>
<td>1.34</td>
</tr>
<tr>
<td>Spring_late</td>
<td></td>
<td>0.59</td>
<td>0.14</td>
<td>1.80</td>
<td>1.38</td>
<td>2.35</td>
</tr>
<tr>
<td>Summer_early</td>
<td></td>
<td>-0.01</td>
<td>0.13</td>
<td>0.99</td>
<td>0.76</td>
<td>1.27</td>
</tr>
<tr>
<td>Summer_mid</td>
<td></td>
<td>0.31</td>
<td>0.13</td>
<td>1.36</td>
<td>1.06</td>
<td>1.74</td>
</tr>
<tr>
<td>Summer_late</td>
<td></td>
<td>0.48</td>
<td>0.14</td>
<td>1.62</td>
<td>1.24</td>
<td>2.12</td>
</tr>
<tr>
<td>Fall_early</td>
<td></td>
<td>0.04</td>
<td>0.13</td>
<td>1.04</td>
<td>0.81</td>
<td>1.34</td>
</tr>
<tr>
<td>Fall_mid</td>
<td></td>
<td>0.25</td>
<td>0.13</td>
<td>1.29</td>
<td>1.00</td>
<td>1.66</td>
</tr>
<tr>
<td>Fall_late</td>
<td></td>
<td>0.39</td>
<td>0.14</td>
<td>1.47</td>
<td>1.12</td>
<td>1.93</td>
</tr>
<tr>
<td>Lactation</td>
<td>1</td>
<td>Baseline</td>
<td></td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-0.19</td>
<td>0.08</td>
<td>0.83</td>
<td>0.72</td>
<td>0.96</td>
<td></td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th></th>
<th>3+</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Lactating-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paddock</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yard</td>
<td>-0.29</td>
<td>0.36</td>
<td>0.75</td>
<td>0.37;1.51</td>
<td></td>
</tr>
<tr>
<td>housing</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indoors</td>
<td>0.45</td>
<td>0.19</td>
<td>1.57</td>
<td>1.08;2.29</td>
<td></td>
</tr>
<tr>
<td>Peak milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early</td>
<td>-0.01</td>
<td>0.06</td>
<td>&lt;0.01</td>
<td>0.99;1.11</td>
<td></td>
</tr>
<tr>
<td>mid</td>
<td>0.06</td>
<td>0.05</td>
<td>1.06</td>
<td>0.96;1.19</td>
<td></td>
</tr>
<tr>
<td>late</td>
<td>0.12</td>
<td>0.05</td>
<td>1.13</td>
<td>1.02;1.25</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Overall P-value for all levels of the variable/time-periods of follow-up
Table 3.5: Results of a random effects poisson regression model on the effect of eprinomectin treatment on the number of inseminations per conception in 1791 cows from confined and semi-confined dairy herds.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Level</th>
<th>Estimate</th>
<th>Std. error</th>
<th>P-value</th>
<th>Count</th>
<th>CI ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td></td>
<td>0.82</td>
<td>0.11</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>Placebo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eprinomectin</td>
<td>0.02</td>
<td>0.03</td>
<td>0.60</td>
<td>1.02</td>
<td>0.96;1.08</td>
</tr>
<tr>
<td></td>
<td>ODR Low</td>
<td>-0.22</td>
<td>0.11</td>
<td>0.05</td>
<td>0.80</td>
<td>0.65;1.00</td>
</tr>
<tr>
<td></td>
<td>ODR High</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Calving Winter</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>0.21</td>
<td>0.05</td>
<td></td>
<td>1.23</td>
<td>1.12;1.36</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>0.10</td>
<td>0.05</td>
<td></td>
<td>1.10</td>
<td>1.00;1.21</td>
</tr>
<tr>
<td></td>
<td>Fall</td>
<td>-0.01</td>
<td>0.05</td>
<td></td>
<td>0.99</td>
<td>0.90;1.10</td>
</tr>
<tr>
<td></td>
<td>CFI (^2)</td>
<td>-0.003</td>
<td>0.001</td>
<td>&lt;0.01</td>
<td>1.00</td>
<td>1.00;1.00</td>
</tr>
<tr>
<td></td>
<td>Peak milk</td>
<td>0.003</td>
<td>0.002</td>
<td>0.09</td>
<td>1.00</td>
<td>1.00;1.00</td>
</tr>
</tbody>
</table>

\(^1\) Overall P-value for all levels of the variable

\(^2\) Calving to first insemination interval (days)
Figure 3.1: Frequency distribution of the calving to conception intervals (from 41 days) of 1186 cows that were treated with eprinomectin in confined and semi-confined dairy herds.
Figure 3.2: Frequency distribution of the calving to conception intervals (from 41 days) of 1195 cows that were treated with placebo in confined and semi-confined dairy herds.
Figure 3.3: Survival curves for calving to first insemination interval from a study with 2381 cows treated with eprinomectin or placebo at calving in confined and semi-confined dairy herds.
Figure 3.4: Survival curves for calving to conception interval from a study with 2381 cows treated with eprinomectin or placebo at calving in confined and semi-confined dairy herds.
Figure 3.5: Plot of the estimated cumulative Cox-regression estimates for optical density ratio (ODR) and for the pointwise 95% confidence limits (CL) against the number of days after calving.
4 Agreement in dairy cow reproduction parameters between data collected by the Quebec and Ontario DHI and that collected by veterinary clinics

4.1 Abstract

The objective of this study was to determine if there was agreement in the calving dates and reproduction indices (calving to first artificial insemination interval, calving to last insemination interval, calving to conception interval and number or inseminations per conception) between dairy herd improvement (DHI) data and data obtained from veterinary clinics in Ontario and Quebec that provided a herd health service to these farms (also called farm data). The proportion of herds and cows within herds whose reproduction records were submitted to DHI, was also determined. The study was performed in 23 herds (1548 cows) from 2 provinces of Canada (Ontario and Quebec) that participated in a clinical trial to evaluate the effect of eprinomectin treatment on reproduction parameters. All herds were enrolled with the DHI organization, and were willing to provide access to those data. They also had computerized reproductive records that were maintained at their veterinary clinic. Out of 1123 cows from Ontario, only 21 % had DHI reproduction records while only 15 % of the 425 cows from Quebec had these records. A total of 6 herds (3 from each province) did not have any DHI records. The concordance correlation coefficient (CCC) between the DHI and farm data calving dates was very good (0.99). There was poor agreement (CCC = 0.49) in the calving to first insemination intervals mainly because of cows whose DHI first insemination intervals were recorded as last insemination intervals (as a
result of inconsistent recording of DHI data). There was good agreement in the last insemination intervals (CCC = 0.93). Two methods were used to determine the calving to conception interval. One was directly from DHI data using last insemination intervals and the pregnancy indicator variable, and the other indirectly by subtracting the gestation length (275 days in this study) from the inter-calving period. Good agreement (above 0.90) was obtained with both methods, indicating that the use of the indirect method is appropriate in the absence of sufficient DHI records. Agreement in the number of inseminations per conception was not good (CCC = 0.74) mainly because of insufficient recording of DHI data. The study shows a need to increase compliance among producers, in the submission of their reproduction records to DHI. The study suggested that DHI calving to first insemination intervals beyond 100 should be used cautiously as they may be invalid. There was no indication of invalidity (across the spectrum) in the calving to last insemination as well as the direct calving to conception intervals. Herd median number of inseminations per conception less than 2 should be used cautiously.
4.2 Introduction

Canada has 3 dairy herd improvement (DHI) organizations, Canwest DHI (services the provinces of British Columbia, Alberta, Saskatchewan, Manitoba and Ontario), Programme d’Analyse des Troupeaux Laitiers du Quebec (PATQL) which services only Quebec and the Atlantic Dairy Livestock Improvement (ADLIC) organisation (services Nova Scotia, New Brunswick and Prince Edward Island). The main aim of these organizations is to help dairy farmers make good management decisions by providing management products and milk recording services to enhance revenues or reduce expenses. They offer a wide range of services to dairy producers from basic milk recording, to sales and support of herd management software, submission of electronic registration applications to breed associations, herd management consulting and continuing education in herd management topics.

The convenience of production data collected by DHI, makes it possible to use these data in epidemiological studies (1-3). This is done with the consent of the producers. For registered producers, the collection of milk production data is compulsory whereas the submission of reproduction data is optional. Most studies have used productivity data (e.g. calving dates, test-day milk production, parity, somatic cell counts, % milk fat and % milk protein). No formal studies have been published validating these records. However, the fact that the recording is done by DHI technicians (independent party) eliminates bias in the recording. Not many studies have used DHI reproduction data. It is not clear whether this is due to the fact that reproduction data services are not used consistently by DHI subscribers or just that the
nature of the records (e.g. calving to conceptional intervals) requires more specialized statistical techniques to model, especially as outcome variables.

Since reproduction records are not consistently provided to DHI, questions arise pertaining their accuracy as well as the level of compliance among registered producers in submitting these records to DHI. The main objective of the present study was to determine the agreement in the calving dates and reproduction indices (calving to first artificial insemination interval, calving to last insemination interval, calving to conception interval and number or inseminations per conception) between DHI data and data obtained from veterinary clinics in Ontario and Quebec that provided a herd health service to these farms (farm data). As well, we determined the proportion of herds and cows within herds whose reproduction records were submitted to DHI.

4.3 Methods and materials

4.3.1 Study animals

The study was performed in 23 herds from 2 provinces of Canada (Ontario and Quebec). The study herds were part of a clinical trial that was carried out to investigate if eprinomectin treatment at calving had any benefits on the calving to first artificial insemination interval, calving to conception interval and number of inseminations per conception in confined and semi-confined dairy herds (4). All herds in the clinical trial had to be enrolled with the DHI organization, and be willing to provide access to those data. Herds used in this study were those that also had computerized reproductive
records maintained at their veterinary clinic. Cows enrolled in the study calved between February 2002 and February 2003.

4.3.2 Reproduction data

Information on calving date, calving to first insemination interval, calving to last insemination interval, calving to conception interval and number of inseminations per conception was obtained from 2 sources (for the same cows). The first source was the Canadian Dairy Herd Management System (CDHMS) database in Montreal that processes information from all DHI organisations in Canada. The calving to conception interval for the DHI data was determined by 2 methods: 1) the number of days to last insemination of all cows deemed to be pregnant according to a pregnancy status variable, 2) subtracting the gestation period from the inter-calving period of cows that calved twice between February 2002 and August 2003. The selected gestation period was one that produced the highest concordance correlation coefficient (CCC) between farm and DHI calving to conception intervals as well as DHI interval data with similar 25th, 50th and 75th percentiles as the farm data-based interval. Another set of data were obtained from computerized records kept at the Farm Service Ambulatory unit at the University of Montreal for the Quebec site and from a collaborating veterinary clinic in Ontario (both provided a routine herd health service to the study herds). Event dates obtained from these data were used to determine all interval reproduction indices. Data from CDHMS can be requested as monthly (TIPS) files or as historical files (with data for longer periods). Enrolment of cows in the clinical trial lasted for a year and a further
6 months of follow-up was required for these cows, so we obtained a historical file with milk production and reproduction data for the period of February 2002 to August 2003. Unlike milk production recordings that are mandatory, collection of reproduction records by DHI is at the producer's discretion. Out of 1548 cows in this study (all with calving dates from the DHI), only 297 had complete information on all the other reproduction indices. It was assumed that the producers did not provide the rest of the information to the DHI.

4.3.3 Statistical analysis

Calving date, calving to first insemination interval, calving to last insemination interval, calving to conception interval and the number of inseminations per conception were summarized by data source. Agreement between the 2 data sources, on calving date and the 2 interval reproduction indices, was assessed using the CCC. A weighted kappa statistic (5) was used to assess the agreement in the number of inseminations per conception between the 2 data sources. Number of inseminations per conception of 4 and above were made into one category. Bland-Altman plots were also used to assess agreement in the calving to first insemination intervals, calving to last insemination intervals and calving to conception intervals. The CCC measures the agreement between 2 values by measuring, not only how each observation deviates from the line fit to the data (precision), but also how far this line deviates from the 45° line through the origin (6). A Bland-Altman plot is a graph that plots the average values of the two data sources against the mean difference between them (7). The 95 % limits of agreement are set at 2
standard deviations of the difference above and below their mean value. All the cows in the study were used to assess the agreement in calving dates between the 2 data sources. However, to assess the agreement in the 4 reproduction indices, only cows without missing values (n = 297) in DHI records, were used. Recommendations on the ranges of DHI indices deemed to be valid were made on the following indices; calving to first insemination intervals, calving to last insemination intervals, calving to conception intervals and number of inseminations per conception considered to be valid. CCC values were obtained at various levels, which were made by restricting the analysis to various cut-points of the interval indices (all values smaller than or equal to the cut-point). The cut-point where the highest CCC was observed was considered to be the value beyond which all DHI values were considered suspect. Stata version 8 (8) was used for all the statistical analysis.

4.4 Results

4.4.1 Study animals

A total of 1548 cows (1123 from 16 herds in Ontario and 425 from 7 herds in Quebec) with DHI and farm calving dates were used for assessing the agreement in calving dates. Only 297 cows (19 %) were used to assess the agreement in reproduction indices. This represented 21 % of Ontario cows and 15 % of the Quebec ones. Generally, the proportion of cows with DHI reproduction records per herd was low (Figure 4.1). In each province, cows from 3 herds did not have any DHI reproduction
records. Out of the 297 cows, 208 were recorded as having conceived in the DHI data (and these were used to determine the agreement in the calving to conception intervals). The rest were recorded as missing, and it was assumed that some of these did not conceive.

4.4.2 Agreement between farm and DHI data

4.4.2.1 Calving date

The CCC in calving dates between the 2 data sources was very high at 0.996 (95% CI: 0.996 – 0.997). Table 4.1 shows that there was exact agreement on calving dates for 92% of the cases. There was no indication that differences in province played a role in the non-agreement of calving dates. There was no indication that the differences were related to particular herds, provinces or calving seasons.

4.4.2.2 Calving to first insemination interval

The 25th, 50th and 75th percentiles of the calving to first insemination interval (days) in the farm data were 60, 69 and 85, respectively. While in the DHI data, the 25th, 50th and 75th percentiles were 65, 81 and 101, respectively. DHI values were generally higher than farm values.

The CCC in the calving to first insemination interval between both data sources was 0.49 (95% CI: 0.41 – 0.57). There was exact agreement in 68% of the cases. Total
agreement was observed in 2 herds and there were almost equal proportions of
disagreements in the remainder of the herds. Most of the differences were close to zero,
but there were a number of them that are far away from zero and it is these that have
brought the CCC down to 0.49 (Figure 4.3). Out of all cows that had one insemination
in the DHI data (same DHI calving to first and last insemination intervals), 57 (84 %
from Ontario and 15 % from Quebec) had their DHI calving to first insemination
intervals erroneously recorded as DHI calving to last insemination intervals. This
implies that these 57 cows had actually been inseminated more than once, but the first
insemination was not recorded, therefore the last was recorded as the first insemination.
The CCC without the 57 cows was 0.73 (95 % CI = 0.67 – 0.79) which is a substantial
increase from the one obtained with the 57 cows included in the analysis. Looking at
Figure 4.2, there is an indication that the differences increased as the average calving to
first insemination intervals increased. This also means that as the average first
insemination intervals increased, the DHI data intervals increased more than the farm
data intervals. Restricting the analysis to DHI intervals less than or equal to 100 days
produced the highest CCC of 0.75.

4.4.2.3 Calving to last insemination interval

The 25th, 50th and 75th percentiles of the calving to last insemination interval
days in the farm data were 77, 109 and 160, respectively. While in the DHI data the
25th, 50th and 75th percentiles were 76, 102 and 136, respectively.
The CCC in the calving to last insemination interval between both data sources was 0.93 (95 % CI: 0.92 – 0.95). There was exact agreement in 86 % of the cases. Figure 4.3 shows that most of the differences were observed beyond a calving to last insemination interval of 90 days. Good agreements (more than 90 %) were observed at all cut-points, therefore, there was no indication of a necessity to restrict the DHI intervals to a certain value in order to optimize the validity of the data.

4.4.2.4 Calving to conception interval (using the DHI calving to last insemination interval and the DHI pregnancy indicator)

A total of 208 cows that had DHI calving to last insemination intervals and were identified as pregnant by the DHI pregnancy indicator variable, were used in this analysis. The 25th, 50th and 75th percentiles of the calving to conception interval (days) in the farm data were 73, 97 and 133, respectively. While in the DHI data the 25th, 50th and 75th percentiles were 73, 98 and 134, respectively.

The CCC in the calving to conception interval between both data sources was 0.95 (95 % CI: 0.93 – 0.96). There was exact agreement in 90 % of the cases and the biggest differences were observed in calving to conception intervals above 90 days (Figure 4.4). Only one cow from Quebec was recorded as pregnant according to the DHI pregnancy indicator variable, the rest were recorded as missing and so these data are reflective of the Ontario situation. There was no indication that poor agreement was associated with particular herds or provinces. Good agreements (more than 90 %) were
observed at all cut-points, therefore, there was no indication of a necessity to restrict the DHI intervals to a certain value in order to optimize the validity of the data.

4.4.2.5 Calving to conception interval (using the DHI inter-calving period)

Many cows used in determining the DHI calving to conception interval did not calve twice during the study period, so the following results are derived from different cows to the ones used above. Out of the 1548 cows used in this study, the dataset for the clinical trial only contained the subsequent DHI calving dates for 295 cows for the following reasons: 1) some cows were culled after the first calving of the study period, 2) assuming an inter-calving period of 13 months, about 900 cows would not have calved by the end of the study (end of August, 2003) so we were not able to determine their inter-calving period, 3) some of the second calving dates had not yet been recorded since a minimum of 8 to 10 tests per year are required by DHI.

The 25th, 50th and 75th percentiles of the calving to conception interval (days) in the farm data were 69, 89 and 120, respectively. Assuming a gestation period of 275 days, the 25th, 50th and 75th percentiles of the DHI calving to conception intervals were, 67 days, 89 days and 118 days, respectively. The CCC in the calving to conception interval between both data sources was 0.94 (95 % CI: 0.92 – 0.95). However, exact agreement was observed in only 4 % of the cases and 47 % were within a range of +/- 5 days. Figure 4.5 shows that the differences were generally close to zero, with a few relatively large differences. There was no indication that there was a province or herd effect on the distribution of non-agreements in the calving to conception interval.
4.4.2.6 Number of inseminations per conception

The 25th, 50th and 75th percentiles of the number of inseminations per conception in the farm data were 1, 2 and 3, respectively. While in the DHI data the 25th, 50th and 75th percentiles were 1, 1 and 2, respectively. The means in farm and DHI data were 2.1 and 1.8, respectively.

The weighted kappa statistic between the farm and DHI data was 0.63 (P < 0.001). There was exact agreement in 71% of the cases. This is depicted by Figure 4.6, which also shows that there were a number of cases where the difference was big. The proportion of non-agreements was almost the same in all herds. In the majority of circumstances, DHI data underestimated the number of inseminations per conception.

4.5 Discussion

Very few (19%) cows with calving dates also had the other reproduction indices recorded. There were variations in the proportions of cows with DHI reproduction records between herds, with 6 herds not having any records. This is an indication that there was generally low compliance among producers in providing their reproduction records to the DHI organisations. In Ontario almost all the herds are electronically connected to the DHI through a network called the Loop. DHI technicians are able to enter data directly into a computer (using Dairy Comp 305 software) connected on the Loop, ensuring efficiency in the data collection process. Out of all herds registered with
Canwest DHI in Ontario, approximately 50% submit their reproduction records regularly to DHI (Dave Kelton, unpublished data). In the present study, the incentive to submit reproduction records may have been low, considering that these data were already being processed and analysed by the veterinary clinic.

The results show that the agreement in calving dates between the farm and DHI data was very good (CCC close to 1). Therefore, differences in calving dates would not likely contribute towards any lack of agreement in the reproduction indices.

The poor agreement (CCC = 0.49) in the calving to first insemination intervals was mostly influenced by the recording of last insemination intervals in the DHI data as first insemination intervals in 57 cows. In these cows, as the average first insemination interval increased, the DHI data intervals increased more than the farm data intervals (Figure 4.2). This is expected since these DHI calving to first insemination intervals were actually last insemination intervals and are therefore bound to increase more than the actual first insemination intervals (as average intervals increase) obtained from the farm data. The fact that most of these 57 cows (84%) were from Ontario should not be interpreted to mean that the magnitude of the recording problem is worse there because we are dealing with small numbers of herds and cows in this study. Study results indicate that the validity of DHI calving to first insemination intervals is better if restricted to values up to 100 days after calving, but was still quite low (CCC = 0.75). Most of the 57 cows mentioned earlier, had calving to first insemination intervals that were greater than 100 days, so this limit effectively removes their effect.

There was very good agreement in the calving to last insemination (CCC = 0.94). Agreement in calving to conception intervals where the DHI intervals were
determined from the last insemination intervals as well as by subtracting the gestation length (275 days) from the calving interval were, 0.95 and 0.94, respectively. The percentage with exact agreement was far less when the interval was derived from the inter-calving period. However, these results indicate that the use of the inter-calving interval (minus the gestation length) is a suitable alternative in determining the calving to conception interval in instances where there may not be enough DHI data. Haddad et al. (unpublished data) used a gestation length of 284 days to calculate the calving to conception intervals in their study. Using this value in the present study produced a CCC of 0.92 (compared to 0.94 when 275 days was used), however the median calving to conception interval was slightly underestimated (80 days versus the 89 in the farm data). When DHI data were downloaded, we did not have the second calving dates of some of the cows that participated in this study. The median calving to conception interval of 89 days is quite short. The reason for this might be that cows that had second calving dates were those that also had short calving to conception intervals. These cows also had short inter-calving intervals (mean = 374 days) while a number of studies reveal means greater than 400 days with mean gestation lengths of 280 days (9;10). Selection of a gestation length of 275 days was based on the CCC (between DHI and farm calving to conception intervals) as well as the gestation length that produced a calving to conception interval distribution similar to that for farm data. This gestation length was observed on few cows and therefore should be used with caution. Eventually, most of these cows will have their calving dates recorded by DHI, so they will have calving to conception intervals this way. There was no indication of non-validity across
the spectrum of DHI calving to conception intervals determined using the calving to last insemination intervals and the pregnancy indicator variable.

Agreement in the number of inseminations per conception was not good (Kappa = 0.63), despite the fact that there was 71% exact agreement between DHI and farm data. This was most likely due underestimation of the number of inseminations in DHI data, presumably as result of producers not giving these data to DHI. If the herd median number of inseminations per conception is less than 2 (farm data median), then questions on the validity of the data should be raised.

The proportion of non-agreements in calving dates and all reproduction indices, between farm and DHI data was almost the same across herds. This is an indication that there was no systematic influence of herd on agreement.

4.6 Conclusions

The calving to first insemination intervals in DHI data were not accurate, mainly because for many cows last inseminations are recorded as first insemination intervals. The agreement in the calving to last insemination and calving to conception intervals was very good. Calving to conception intervals can be determined by subtracting the gestation length (275 days in this study) from the inter-calving interval in instances where calving to last insemination data are not recorded in the DHI records. There was not very good agreement in the number of inseminations per conception most likely because not enough data were submitted to DHI by the producers. The study shows a need to increase compliance among producers, in the submission of their reproduction
records to DHI. A larger study with random sampling of herds from all the provinces of Canada would give more representative results on the validation of DHI reproduction records in Canada.
4.7 References


Table 4.1: Percentage distribution of the differences in calving dates between DHI and farm data in a study that evaluated the agreement in the reproduction indices obtained from DHI and farm data

<table>
<thead>
<tr>
<th>Difference (days)</th>
<th>% with difference</th>
<th>Cumulative %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
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<td>97</td>
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<td>4 – 10</td>
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<td>98</td>
</tr>
<tr>
<td>remainder</td>
<td>2</td>
<td>100</td>
</tr>
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</table>
Figure 4.1: Herd distribution of the proportion of cows that had DHI reproduction indices from a total of 1548 cows from 23 herds in Quebec and Ontario that had reproduction records obtained from veterinary clinics that provide them a herd health service.
Figure 4.2: Bland-Altman plot showing the distribution of the differences between the calving to first insemination intervals in farm and DHI data (farm minus DHI) in a study that evaluated the agreement in the reproduction indices obtained from farm and DHI data.
Figure 4.3: Bland-Altman plot showing the distribution of the differences between the calving to last insemination intervals in farm and DHI data (farm minus DHI) in a study that evaluated the agreement in the reproduction indices obtained from DHI and farm data.
Figure 4.4: Bland-Altman plot showing the distribution of the differences between the calving to conception intervals in DHI (obtained using DHI calving to last insemination intervals) and farm data (farm minus DHI) in a study that evaluated the agreement in the reproduction indices obtained from DHI and farm data.
Figure 4.5: Bland-Altman plot showing the distribution of the differences between the calving to conception intervals in DHI (obtained using DHI inter-calving intervals) and farm data (farm minus DHI) in a study that evaluated the agreement in the reproduction indices obtained from DHI and farm data.
Figure 4.6: Frequency distribution of the differences between the number of inseminations per conception in DHI and farm data in a study that evaluated the agreement in the reproduction indices obtained from DHI and farm data (n = 297 cows)
5 Evaluation of the effect of internal parasite burden on the energy status of peri-parturient dairy cattle

5.1 Abstract

This study was conducted to investigate the association between parasite burden and energy status of dairy cows around calving and the effect of deworming. Milk samples were collected from 159 cows that were in late lactation and then tested with an *Ostertagia ostertagi* ELISA which generated optical density ratios (ODR) as measures of gastro-intestinal nematode parasite burden. The cows were treated with eprinomectin pour-on 2 weeks before the expected calving date. Blood samples were collected before and after calving for the determination of serum non-esterified fatty acid (NEFA) and beta-hydroxybutyrate (BHB), respectively. Cows with BHB levels ≥ 1200 μmols/L were classified as having subclinical ketosis. The association between pre-calving NEFA levels and subclinical ketosis was evaluated using a mixed effects logistic regression model. A linear mixed effects model was used to evaluate the relationship between NEFA levels (outcome) and ODR and eprinomectin treatment. A similar model was used to evaluate the association between BHB levels and ODR and treatment. The odds of developing subclinical ketosis in cows with a mean NEFA level of 0.43 mEq/L (75th percentile) were 2.5 times higher than those of cows with a mean NEFA of 0.17 mEq/L (25th percentile). Treatment of cows with eprinomectin before calving was not associated with peri-parturient energy balance. However, there was evidence that BHB levels increased when ODR values in the previous lactation were > 0.40. A larger study...
conducted in pastured herds may be able to demonstrate a significant relationship between endectocide treatment and peri-parturient energy balance.
5.2 Introduction

Many studies have shown that de-worming adult dairy cows has beneficial effects on milk production. A meta-analysis done by Sanchez et al. (1) reported a mean milk production increase of 0.46 kg/cow per day in 75 clinical trials, although an adjustment to eliminate publication bias reduced this value to 0.35 kg/cow per day.

Around the time of calving there is a depression of feed intake in dairy cows. As a result the cow is unable to meet her energy demands for maintenance, growth and milk production resulting in a state of negative energy balance (2). Mobilization of the cow’s fat reserves beginning about 3 weeks before calving results in a rise in the blood levels of non-esterified fatty acids (NEFA), which reach a peak level at calving. High NEFA levels are also associated with an increased tendency to develop fatty liver (3). The cow’s homeorrhetic drive for milk production after calving imposes high energy demands during a period of reduced energy intake thus worsening the state of negative energy balance and predisposing the cow to subclinical or clinical ketosis. There are 2 theories regarding the development of ketosis in cows. One is that fatty liver results in impaired liver function, which leads to the production of ketone bodies (beta-hydroxybutyrate (BHB), acetone and acetoacetate). The other is that ketosis can occur without the development of fatty liver. In addition to ketosis, negative energy balance is associated with other peri-parturient diseases/disorders such as milk fever, displaced abomasum, retained placenta and mastitis all of which have a negative impact on milk production and reproduction (4). Since NEFA and BHB are indicators of negative

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energy balance, they are commonly used to monitor the metabolic status of the cow, with the latter being an indicator of subclinical ketosis.

The peri-parturient depression in feed intake may be exacerbated by decreased intake caused by gastro-intestinal nematode parasites in infected cows (5). In temperate areas, *Ostertagia ostertagi* has been reported as the most prevalent parasite causing important economic losses in young stock and adult dairy cows (6;7). A study by Sanchez et al. (unpublished data) evaluated if a crude antigen *Ostertagia ostertagi* ELISA test performed on late-lactation milk samples, was able to identify cows whose milk production would benefit from eprinomectin treatment at calving. The study found a higher treatment effect in high parasite burden cows than in those with a low parasite burden. These results implied that removal of parasites by eprinomectin treatment was able to mitigate their negative impact on milk production.

The objective of this study was to investigate if treatment of cows with eprinomectin before calving had any effect on energy balance pre- and post-calving. The relationship between parasite burden (as determined by milk antibody levels) at the end of one lactation and energy balance in the subsequent peri-parturient period was also evaluated.

5.3 Materials and methods

5.3.1 Study animals
This study was conducted in order to investigate if de-worming dairy cows 2 weeks before the expected calving date would reduce the negative energy balance caused by gastrointestinal nematodes. A total of 159 cows entering their second or above lactation participated in this study. The cows came from three dairy herds in Prince Edward Island that were participants of a larger study that investigated the impact of eprinomectin treatment around calving on milk production in herds with limited exposure to pasture (8). These herds were defined as semi-confined, meaning that outdoor exposure for the lactating and dry cows was limited to a yard or paddock; nevertheless, the cows were still fed a ration that met all of their nutritional requirements (i.e. no reliance on pasture to meet any nutritional requirements).

5.3.2 Treatment

The dose of eprinomectin pour-on (5 mg/ml per cow) was calculated on the basis of a 725 kg cow applied at 500 μg/kg (1 ml/10 kg) resulting in a dose per cow of 72.5 ml. Eprinomectin was dispensed as individual doses into brown colored plastic bottles and an equal quantity of placebo (mineral oil) was dispensed in a similar way. Treatment was administered by pouring the entire contents of the bottle along the dorsal midline of the animal. Cows were treated 2 weeks before expected date of calving and the cow’s name, treatment date, calving date and bottle number were recorded. Production records, including test-day milk yield and parity, were obtained from the Canadian Dairy Herd Management System database in Montreal.
5.3.3 Sampling

At the time of treatment, a blood sample was obtained from the coccygeal vein using a 10 ml vacutainer tube and the cow was assigned a body condition score (BCS) based on the five-point system (9). Additional blood samples were obtained 7 days apart, until calving. Two more blood and milk samples were collected a week apart after calving (the first sample between 1 and 7 days and the second between 8 and 15 days). Blood samples were centrifuged for 10 min at 16,000* g and at least 2 ml of serum was pipetted into plastic vials that were kept frozen at -20 °C until analysis. All blood samples were sent to the Ontario Veterinary College where the NEFA concentration was determined in pre-calving samples and BHB concentration in post-calving samples. The samples were analyzed on a HITACHI 911 machine using RANDOX kits for BHB (kinetic enzymatic method) and NEFA (colorimetric method). One or more late-lactation (after 150 days in milk (DIM)) milk samples from the previous lactation which were collected by the Atlantic Dairy Livestock Improvement Corporation, were tested with an indirect microtitre ELISA (10) in order to measure the gastrointestinal nematode burden. Results were recorded as optical density ratios (ODR) and the mean value for each cow was computed.

5.3.4 Statistical analysis

5.3.4.1 Descriptive statistics
The study period was divided into 4 calving seasons: winter (January to March), spring (April to June), summer (July to September) and fall (October to December). Parity was classified into 2 classes: second lactation or third lactation and above. Distribution of cows according to treatment, herd, parity and calving season were summarized. Peak milk production, ELISA ODR, NEFA and BHB were also summarized.

5.3.4.2 Association between pre-calving NEFA and ketosis

BHB values were dichotomized using a cut-point of 1200 μmol/L (11) with values 1200 μmol/L and above indicating subclinical ketosis. A random effects logistic regression model was fit to evaluate if pre-calving mean NEFA values could be used to predict post-calving subclinical ketosis. Herd was included in the model as a fixed effect and cow was included as a random effect. Other predictors (fixed effects) included in the model were mean NEFA, ODR, parity, peak milk production, body condition score (BCS), days in milk (DIM) and calving season. The residuals of the cow random effects followed a normal distribution.

5.3.4.3 Effect of treatment and parasites on energy balance

Unconditional associations between the outcomes (BHB and NEFA) and their respective potential predictors were evaluated using separate linear regression models. Three separate models were evaluated: 1) The effect of treatment on the NEFA
concentration of blood samples collected at the second and subsequent sampling (the first sample was collected at the time of treatment). 2) The effect of ODR and treatment on the NEFA concentration of all blood samples. 3) The effect of ODR and treatment on BHB concentration. Interactions between treatment and all other predictors were evaluated. All models were linear mixed effect models where cow was included as a random effect in order to correct for within-cow clustering (12). Since there were only 3 herds, herd was included in the model as a fixed effect. The following predictors were used in Model 1 and 2: treatment, ODR, calving season, parity, peak milk production and BCS. The predictors in model 3 were treatment, NEFA measurement, ODR, calving season, parity, peak milk production and BCS. Separate models that differed on the forms of the NEFA variable (mean NEFA, maximum NEFA and first NEFA) were compared to investigate which form best predicted BHB levels. In all models, the linear relationship between the outcomes and individual continuous predictors was evaluated. Quadratic terms of all predictors that were not linearly associated with the outcome variable were evaluated in the model. Quadratic terms were made by first centering the predictors (so as to avoid collinearity with their respective linear terms) and then squaring them (12). Since only 2 BHB measurements were made on each cow, all linear mixed models assumed a compound symmetry correlation structure in order to account for any correlation between the two measurements. A backward-stepwise method was used to build the models. At each step, the variable with the highest P-value was eliminated until all remaining variables had a P < 0.05. Treatment and ODR were not eliminated from the models and the potential role of eliminated variables as confounders was considered. Variance components for between and within-cow variation were used.
to determine the proportion of variation remaining at both levels after accounting for the significant predictors. The assumptions of normality of residuals and homoscedasticity were checked and a box-cox transformation on the outcome was used when the assumptions were violated.

Stata 8 (13) was used for all the descriptive statistics and the random effects logistic regression model while MLwiN (14) was used for the linear mixed effects models.

5.4 Results

5.4.1 Descriptive statistics

Out of 159 cows that were treated and sampled, six did not have production data. All descriptive statistics were performed on 153 treated cows that had production records. Table 5.1 shows that 57% of the cows received eprinomectin. Since this study used only 153 cows out of 6100 cows in the overall study and also excluded first parity cows, it was not unusual to have a slight imbalance between eprinomectin and placebo treated cows just by chance. Table 5.2 shows that late-lactation ODR values were generally low (median = 0.31). A total of 354 NEFA and 295 BHB measurements were used in the analysis. A mean of 2 blood samples (minimum = 1 and maximum = 6) per cow, were collected for NEFA evaluation. Figures 5.1 and 5.2 show that most NEFA and BHB measurements were below 0.50 mEq/L and 1000 μmol/L, respectively. Figure 5.3 shows a frequency distribution of ODR values.
5.4.2 Association between pre-calving NEFA and subclinical ketosis

A total of 293 records from 152 cows were used in this analysis and 36 of these cows (43 BHB measurements) were classified having subclinical ketosis. Since there were only 3 cows that calved in the spring, these were joined with the ones that calved in the summer for this analysis. The mean NEFA ($P < 0.01$) and calving season ($P < 0.01$) were significantly associated with subclinical ketosis (Table 5.3). The other predictors were not significant and were subsequently dropped during model building. The odds of developing subclinical ketosis in a cow having a mean NEFA of 0.43 (75\textsuperscript{th} percentile) were 2.5 times (95 \% CI: 1.4 – 4.1) higher that for a cow with a mean NEFA of 0.17 (25\textsuperscript{th} percentile).

5.4.3 Effect of treatment and parasite burden on NEFA

A total of 144 cows (331 records) with complete records were used in the analysis. The following unconditional associations with NEFA were significant: Days before calving ($P < 0.01$), peak milk production ($P < 0.01$) and calving season ($P < 0.01$). Treatment ($P = 0.54$), BCS ($P = 0.21$), ODR ($P = 0.89$) and parity ($P = 0.23$) were not significant.

A square-root transformation was used on NEFA values in order to satisfy the model assumptions. Number of days before calving was not linearly associated with NEFA, therefore, its quadratic term was evaluated in the model. Number of days before...
calving was centred by subtracting all values with 10 days. There was no indication that treatment was associated with NEFA levels, in a model that excluded NEFA values from the first blood sample. Table 5.4 shows results of the model that included all NEFA measurements. The following predictors were significant: calving season (P = 0.03), linear term for days to calving (P < 0.01), quadratic term for days to calving (P < 0.01) and herd (P < 0.01). Treatment (P = 0.6) and ODR (P = 0.94) were not significantly associated with NEFA. Peak milk production, BCS and parity were dropped from the analysis during model building. An average cow was defined as a cow: from herd 1, treated with placebo, ODR of 0.36 and calved in winter. Figure 5.4 shows that NEFA values started increasing 20 days before calving and reached highest levels around the time of calving. First order interactions between treatment and all variables in the final model were not significant. The between-cow variance was lower (0.01) than the within-cow variance (0.02) and contributed 29 % of the variation in NEFA that remained after accounting for all the predictors in the final model. The correlation between NEFA measurements on the same cow was 0.29.

5.4.4 Effect of treatment and parasite burden on BHB

A total of 142 cows (273 records) that had complete records were used in the analysis. The following unconditional associations with BHB were significant: BCS (P < 0.01), NEFA (P < 0.01), peak milk production (P = 0.04), parity (P = 0.04), calving season (P < 0.01). Treatment (P = 0.86), DIM (P = 0.10) and ODR (P = 0.13) were not significant.
BHB values were ln-transformed in order to satisfy the assumptions of normality of residuals and homoscedasticity. There were very minor differences in the models that were fit with different NEFA variables. The results of the model with mean NEFA are presented (Table 5.5). ODR and DIM did were not linearly associated with BHB, therefore their quadratic terms were evaluated in the model. ODR and DIM were centred by subtracting all values with 0.36 and 8 days, respectively. The following predictors were significant: quadratic ODR term (P = 0.02), peak milk production (P < 0.01), calving season (P < 0.01), mean NEFA (P < 0.01), quadratic DIM term (P < 0.01) and herd (P = 0.04). Treatment was not significant (P = 0.90). All first order interactions between treatment and all predictors were not significant. Parity and BCS proved not to be significant and were dropped during model building. There was an initial drop in BHB levels, however they started to increase at an ODR value of 0.4 (Figure 5.5). An average cow was defined as a cow that had the following characteristics: from herd 1, a mean NEFA concentration of 0.36, calved in winter, treated with placebo and peaked at 35 kg of milk, 8 days in milk and an ODR of 0.36. One kg increase in peak milk production was associated with an increase in lnBHB concentration of 0.02. An increase in mean NEFA in an average cow (except for varying mean NEFA values) between the 25th percentile (0.16) and the 75th percentile (0.43) corresponded to an increase in BHB levels of 88. Cows calving in the winter had the highest BHB levels while those calving in the fall had the lowest. Figure 5.6 shows that the BHB levels of an average cow (except for varying DIM) peaked 10 days after calving and then started decreasing. The between-cow variance was lower (0.05) than the within-cow variance (0.16) and contributed 21% of the variation in BHB that remained after accounting for all the
predictors in the final model. The correlation between BHB measurements on the same cow was 0.21.

5.5 Discussion

This study did not provide any indication that treatment of cows with eprinomectin before calving influenced the peri-parturient energy balance. However, BHB levels increased when ODR values at the previous lactation were greater than 0.4. There is no explanation of the drop in BHB when ODR was less than 0.40. This result gives an indication that high ODR values are associated with a tendency towards larger negative energy balances. A study that evaluated whether late-lactation ODR could be used to identify cows that would benefit from eprinomectin treatment at calving found that a positive treatment response was associated with cows that had ODR values greater than 0.40 (Sanchez et al., unpublished data). It is interesting that this ODR value is similar to the one associated with increased BHB values in the present study. The fact that there was a significant quadratic ODR term but no significant treatment effect may be due to the overall low parasite burdens (evidenced by generally low ELISA ODR values) that did not warrant endectocide treatment. In terms of the treatment effect, the results obtained in this study are consistent with those reported in 2 papers based on all herds in the clinical trial that showed that the milk production (8) and reproduction (Sithole et al., unpublished data) of cows from confined and semi-confined dairy herds were not associated with eprinomectin treatment at calving. However, there was some indication from the milk production study, that ELISA performed on bulk milk samples...
was able to identify herds that would benefit from endectocide treatment. From the results of the studies by Nodvedt et al. (15) and Sanchez et al. (16) that were conducted on pastured dairy herds, there is reason to believe that a study of a similar nature to this one but carried out on pastured herds may show that parasites are significantly associated with increased negative energy balance around calving.

Peak milk production was included in the models because of its known influence on peri-parturient energy metabolism, however, on a timescale it was measured after the determination of NEFA and BHB serum levels. Consistent with literature, BHB data showed that high peaking cows were associated with an increased tendency to develop a negative energy balance. Other studies have shown a significant association between negative energy balance and parity and BCS. This study did not find these associations, most likely because of the small sample size. Cows calving in winter had the highest BHB levels while those calving in the fall had the lowest. This is in agreement with a study by Grohn et al. (17) who reported that the risk of clinical ketosis was higher during the indoor feeding season likely as a result of cows calving with a high BCS. In contrast, a Canadian study showed that both highest BHB levels and highest subclinical ketosis prevalence occurred in the summer (18). As expected, mean NEFA values were significantly associated with BHB levels post-calving. The odds of developing subclinical ketosis in a cow with a mean NEFA value of 0.43 was 2.5 times higher than that of a cow with a mean NEFA of 0.17. This underlines the importance of implementing preventive management strategies before calving. This study showed that peak BHB levels are reached 10 days after calving. This is in agreement with recent work that suggests that the peak prevalence of hyperketonaemia occurs in the first 2
weeks post-calving (19). Consistent with literature findings, this study showed that NEFA levels started increasing about 3 weeks prior to calving, reaching maximum levels at calving.

Both NEFA and BHB models showed that the proportion of variation in BHB or NEFA attributed to differences between cows was much less than that between measurements within a cow. A study by Dohoo and Martin (20) found that the heritability of subclinical ketosis in Canadian dairy cows was low. This may partially explain the low variation in BHB and NEFA levels between cows. The within-cow variation in BHB and NEFA measurements is a reflection to the rapidly changing metabolic energy status of cows in the peri-parturient period.

5.6 Conclusions

The study did not demonstrate any association between eprinomectin treatment at calving and peri-parturient energy balance as determined by both serum NEFA and BHB levels. However, it did provide an indication that late lactation parasite burden was associated with an increase in BHB levels at ODR values greater than 0.40. The low parasite burdens in the study animals may not have warranted endectocide treatment. A study conducted in pastured herds may be able to demonstrate a significant relationship between endectocide treatment and peri-parturient energy balance, hence providing a more solid basis for the timing of treatment around calving.
5.7 References


Table 5.1: Distribution of 153 cows treated with eprinomectin or placebo before calving according to treatment, parity, calving season and herd.

<table>
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<th>Variable</th>
<th>Level</th>
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<td>Parity</td>
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<td></td>
<td>3+</td>
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<td>TOTAL</td>
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Table 5.2: Median, 25th and 75th percentiles of mean cow ODR, mean cow BHB, NEFA, and peak milk production in 153 cows treated with either eprinomectin or placebo before calving

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<th>Variable</th>
<th>Median</th>
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<th>75th percentile</th>
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<tr>
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<td>NEFA</td>
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<td>Peak milk yield (kg)</td>
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Table 5.3: Results of a random effects logistic regression model to evaluate the association between pre-calving serum levels of non-esterified fatty acids (NEFA) and post-calving subclinical ketosis defined by BHB levels above 1200 μmol/L.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Level</th>
<th>Estimate</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td></td>
<td>-2.14</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>Mean NEFA</td>
<td></td>
<td>3.49</td>
<td>1.03</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Calving season</td>
<td>winter</td>
<td>basic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>summer</td>
<td>-1.00</td>
<td>0.49</td>
<td>&lt;0.01</td>
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<tr>
<td></td>
<td>fall</td>
<td>-3.20</td>
<td>0.76</td>
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Table 5.4: Linear mixed effects model evaluating the effect of deworming cows with eprinomectin before calving on non-esterified fatty acid (NEFA) levels measured before calving in 144 cows (331 records).

<table>
<thead>
<tr>
<th>Variable</th>
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<th>Estimate</th>
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<th>P-value</th>
</tr>
</thead>
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<tr>
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<td>0.04</td>
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<tr>
<td>Treatment</td>
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<td>Baseline</td>
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<td></td>
</tr>
<tr>
<td>Eprinomectin</td>
<td></td>
<td>0.01</td>
<td>0.02</td>
<td>0.60</td>
</tr>
<tr>
<td>ODR</td>
<td></td>
<td>-0.003</td>
<td>0.06</td>
<td>0.94</td>
</tr>
<tr>
<td>Days to</td>
<td></td>
<td>-0.01</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>calving_linear</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days to</td>
<td></td>
<td>0.0004</td>
<td>0.0001</td>
<td>&lt;0.01</td>
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<tr>
<td>calving_quadratic</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Calving season</td>
<td>Winter</td>
<td>Baseline</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>Spring</td>
<td></td>
<td>-0.14</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td></td>
<td>0.06</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Fall</td>
<td></td>
<td>-0.02</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Herd</td>
<td>1</td>
<td>Baseline</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>-0.03</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>0.07</td>
<td>0.03</td>
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Table 5.5: Linear mixed effects model evaluating the effect of deworming cows with eprinomectin before calving on the beta-hydroxybutyrate (ln BHB) levels measured after calving in 142 cows (273 records).

<table>
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<th>Estimate</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
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<td>Treatment</td>
<td>Placebo</td>
<td>Baseline</td>
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<td></td>
<td>Eprinomectin</td>
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<td>0.06</td>
<td>0.88</td>
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<td>ODR_linear</td>
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<td>-0.18</td>
<td>0.20</td>
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<td>ODR_quadratic</td>
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<td>1.42</td>
<td>0.62</td>
<td>0.02</td>
</tr>
<tr>
<td>Calving season</td>
<td>Winter</td>
<td>Baseline</td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>-0.08</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>-0.21</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fall</td>
<td>-0.43</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Mean NEFA</td>
<td></td>
<td>0.47</td>
<td>0.17</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Herd</td>
<td>1</td>
<td>Baseline</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.08</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.18</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Peak milk</td>
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<td>0.02</td>
<td>0.004</td>
<td>&lt;0.01</td>
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<tr>
<td>DIM_linear</td>
<td></td>
<td>0.01</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>DIM_quadratic</td>
<td></td>
<td>-0.003</td>
<td>0.001</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Figure 5.1: Frequency distribution of pre-calving serum NEFA levels in a study to evaluate if eprinomectin treatment and gastro-intestinal nematode worm burden are associated with peri-parturient energy balance in dairy cows (n = 354 samples)
Figure 5.2: Frequency distribution of post-calving serum BHB levels in a study to evaluate if eprinomectin treatment and gastro-intestinal nematode worm burden are associated with peri-parturient energy balance in dairy cows (n = 295 samples)
Figure 5.3: Frequency distribution of late-lactation optical density ratio values in a study to evaluate if eprinomectin treatment and gastro-intestinal nematode worm burden are associated with peri-parturient energy balance in 153 dairy cows.
Figure 5.4: Predicted effect (from a linear fixed effects model) of the number of days before calving on pre-calving NEFA serum levels in dairy cows (from model in Table 4)

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Figure 5.5: Predicted effect (from a linear fixed effects model) of late-lactation parasite worm burden (measured using an ELISA) on post-calving BHB serum levels in dairy cows (from model in Table 5)
Figure 5.6: Predicted effect (from a linear fixed effects model) of the number of days after calving on post-calving BHB serum levels in dairy cows (from model in Table 5)
6 Stability of *Ostertagia ostertagi* ELISA microtitre plates over time

6.1 Abstract

The objective of this study was to assess the stability of ELISA plates prepared with one of 3 blocking agents and used with one of 2 conjugates at various time intervals after preparation of the plates. Two of the blocking agents used were commercially available: one termed Stabilguard (bl_stab) and one manufactured by SVANOVA Biotek AB Inc. (bl_svan). The third blocking agent used was bovine serum albumin (bl_bsa). A polyclonal rabbit anti-bovine IgG (con_poly) and an anti-bovine IgG monoclonal (con_mono) conjugate were used. Eighteen composite individual cow milk samples collected late in lactation (200 - 400 days in milk) were used. An indirect microtitre plate ELISA that used the *Ostertagia ostertagi* antigen was used to quantify antibodies against the parasite, present in the milk samples. Each of 6 blocking agent/conjugate combinations (called systems) were used to test 18 milk sub-samples at 1, 4 and 24 weeks after blocking the plates. Plates blocked with bl_stab and bl_svan were kept at room temperature and an additional set were incubated at 37 °C so as to mimic long term storage (about 1 year) and tested only once at 4 weeks. Those blocked with bl_bsa were frozen at -20 °C. Concordance correlation coefficients (CCC) and reproducibility were used to assess the agreement between test results conducted on the same milk sample at the various test-times using a particular system. Generally, there was good agreement between tests conducted at different times for all systems. However, the bl_svan-con_mono and bl_bsa-con_poly systems had the best agreement.
with overall CCC values of 96% and 93%, respectively. The bl_svan-con_poly system had the lowest CCC of 75%. The CCC and reproducibility ranked the systems in a similar way. The high CCC between tests done using plates kept at room temperature and ones incubated at 37 °C, suggested that plates would be stable up to a year after blocking. The storage of plates blocked with bl_svan and bl_stab agents under room temperature, makes them more convenient to use and transport relative to bl_bsa-blocked plates that have to be frozen.
6.2 Introduction

Fecal egg counts are an unreliable measure of parasite worm burden in adults animals (1;2). A non-competitive enzyme-linked immuno-sorbent (ELISA) that uses a crude adult worm *Ostertagia ostertagi* antigen (3) has been shown to be a better measure of worm burden in a number of studies (1;2). Sanchez et al. (4) evaluated the ELISA using serum and milk samples from adult dairy cows. The study found a high within and between plate repeatability when the ELISA values were expressed as optical density ratio (ODR) values compared to when raw optical density values were used. No difference in ODR was found between 2 batches of crude antigen. A bovine serum albumin block and a rabbit anti-bovine polyclonal IgG conjugate were used in the study (4).

Microtitre plates that are blocked with bovine serum antigen have to be kept frozen at -20 °C. This method of storage is not very convenient, especially when the plates have to be transported. Plates blocked with other blocking agents can be dried and stored at room temperature making them easier to store and transport. However, the stability of these blocking agents (agreement between test results of the same sample over time) has not been evaluated.

Anti-bovine IgG monoclonal or polyclonal conjugates are routinely used for ELISAs. However, monoclonal conjugates have been reported to give minimal background reactions owing to a better specificity compared to polyclonal conjugates (5).
The objective of this study was to assess the stability of *O. ostertagi* ELISA plates prepared with one of 3 blocking agents, and used with one of 2 conjugates, at various time intervals after preparation of the plates.

6.3 Materials and Methods

6.3.1 Test materials and milk samples

Three blocking agents and 2 conjugates were used at various time intervals after coating the plates. The 3 blocking agents were: 1) bovine serum albumin (bl_bsa), 2) Stabilguard (bl_stab) manufactured by SurModics Inc. and 3) one manufactured by SVANOVA Biotech AB Inc. (bl_svan). A polyclonal rabbit anti-bovine IgG (con_poly) and an anti-bovine IgG monoclonal (con_mono) conjugate were used. Figure 1 and 2 show schematic presentations of the experimental design for the bl_bsa blocking agent and for the bl_stab and bl_svan blocking agents, respectively.

Eighteen composite individual cow milk samples were used in this study. These samples were collected from cows that were late in lactation (200 - 400 days in milk) and participated in a clinical trial to evaluate the ELISA for identification of cows whose milk production would benefit from eprinomectin treatment at calving (Sanchez et al., unpublished data). Using the ODR values from that study, the samples were classified as high, medium and low ODR to ensure a range of values in the study. Eight high ODR, 5 medium ODR and 5 low ODR samples were selected. Each milk sample was divided into 8 sub-samples that were stored in 1ml plastic vials. The sub-samples were
centrifuged at 16,000*g for 4 minutes. Milk fat was removed using a spatula and the underlying supernatant was obtained and frozen at –20 °C.

6.3.2 Preparation of plates

The adult worm *Ostertagia* antigen used for the ELISA was prepared using a method described by Sanchez et al. (4). Modular plates were used in the study so as to ensure flexibility and avoid wastage of both plates and ELISA reagents. Each modular plate had 8 removable Removawell strips (Dynex Technologies Inc.), each with 12 flat-bottomed wells. Two strips (24 wells) were assigned to each of the blocking agent/conjugate/time combination evaluated. These were used for 18 samples, 2 positive controls, 2 negative controls and 2 blanks. The whole ELISA technique was carried out according to Sanchez et al. (2002). A total of 6 plates were coated with antigen.

Two plates (16 strips) were blocked with bl_bsa (200 μl/well), frozen and kept at –20 °C to prevent bacterial growth. Another 2 plates had 200 μl of bl_stab titrated into each well and then aspirated. The plates were then dried in the incubator at 37 °C for 2 hours. 4 strips were then wrapped with plastic and incubated at 37 °C until the ELISAs were performed. Another 12 strips were wrapped in a similar way and stored at room temperature. Similar blocking procedures and strip storage conditions to the one described for bl_stab were performed using bl_svan on the remaining 2 plates.

6.3.3 ELISA tests

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ELISAs were performed on sub-samples from the original 18 cow milk samples at 1 week, 4 weeks and 24 weeks after blocking, using all conjugate-blocking agent combinations. At any test-time, 2 strips (24 wells) blocked with the same agent were used. Therefore, a total of 6 strips (2 per blocking agent) were placed into a modular plate, for each test-time evaluation. One modular plate was used for each of the 2 conjugates evaluated. The exception was for the 4-week evaluation when each plate had 8 strips because of additional tests performed using bl_stab and bl_svan strips that had been incubated at 37 °C. All samples were read using a spectrophotometer (Spectramax 340, Biotek-Instruments Inc.) at wavelengths 405 (L1) and 490 (L2).

6.3.4 Statistical analysis

6.3.4.1 Descriptive statistics

Combinations of blocking agent and conjugate were called systems. A total of 6 systems were formed: bl_bsa-con_mono, bl_bsa-con_poly, bl_svan-con_mono, bl_svan-con_poly, bl_stab-con_mono and bl_stab-con_poly. The ODR mean, range and standard deviation of high, low and medium ODR samples were summarized for all systems at the respective test times.

6.3.4.2 ANOVA
An ANOVA model was run to investigate the relationship between the response variable ODR and the factors test-time, milk sample, and system. The model included all first-order interactions. The interaction between test time and system was evaluated by an interaction graph. The deletion t-test and Cooks’ distance were used to identify outlying and influential observations, respectively. The assumption of normality was evaluated by the analysis of the residuals.

In addition, a random effects ANOVA model with random effects of time (and all interactions with time) was run to estimate repeatability and reproducibility, using the following formulas (6):

\[
\text{repeatability} = 2.83 \times (\sigma^2(\text{error}))^{1/2}
\]
\[
\text{reproducibility} = 2.83 \times (\sigma^2(\text{error}) + \sigma^2(\text{time}) + \sigma^2(\text{time*system}))^{1/2}
\]

where \( \sigma^2 \) is the variance.

Repeatability gives a value that is not exceeded (with 95% confidence) by the difference between any 2 ODR values of the same sample, measured using the same system at the same time.

Reproducibility gives a value that is not exceeded (with 95% confidence) by the difference between any 2 ODR values of the same sample, measured using the same system at different times. Due to the lack of replication for each system and time combination, the estimated repeatability is based on an assumption of negligible second-order interaction between the factors.
6.3.4.3 Agreement between test-times within a system.

The concordance correlation coefficient (CCC) (7) and reproducibility were used to evaluate the agreement of the tests carried out using the same system at different times. The CCC measures the agreement between results obtained at 2 or more time-points by measuring, not only how each observation deviates from the line fit to the data (precision), but also how far this line deviates from the 45° line through the origin (accuracy). CCC was determined for tests between any 2 time intervals within a system as well as overall across the 3 test-times.

An ANOVA model with additive effects of time and sample was used to compute the separate reproducibilities (across time points) for each system; the formula used is that of the repeatability given above (1). The reproducibilities of bl Starr and bl_svan systems whose plates were incubated at 37°C were not determined since they were only tested at 4 weeks.

Three Bland-Altman plots (8) were made for the system with the best agreement across test-times, each showing the variation in ODR differences between samples measured at 2 test-times. Minitab 13 for Windows (9) was used for the ANOVA random effects model, while Stata 8 (10) was used for rest of the analyses.

6.4 Results

6.4.1 Descriptive statistics

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There was a wide range of ODR values in all systems at all the test times, as expected (Table 6.1). In most systems, the mean ODR values for the last test-time (24 weeks) were higher than the ones obtained in the first 2 times. The variation in mean ODR over time was smallest for the bl_svan-con_mono system.

6.4.2 ANOVA model

All the factors and their first-order interaction terms (with the exception of sample*time) were significantly related to ODR (Table 6.2). Figure 6.3 shows the interaction plot of test time and system. Generally, the variation of mean ODR with time within a system was not as big as the one between systems within a given test-time. The only exception was the bl_svan-con_poly system, where there was a considerable increase in mean ODR between test times 1 week and 4 weeks. The variation in mean ODR between systems increased with increasing test-times. According to the percentage of variation (expressed as sequential sum of squares) in ODR attributed by the factors in Table 6.2, it is clear that most of the variation was explained by the differences in samples. The rest of the factors contributed little variation. The same table shows that the type of system used explained relatively more variation in ODR than the test-times (as in Figure 6.3). Repeatability and reproducibility values obtained from the random effects ANOVA model are shown in Table 6.4. It is clear that both values are very close.

There was one observation whose ODR was found to be both outlying and influential. This was a high-ODR sample that was tested at 4 weeks using the bl_bsa-con_poly system and had an ODR of 0.22 which was far less than other sub-samples.
which generally had ODR greater than 1) tested using the same system as well as other systems. The authors were convinced that this was an erroneous value, and it was excluded from all analyses. There was no indication that the assumption of normality of residuals was violated.

6.4.3 Agreement between test-times within a system

Table 6.3 shows the CCC of pairs of test-times within a system, as well as the CCC for all the test-times within a system. Generally, the CCCs were quite high. The bl_svan-con_mono and the bl_bsa-con_poly systems had the highest CCC (and the most narrow confidence intervals), followed by the bl_stab-con_mono system. The bl_svan-con_poly system had the lowest CCC. There was generally good agreement between the bl_svan and bl_stab systems, whose plates were incubated at room temperature and those incubated at 37 °C. The agreement between samples tested at 4 and 24 weeks was generally good in all systems. In all systems, the pair-wise CCC of tests done between 4 and 24 weeks was higher than the other 2 (1 vs 4 and 1 vs 24).

Results of the reproducibility values of each system obtained by performing separate ANOVA models are shown in Table 6.4. Generally, there were no dramatic differences in the reproducibility values. The bl_svan-con_mono system had the best reproducibility between any two measurements at any test times followed by the bl_bsa-con_poly system. The one with the least reproducibility was the bl_svan-con_poly system.
The 2 methods (CCC and reproducibility) ranked the systems in a similar way. With the exception of bl_bsa systems, ELISA tests run in combination with the monoclonal conjugate had better concordance and repeatability than those tests where the polyclonal conjugate was used.

Each Bland-Altman plot in Figure 6.4 shows the differences between ODR values of the same sample (using the bl_svan-con_mono system) measured at 2 test-times against the mean ODR of both tests. The line in the middle is the mean difference and the 2 lines above and below the mean represent the values which would enclose 95% of the observations. It is evident that the mean ODR difference between 2 tests in each plot is very close to zero, indicating that the values are very similar. Another noteworthy point is that the variation in the differences does not seem to depend on whether the sample has a high or low ODR. In general, two observations would be expected to be no more than 0.2 units apart.

6.5 Discussion

The huge variation (85%) that was accounted for by differences in samples was expected, since there was deliberate selection of samples with a wide variation in ODR. The small variation in ODR contributed by test-time indicates that plates blocked with all the agents are generally stable for a period of at least 6 months after blocking. This was verified by the generally high CCC values obtained across test-times for all systems where blocked plates were kept at room temperature. Generally, the good agreement between 2 ODR measurements taken at different time points using a particular system,
was evidenced by the overall repeatability and reproducibility values (Table 6.4) that were very close.

The CCC and individual system reproducibility ranked the ELISA systems in a similar manner. The two methods are complimentary because CCC assesses the degree of closeness between ODR values, while reproducibility (and repeatability) gives the actual magnitude of differences among results from the same sample. Tests performed using plates blocked with bl_svan showed the best stability over time when an anti-bovine IgG monoclonal conjugate was used. Two other systems that showed good stability were the plates blocked with bl_bsa where a polyclonal conjugate was used, and the plates blocked with bl_stab where a monoclonal conjugate was used. The only system that showed relatively low stability was when tests were performed using the bl_svan block and the polyclonal conjugate. There was better concordance and reproducibility when bl_stab and bl_svan blocking agents were used in combination with the monoclonal conjugate, than with the polyclonal conjugate. This may be due to better specificity of the monoclonal conjugate in binding with bovine IgG. Non-specific binding that occurs more frequently when polyclonal conjugate is used may introduce more variation in ODR between test-times.

Incubation of plates at 37 °C for 4 weeks is believed to mimic long term (about 1 year) storage at room temperature. Results of tests performed using plates blocked with either bl_stab or bl_svan, and incubated at 37 °C, were generally similar to those performed using plates blocked with the same blocking agents and incubated at room temperature. Therefore, plates blocked with bl_stab and bl_svan can be stored for periods longer than 6 months to a year without affecting ELISA results.
ELISA microtitre plates possess a high affinity for proteins and in order to prevent non-specific binding of antibodies on parts of the plates not coated with antigen, blocking agents (normally protein) are used. Commonly used blocking agents include BSA, casein, skim milk, fish skin gelatin, horse serum and goat serum. These proteins have different physico-chemical properties, which may explain the differences in their blocking abilities (11). Use of blocking agents with poor blocking properties is bound to result in a lower agreement in ODR between samples tested at different times. The greater the degree of non-specific binding, the more variable will be the results of tests done at different times.

6.6 Conclusions

All blocking agents produced results that were generally consistent over time. Using mono-clonal conjugate in tests conducted on plates blocked with bl_svan produced the most stable results over 24 weeks. This was closely followed by using a polyclonal conjugate on bsa-blocked plates, and using a monoclonal conjugate on plates blocked with bl_stab. The storage of plates blocked with bl_svan and bl_stab agents at room temperature, makes them more convenient to use and transport relative to bsa-blocked plates that have to be frozen.
6.7 References


Table 6.1: Means and ranges of ODR values from 18 milk samples tested at 3 time periods using 6 ELISA systems.

<table>
<thead>
<tr>
<th>System</th>
<th>Statistic</th>
<th>1 wk</th>
<th>4 wks</th>
<th>24 wks</th>
<th>4 wks (37°C)</th>
<th>System mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) bl_bsa-</td>
<td>mean</td>
<td>0.57</td>
<td>0.58</td>
<td>0.67</td>
<td></td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>0.12;1.18</td>
<td>0.14;1.46</td>
<td>0.18;1.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) bl_bsa-</td>
<td>mean</td>
<td>0.61</td>
<td>0.56</td>
<td>0.69</td>
<td></td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>0.08;1.19</td>
<td>-0.08;1.25</td>
<td>0.04;1.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3) bl_stab-</td>
<td>mean</td>
<td>0.67</td>
<td>0.64</td>
<td>0.77</td>
<td>0.84</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>0.27;1.16</td>
<td>0.07;1.38</td>
<td>0.09;1.71</td>
<td>0.19;1.94</td>
<td></td>
</tr>
<tr>
<td>4) bl_stab-</td>
<td>mean</td>
<td>0.54</td>
<td>0.44</td>
<td>0.41</td>
<td>0.49</td>
<td>0.47</td>
</tr>
<tr>
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<td>range</td>
<td>-0.05;1.31</td>
<td>-0.10;1.15</td>
<td>-0.02;1.06</td>
<td>0.01;1.11</td>
<td></td>
</tr>
<tr>
<td>5) bl_svan-</td>
<td>mean</td>
<td>0.47</td>
<td>0.72</td>
<td>0.82</td>
<td>0.76</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>-0.06;1.28</td>
<td>0.17;1.62</td>
<td>0.32;1.51</td>
<td>0.21;1.67</td>
<td></td>
</tr>
<tr>
<td>6) bl_svan-</td>
<td>mean</td>
<td>0.55</td>
<td>0.52</td>
<td>0.57</td>
<td>0.53</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>-0.05;1.43</td>
<td>-0.09;1.37</td>
<td>-0.05;0.54</td>
<td>-0.09;1.25</td>
<td></td>
</tr>
</tbody>
</table>

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Table 6.2: Results of the overall ANOVA model performed to investigate the association between ODR and the factors sample, time and system and their first order interaction terms

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SSS$^1$</th>
<th>% of SSS</th>
<th>P-value</th>
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<tr>
<td>sample</td>
<td>40.79</td>
<td>85</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>time</td>
<td>0.47</td>
<td>1</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>system</td>
<td>1.94</td>
<td>4</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>time*system</td>
<td>1.27</td>
<td>3</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>time*sampel</td>
<td>0.32</td>
<td>2</td>
<td>0.69</td>
</tr>
<tr>
<td>system*sampel</td>
<td>1.39</td>
<td>3</td>
<td>0.02</td>
</tr>
<tr>
<td>error</td>
<td>1.86</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>TOTAL SSS</td>
<td>48.04</td>
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<td></td>
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</table>

$^1$ Sequential sum of squares
Table 6.3: Concordance correlation coefficients of ODR values from 18 milk samples tested at 3 time periods using a specific ELISA system (total of 6 systems).

<table>
<thead>
<tr>
<th>System</th>
<th>Test-times</th>
<th>1² vs 4</th>
<th>1 vs 24</th>
<th>4 vs 24</th>
<th>1 vs 4(37)⁻¹</th>
<th>4 vs 4(37)</th>
<th>24 vs 4(37)</th>
<th>All times ⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) bl_bsa-</td>
<td>con_poly</td>
<td>0.91</td>
<td>0.90</td>
<td>0.97</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>(0.82-1.00) (0.81-0.99)</td>
<td>(0.93-1.00)</td>
<td>(0.88-0.95)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) bl_bsa-</td>
<td>con_mono</td>
<td>0.86</td>
<td>0.87</td>
<td>0.91</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>(0.75-0.98) (0.77-0.98)</td>
<td>(0.84-0.99)</td>
<td>(0.82-0.93)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3) bl_stab-</td>
<td>con_poly</td>
<td>0.85</td>
<td>0.77</td>
<td>0.91</td>
<td>0.68</td>
<td>0.82</td>
<td>0.96</td>
<td>0.85</td>
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<tr>
<td></td>
<td>(0.72-0.98) (0.59-0.95)</td>
<td>(0.85-0.98)</td>
<td>(0.76-0.90)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4) bl_stab-</td>
<td>con_mono</td>
<td>0.88</td>
<td>0.86</td>
<td>0.97</td>
<td>0.92</td>
<td>0.97</td>
<td>0.94</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>(0.77-0.99) (0.74-0.97)</td>
<td>(0.95-1.00)</td>
<td>(0.85-0.99)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5) bl_svan-</td>
<td>con_poly</td>
<td>0.77</td>
<td>0.60</td>
<td>0.93</td>
<td>0.65</td>
<td>0.92</td>
<td>0.91</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>(0.62-0.92) (0.42-0.79)</td>
<td>(0.86-0.99)</td>
<td>(0.60-0.84)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6) bl_svan-</td>
<td>con_mono</td>
<td>0.97</td>
<td>0.95</td>
<td>0.96</td>
<td>0.97</td>
<td>0.97</td>
<td>0.93</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>(0.95-1.00) (0.90-1.00)</td>
<td>(0.92-1.00)</td>
<td>(0.92-0.98)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1 Block/conjugate combination
2 Concordance correlation between tests done at 1 week and 4 weeks
3 Blocked plates stored at 37 °C and used after 4 weeks
4 Excludes tests done using plates incubated at 37 °C
Table 6.4: Reproducibilities (from separate ANOVA models) of ODR values from 18 milk samples tested using a specific ELISA system at 3 test periods and the repeatability and reproducibility from the overall random effects ANOVA model

<table>
<thead>
<tr>
<th>System</th>
<th>Reproducibility</th>
<th>Repeatability</th>
</tr>
</thead>
<tbody>
<tr>
<td>bl_bsa-con_poly</td>
<td>0.26</td>
<td>-</td>
</tr>
<tr>
<td>bl_bsa-conMono</td>
<td>0.36</td>
<td>-</td>
</tr>
<tr>
<td>bl_stab-con_poly</td>
<td>0.40</td>
<td>-</td>
</tr>
<tr>
<td>bl_stab-conMono</td>
<td>0.34</td>
<td>-</td>
</tr>
<tr>
<td>bl_svan-con_poly</td>
<td>0.59</td>
<td>-</td>
</tr>
<tr>
<td>bl_svan-conMono</td>
<td>0.22</td>
<td>-</td>
</tr>
<tr>
<td>Overall</td>
<td>0.39</td>
<td>0.30</td>
</tr>
</tbody>
</table>
Figure 6.1: Schematic presentation of the experimental design where an ELISA was performed on 18 milk samples using the Bsa (bovine serum albumin) blocking agent and the monoclonal (con_m getRandomText() on) and polyclonal (con_poly) conjugates.
Figure 6.2: Schematic presentation of the experimental design where an ELISA test was performed on 18 milk samples using the Stab (Stabilguard) and Svan (Svanova) blocking agents and monoclonal (con Mono) and polyclonal (con Poly) conjugates.
Figure 6.3: Relationship between test-times and ODR of 6 ELISA systems in a study to assess the stability of 3 blocking agents.
Figure 6.4: Bland-Altman plots of the difference in ODR values between 2 test-times against the mean of the test times using ELISA plates blocked with bl_svan and the monoclonal conjugate.

1 vs 4 weeks

4 vs 24 weeks

1 vs 24 weeks

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Assessing the agreement between *Ostertagia ostertagi* ELISA tests performed using the crude adult antigen and the adult and larval stage 4 excretory/secretory antigens

7.1 Abstract

The objective of this study was to determine the agreement between ELISAs conducted using 3 *O. ostertagia* antigens: crude adult worm, larval stage 4 (L4) excretory/secretory (ES) and adult ES. This study was carried out on 289 Holstein Friesian cows from 5 herds in Prince Edward Island and 1 herd in Nova Scotia. Composite milk samples of these cows were collected (between May and September 2002) from the respective provincial laboratories and sent to the Atlantic Veterinary College where each sample was tested for antibodies to *O. ostertagi* using and indirect microtitre ELISA test. Results were expressed as optical density ratio (ODR) values. Each milk sample was tested with 3 ELISA tests, with each test using a different *O. ostertagia* antigen. There was a slight rise in ODR values of both adult antigens between May and August, with higher values obtained using the adult ES antigen. L4 ES ODR values were generally higher than those for both adult antigens during the study period. There was a more dramatic rise in L4 ES ODR values between May and August. Rises in ODR in May and end of July coincided with periods of mass maturation of L4 to adult worms. The concordance correlation coefficient (CCC) between tests performed using both ES and the crude antigens were low. The highest Pearson correlation coefficient was obtained between ODR values from the adult ES and crude adult Ag.
The highest CCC was observed between tests done using both ES antigens and the highest ODR values were observed when the L4 ES antigen was used. Bland-Altman plots showed that the variation in ODR differences between tests performed with the 2 adult antigens and those performed using the L4 ES, was not the same across the ODR spectrum. At low ODR values, L4 ES values were still higher than crude adult values but lower than the adult ES ones. However, at high ODR values L4 ES tests had the highest ODR values. Generally, the study results suggest that the antibody response (detectable by the ELISA) is mainly directed against ES antigens (especially L4) than the crude adult worm antigen.
7.2 Introduction

Gastrointestinal nematode parasites have detrimental effects on milk production and reproduction in adult dairy cows (1;2). *Ostertagia ostertagi* is one of the most important gastrointestinal nematode parasites of cattle in temperate climatic areas (3). Fecal egg counts (FEC) have been used for a long time to measure parasite burden but are an inaccurate measure of worm burden in adult animals (3;4). An ELISA using crude adult *O. ostertagi* antigen (5) has been used to evaluate worm burden in several studies (6;7) and has been shown to be a better measure of worm burdens than the FEC. The ELISA has shown promise as a tool to predict a milk production or reproduction response to endectocide treatment in dairy cows (2;8).

When compared with viral, protozoal and bacterial serology it has taken a longer time to develop suitable antigens from parasitic nematodes. Now that *in-vitro* culture techniques for nematodes have been developed, it is possible to obtain, not only a wider variety of antigens, but antigens that are pure and not contaminated by host material or other undefined components (9). Recombinant DNA technology has also enabled the generation, isolation and characterization of antigens associated with different life stages of parasites.

In addition to crude worm (somatic) antigens derived from whole intact worms there are also excretory/secretory (ES) antigens. Crude worm extracts are complex mixtures of antigens and many of these antigens may fail to evoke a serological response in-vivo until the worm dies and they are presented to the adaptive immune system. Therefore, these antigens may play a minor role in stimulating the host’s
response. ES antigens are comprised partly of proteases that are released by nematode parasites to facilitate a parasitic lifestyle, including tissue penetration, digestion of host tissue for nutrition and evasion of the immune system. ES antigens are superior at stimulating the host's immune system compared to crude antigens. Immune responses to ES antigens are believed to be more closely related temporally to active infection than anti-whole worm responses, which may persist following death of the parasite (9).

Canals and Gasbarre (10) found that *O. ostertagi* ES antigens are more immunogenic and demonstrate less cross-reactivity than crude antigens. The use of ES antigens may also enable better representation of antigenic proteins on ELISA plates with the potential to interfere with binding of specific proteins. In addition, use of ES antigen may result in more stable coating than crude antigen. Antigenic differences between the developmental stages of *O. ostertagi* have also been demonstrated (10;11).

The repeatability of a crude antigen ELISA test has been evaluated and has been found to be generally good (12;13). The objective of this study was to determine the agreement between this ELISA and ELISAs using ES antigens from the larval stage 4 (L4) and adult worms.

### 7.3 Materials and methods

#### 7.3.1 Study animals

This study was carried out on 289 Holstein Friesian cows from 5 herds in Prince Edward Island and 1 herd in Nova Scotia. The study herds were part of a clinical trial
that was carried out to investigate if eprinomectin treatment at calving had any benefits on milk production in dairy cows that had no or limited pasture exposure (8).

Participating herds were either totally confined or semi-confined. Totally confined herds were defined as herds in which lactating and dry cows were housed throughout the summer and had no outdoor exposure at all. Semi-confined herds were defined as herds that had limited outdoor exposure of lactating and/or dry cows to a yard or paddock but which still fed a ration that met all their nutritional requirements. The composition and quantity of stored feeds (components or total mixed ration) fed to lactating or dry cows was not changed when the cows went outside.

7.3.2 Milk samples

Composite individual cow milk samples were collected from the farms between May 2002 and September 2002, during routine test-day milk production recordings. They were immediately preserved with bronopol and then collected from the provincial dairy laboratories in Prince Edward Island and Nova Scotia after routine testing for fat, protein, SCC and milk urea nitrogen. The samples were then frozen at -20 °C and sent to the Atlantic Veterinary College where they were thawed and centrifuged at 16,000 x g for 4 minutes. The fat fraction was removed and the skim milk was stored at -20°C until it was tested for parasite antibodies using ELISA.

7.3.3 ELISA
An indirect *O. ostertagi* ELISA was performed on all milk samples and the results were expressed as optical density ratio (ODR) values (12). Each milk sample was tested using 3 ELISAs, each with a different *O. ostertagi* antigen. The 3 antigen types used to coat the plates were: 1) crude adult, 2) adult ES and 3) L4 ES. Crude antigen preparation was done according the method used by Sanchez et al. (2002a) while that of both ES antigens was done according to (14). Adult ES and L4 ES antigen were used at concentrations of 0.5 µg/ml and 1µg/ml, respectively. Tests were conducted for a period of 3 weeks (between August and September 2002) with the 3 tests being done on each sample on the same day.

7.3.4 Descriptive statistics

Data on parity and calving date was obtained from the Canadian Dairy Herd Management System database in Montreal. Parity was classified into 3 classes: first lactation, second lactation or third lactation and above. The study period was divided into 4 calving seasons: winter (January - March), spring (April - June), summer (July - September) and fall (October - December). A categorical variable based on the housing of lactating cows was generated. The 3 housing categories were pasture/paddock, gravel yard and confined. The distribution of cows by lactating-cow housing, calving season and parity were summarized. ODR distribution was summarized according to antigen type. Temporal distributions of kernel smoothed (weighted by the number of samples at a point in time) ODR values by antigen type were summarized in a plot.
7.3.5 Agreement between ELISA tests performed using the 3 antigens

Pair-wise agreement between ELISAs based on the 3 antigen types was assessed using the concordance correlation coefficient (CCC), Pearson correlation coefficient and Bland-Altman plots. The CCC measures the agreement between results obtained at 2 tests by measuring, not only how each observation deviates from the line fit to the data (precision), but also how far this line deviates from the $45^\circ$ line through the origin (15). The Pearson correlation measures the strength of the relationship between two variables regardless of the change in scale of the measurements (15). A Bland-Altman plot is a graph that plots the average values of the two tests against the mean difference between them (16). The 95% limits of agreement are set at 2 standard deviations of the difference above and below their mean value. All 3 measures of agreement were applied to the whole data set. They were also applied at each herd, calving season, parity and lactating-cow housing type to investigate any association between these variables and agreement between antigen types. Stata version 8 (17) was used for all the statistical analysis.

7.4 Results

7.4.1 Descriptive statistics

Of 289 cows that had late lactation milk samples tested with the ELISA (using crude and adult ES antigen), 170 (60%) had production records. Since there was a
limited amount of the L4 ES antigen we were only able to test milk samples from 236 cows (145 of these had production records) with the antigen. Table 7.1 shows that the number of cows at all levels of the variables, was generally low.

The median ODR and 25th and 75th percentile ranges for each antigen type were as follows: 1) Crude adult antigen – 0.34 (0.19 to 0.46), 2) Adult ES antigen – 0.72 (0.57 to 0.87), 3) L4 ES antigen – 0.67 (0.49 to 0.88). From the frequency distributions of the ODR values of tests done using the 3 antigen types (Figure 7.1), it is evident that L4 ES antigen tests have the widest range as well as the highest ODR values. Figure 7.2 shows that, generally, L4 ES ODR values were the highest in all months except in May and the lowest values were observed in the crude adult ELISA. There was a rise in L4 ES ODR values between May and June after which they remained constant and then rose (almost abruptly) again at the end of July. The lines representing the adult antigens are almost parallel, with higher ODR values obtained with the ES antigen. Although there was a slight rise in the ODR values in the warmer months, it was not as pronounced as those for the L4 ES. Owing to limited amounts of the L4 ES antigen, samples collected in September were not tested using this antigen. Generally, for all antigens, the highest ODR values were observed in the warmer and wetter months of August and September.

7.4.2 Overall agreement between ELISA tests performed using the 3 antigens

7.4.2.1 Crude adult versus adult ES
The CCC value for ELISAs performed using the crude adult and adult ES antigen was 0.31 (95% CI: 0.26 – 0.35). The Pearson correlation coefficient was 0.80. The ODR values for the adult ES antigen were generally larger (negative differences) than the ones for the crude adult antigen tests (Figure 7.3). The variation in the differences was generally similar across the range of ODR values.

7.4.2.2 Crude adult versus L4 ES

The CCC value for ELISAs performed using the crude adult and L4 ES antigens was 0.30 (95% CI: 0.24 – 0.36). The Pearson correlation coefficient was 0.59. The ODR values for the L4 ES antigen tests were generally larger (negative differences) than the ones for the crude adult antigen tests (Figure 7.4). The variation in the differences differed across the range of ODR values with larger differences being observed in samples with high ODR values.

7.4.2.3 Adult ES versus L4 ES

The CCC value for ELISAs performed using the crude adult and L4 ES antigen was 0.56 (95% CI: 0.49 – 0.63). The Pearson correlation coefficient was 0.63. The variation in the differences differed across the range of ODR values. The ODR values of adult ES tests were generally higher than L4 ES tests at low ODR values (Figure 7.5). However, at high ODR values, L4 ES ODR values were higher than the adult ES values.
7.4.3 *Agreements between tests by production variables*

Agreements between tests by herd, calving season, parity and lactating-cow housing types did not reveal anything different from the overall agreements.

7.5 *Discussion*

ODR values obtained using the L4 ES antigen were generally highest across the sample collection period except for May (Figure 7.2). ODR values obtained using the crude adult antigen were the lowest. The number of adult worms in the abomasum, have been reported to increase in the warmer months while those of the L4 stage go down due to faster maturation of L4 to adults (3). Figure 7.2 shows that the rise in adult antigen ODR values (which reflects rising antibody levels) is not as pronounced as that observed with the L4 ES antigen. This suggests that L4 ES antigens may be more immunogenic than the crude adult worm antigen. This may also be a result of better representation of protein antigens on ELISA plates coated with ES than in those coated with crude adult worm antigen, which is likely to have a higher proportion of non-specific proteins. The parallel distribution of adult antigen ODR values is a reflection that both antigens come from one source. The distribution of L4 ODR values over time seems to be associated with the development of L4 to adults in the abomasum. The initial rise in L4 ES ODR values may be due to the resumption in the development of inhibited L4 into adults, which ends around June. During June and July, the hot weather (and less rainfall) does not favor the survival of L3 (infective stage) in the pasture, therefore there is a reduction
in the number of L4, and hence very few develop into adults. This may explain why
ODR values remain constant during this period. The abrupt rise at the end of July
coincides with the beginning of wet weather, which favors transmission of L3 which
develop to L4, which in turn molt into adults without undergoing inhibition (since the
weather is still warm).

The CCC tells us how close 2 (or more) observations are to each other (takes
into account the scale of the measurements) whereas the Pearson correlation measures
the magnitude of the variation in one variable that explains the variation in another
variable (i.e. 2 measurements may be very different in scale and have a high Pearson
correlation but a low CCC). Bland-Altman plots are graphic presentations of the
magnitude and distribution of the differences between 2 measurements. They also give a
general indication as to which of the 2 measurements is greater in scale.

Overall, pair-wise CCC between tests conducted using both ES antigen and the
crude adult antigen were low. The ODR values obtained using both ES antigens were
generally higher than those obtained using the crude adult antigen. This result is
consistent with the study by Canals and Gasbarre (10) where a stronger reaction (high
ODR) was observed when the ES antigen was used. This is further evidence that ES
antigens may be more immunogenic than crude antigens as observed in other studies,
given that they are more exposed to the host’s immune system than the crude antigens.
The highest (0.80) pair-wise Pearson correlation (not CCC) was observed between tests
performed using the crude adult Ag and the adult ES antigen. This is consistent with
expectations since both antigens reflect adult parasite burden, hence variation in one is
like to influence the variation in the other antigen.

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The concordance between tests performed using the adult ES and L4 ES antigens was the greatest. These preparations are both ES antigens and therefore likely to have similar immunogenic properties when compared to crude antigen. The highest ODR values were obtained using the L4 ES antigen (Figure 7.3). This is also shown by the Bland-Altman plots shown in Figures 7.4 and 7.5 where the differences between tests performed using the L4 ES Ag and the other antigens is greater at high ODR values than at low ones. At low ODR values, L4 ES values were still higher than crude adult values but lower than the adult ES ones (Figures 7.4 and 7.5). However, at high ODR values L4 ES tests have the highest ODR values. A greater L4 ES antigen immunogenicity is expected since the L4 stage is more exposed to the host as it resides in the abomasal mucosal glands (the adult stage is just attached to the abomasal mucosal surface), sometimes for very long periods. Their emergence from gastric glands as they mature into adult worms is associated with an increase in the permeability of surrounding blood capillaries, which may facilitate the entry of ES material into the bloodstream, resulting in a more pronounced immune response. This may partly explain the temporal distribution of L4 ES ODR values obtained in this study (Figure 7.2), as well as the spring rise in ODR values observed by Sanchez et al. (7).

The small number of samples in each of the herds, calving seasons, parity groups and lactating-cow housing types may not have been sufficient in effectively evaluating their influence on the agreement between the ELISA tests.

The ultimate aim is to be able to use the ELISA as a herd health management tool, to determine which cows or herds will benefit (from a production perspective) from endectocide treatment. We did a preliminary analysis in this study, to get an idea which
of the three antigens best predicts the milk production response to eprinomectin treatment. Due to the small sample size (5 herds), we were unable to get a good assessment of the association between milk production and the ELISA tests using the 3 antigens.

7.6 Conclusions

The results of the study show that the agreement between tests performed using the crude adult, adult ES and L4 ES antigens were generally low. However, the highest CCC was observed between tests done using both ES antigens. There was some indication that rises in ODR (using L4 ES ELISA) were associated with periods (spring and early fall) of mass development of L4 to adult worms. The study provided further evidence (using field milk samples as opposed to serum) that ES antigens, especially L4, are better at stimulating the humoral immune system than the crude antigens. However, their ability to predict a milk production or reproduction response to endectocide treatment needs to be evaluated and compared with the crude adult antigen.
7.7 References


(13) Sithole F, Dohoo IR, Markham F, Sanchez J, Strhyn H. An evaluation of the stability of Ostertagia ostertagi ELISA microtitre plates over time. (accepted by Vet Parasitol, 2005).


Table 7.1: Distribution of cows with production data by antigen type, calving season and parity.

<table>
<thead>
<tr>
<th>Variable</th>
<th>level</th>
<th>number of cows</th>
</tr>
</thead>
<tbody>
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<td>Antigen type</td>
<td>Crude adult</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>Adult ES</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>L4 ES</td>
<td>145</td>
</tr>
<tr>
<td>Calving season</td>
<td>Winter</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Fall</td>
<td>74</td>
</tr>
<tr>
<td>Parity</td>
<td>2</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>3+</td>
<td>105</td>
</tr>
<tr>
<td>Lactating-cow housing</td>
<td>outdoor</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>indoor</td>
<td>121</td>
</tr>
</tbody>
</table>
Figure 7.1: Frequency distributions of ODR values from 289 milk samples, each tested with 3 *Ostertagia ostertagi* indirect ELISA tests based on 3 antigens: crude adult, adult excretory-secretory (ES), larval stage 4 (L4) ES.
Figure 7.2: Temporal distribution of optical density ratio values (kernel smoothed) obtained by testing each milk sample with the *Ostertagia ostertagi* crude adult worm ELISA (289 samples), adult excretory/secretory (ES) ELISA (289 samples) and the larval stage 4 (L4) ES ELISA (236 samples).
Figure 7.3: Bland-Altman plot showing the distribution of the differences between the *Ostertagia ostertagi* ELISA optical density ratio (ODR) values (across the average ODR range) of milk samples tested using the crude adult worm antigen and the adult excretory-secretory antigen.
Figure 7.4: Bland-Altman plot showing the distribution of the differences between the *Ostertagia ostertagi* ELISA optical density ratio (ODR) values (across the average ODR range) of milk samples tested using the crude adult worm antigen and the larval stage 4 excretory-secretory antigen.
Figure 7.5: Bland-Altman plot showing the distribution of the differences between the *Ostertagia ostertagi* ELISA optical density ratio (ODR) values (across the average ODR range) of milk samples tested using the adult and the larval stage 4 excretory-secretory antigens.
8 Summary

8.1 Study design

The study was a randomized clinical trial that was performed in 65 herds between February 2002 and February 2003. There were 5, 9, 16, 12 and 11 dairy herds enrolled in the Canadian provinces of Prince Edward Island, Nova Scotia, Ontario, Quebec and Saskatchewan, respectively and 12 herds in Minnesota, USA. Cows in this study were Holstein Friesian and classified as either totally or semi-confined. Totally-confined herds were defined as herds in which lactating and dry cows were housed throughout the summer and had no outdoor exposure at all. Outdoor exposure for the lactating and dry cows in the semi-confined herds was limited to a yard or paddock; nevertheless, all cows were fed a ration that met all of their nutritional requirements. In other words, the composition and quantity of stored feeds (components or total mixed ration) fed to lactating or dry cows was not changed when the cows went outside. During the study period, 4 follow-up visits and monthly phone calls were made to ensure that the study protocol was being followed. Data related to herd size, cow and heifer housing and other management practices were recorded.

The dose of eprinomectin pour-on (5 mg/ml) per cow was calculated on the basis of a 725 kg cow applied at 500 µg/kg (1 ml/10 kg) resulting in a dose per cow of 72.5 ml. Eprinomectin was dispensed as individual doses into brown colored plastic bottles and an equal quantity of placebo (mineral oil) was dispensed in a similar way.
Treatment of cows or heifers was to be done between 3 weeks before the expected calving date and up to 1 week after calving.

Fecal samples were collected from 4 first-parity and 4 second- and above-parity milking-cows that had not been treated at calving. FEC were determined using the modified Wisconsin sugar flotation technique. Monthly bulk tank milk samples from each herd were collected between March 2002 and February 2003. All milk samples were sent to the Atlantic Veterinary College where the ELISA was performed. A mange score (0 to 3) for each cow was determined and recorded at the time of treatment by the producer according to a mange-scoring chart.

Production records for the Canadian sites were obtained from the Canadian Dairy Herd Management System database in Montreal. The records for the Minnesota herds were obtained from United States National Dairy Herd Improvement.

8.2 Results and conclusions of the individual studies

8.2.1 Effect of GI nematodes on milk production

The objective of this study was to determine the effect of endectocide treatment at calving in herds that were totally- or semi-confined during the summer. The secondary objective was to determine if either FEC or the ELISA was able to identify herds in which a treatment effect would be expected. In general, FEC were very low (mean = 1 EPG, range = 0 to 27). Mean herd bulk milk ELISA ODR values for the whole year ranged between 0.22 and 0.80. The ODR values were dichotomized into
high and low using a cut-point of 0.5. Overall, there was no significant effect of
treatment. Cows in low-ODR herds that received the placebo produced 2.8 kg of milk
more than cows in high-ODR herds that received the placebo. This provides continuing
evidence that a high ODR is associated with lower production. There was a marginally
significant interaction between treatment and ODR, which suggested a larger treatment
effect in high-ODR herds than in low-ODR herds. The confidence intervals for the
treatment effects (kg/day per cow of milk) in both high-ODR herds (-0.33 to 1.10) and
in low-ODR herds (-0.53 to 0.14) were both wide and included zero. Consequently, this
study failed to show a beneficial effect of eprinomectin treatment in totally- and semi-
confined herds. However, it did provide some slight evidence that the ELISA test may
be more effective than FEC at being able to identify herds in which a positive treatment
effect might be expected.

8.2.2 Effect of GI nematodes on reproduction

The primary objective of this study was to investigate if treatment of cows with
eprinomectin around calving had any beneficial effects on the calving to first artificial
insemination interval, calving to conception interval, and number of services per
conception in totally- and semi-confined dairy herds. The secondary objective was to
evaluate how well the bulk milk ELISA was able to identify herds whose cows’ calving
to conception intervals would benefit from eprinomectin treatment. The study was
carried out in 35 herds (2381 cows) that had electronic reproduction records. The herds
were located in Quebec, Ontario and Minnesota (USA) and participated in the clinical
trial. Overall, there was no significant effect of treatment on the 3 indices of reproductive performance and the ELISA was not able to detect herds in which the calving to conception intervals would decrease as a consequence of eprinomectin treatment. These results were most likely due to the low parasite burdens of the cows.

8.2.3 DHI reproduction data validation

The DHI enables the convenient collection of production data for conducting epidemiologic studies. However, unlike milk production data, it is not compulsory for producers to provide reproduction data to DHI. The goal of this study was to evaluate the level of compliance amongst producers in Quebec and Ontario, in submitting data to DHI as well as validating these data with the ultimate aim of using them in evaluating the effect of eprinomectin on reproduction (Chapter 3). The primary objective of this study was to determine the agreement in the calving dates and reproduction indices (calving to first artificial insemination interval, calving to last insemination interval, calving to conception interval and number or inseminations per conception) between DHI data and data obtained from veterinary clinics in Ontario and Quebec that provided a herd health service to these farms (also called farm data). The proportion of herds and cows within herds whose reproduction records were submitted to DHI, was also determined. The study was performed in 23 herds (1548 cows) from 2 provinces of Canada (Ontario and Quebec) that participated in the clinical trial. Out of 1123 cows from Ontario, only 21 % had DHI reproduction records while only 15 % of the 425 cows from Quebec had these records. A total of 6 herds (3 from each province) did not
have any DHI records. The agreement (between farm and DHI data) in calving dates, calving to last insemination and calving to conception intervals was good. Two methods were used to determine the calving to conception interval. One was directly from DHI data using last insemination intervals and the pregnancy indicator variable, and the other indirectly by subtracting the gestation length (275 days in this study) from the inter-calving period. Good agreement (above 0.90) was obtained with both methods, indicating that the use of the indirect method is appropriate in the absence of sufficient DHI records. Agreement in the calving to first insemination intervals and the number of inseminations per conception was not good, mainly because of insufficient recording of DHI data. From the study results it is recommended that DHI calving to first insemination intervals beyond 100 should be used cautiously as they may be invalid. The DHI number of inseminations per conception in herds with a median less than 2 should be used cautiously. The study demonstrates a need to increase compliance among producers in their submission of reproduction records to DHI. Due to the limited availability of all the reproduction intervals, as well as the second calving dates (to enable the determination of the inter-calving period), DHI data was not used to evaluate the effect of eprinomectin on reproduction. All registered herds in Ontario are connected electronically with Canwest DHI through a network called the Loop. Approximately 50 % of these herds consistently submit their reproduction records to Canwest. It is hoped that the convenience provided by the Loop will facilitate compliance in the submission of reproduction records to DHI.
8.2.4 Effect of deworming on peri-parturient energy balance

As a result of the depression in feed intake around calving, dairy cattle are prone to get into a state of negative energy balance. Since GI nematodes also cause a depression in feed intake, the goal of the study was evaluate if removing worms before calving by treatment with eprinomectin treatment, would improve the energy status of the cows. The relationship between parasite burden at the end of one lactation and energy balance in the subsequent peri-parturient period was also evaluated. Three semi-confined herds participated in the study. Milk samples were collected from 159 cows that were late in their lactation (200 – 400 days in milk) and tested with the ELISA. The cows were then treated with eprinomectin pour-on 2 weeks before the expected calving date and at least 2 blood samples were collected before calving (for the determination of serum NEFA concentration and 2 more post-calving (for BHB measurement). Cows with BHB levels ≥ 1200 μmols/L were classified as having subclinical ketosis. The study found that the odds of developing subclinical ketosis in cows with a mean NEFA level of 0.43 mEq/L (75th percentile) were 2.5 times higher than those of cows with a mean NEFA of 0.17 mEq/L (25th percentile). This study did not provide any indication that treatment of cows with eprinomectin before calving was associated with peri-parturient energy balance. However, there was evidence that BHB levels increased when ODR values in the previous lactation were greater than 0.40. The low parasite burdens in the study animals may not have warranted endectocide treatment and a bigger study conducted in pastured herds may find a significant treatment effect.
8.2.5 Comparison of ELISA tests using 3 blocking agents and 2 conjugates

For the wide scale adoption of the ELISA, plates that can be stored at room temperature for a considerable amount of time are needed. Use of blocking agents (stabilizers) that enable plates to be more at room temperature can facilitate this convenience. The objective of this study was to assess the stability of ELISA plates prepared with one of 3 blocking agents and used with one of 2 conjugates at various time intervals after preparation of the plates. Two of the blocking agents used were commercially available: one termed Stabilguard (bl stab) and one manufactured by SVANOVA Biotek AB Inc. (bl_svan). The third blocking agent used was bovine serum albumin (bl_bsa). A polyclonal rabbit anti-bovine IgG (con_poly) and an anti-bovine IgG monoclonal (con_mono) conjugate were used. Eighteen composite individual cow milk samples collected late in lactation (200 - 400 days in milk) were used in this study. Each of 6 blocking agent/conjugate combinations (called systems) were used to test 18 milk sub-samples at 1, 4 and 24 weeks after blocking the plates. Plates blocked with bl_stab and bl_svan were kept at room temperature and an additional set were incubated at 37 °C so as to mimic long term storage (about 1 year) and tested only once at 4 weeks. Those blocked with bl_bsa were frozen at -20 °C. Generally, there was good agreement between tests conducted at different times for all systems. However, the bl_svan-con_mono and bl_bsa-con_poly systems had the best agreement with overall CCC values of 96% and 93%, respectively. The bl_svan-con_poly system had the lowest CCC of 75%. The CCC and reproducibility ranked the systems in a similar way. The high CCC between tests done using plates kept at room temperature and ones incubated...
at 37 °C, suggested that plates would be stable up to a year after blocking. The storage of plates blocked with bl_svan and bl_stab agents under room temperature, makes them more convenient to use and transport relative to bl_bsa-blocked plates that have to be frozen. Therefore, use of these plates that can be stored at room temperature sets the stage for the wide scale adoption of the ELISA.

8.2.6 Agreement between 3 O. ostertagi antigen ELISAs

Many studies have used the crude adult O. ostertagi antigen in ELISAs to measure worm burden. Lately, there has been evidence that the immune response may be directed more towards excretory/secretory (ES) antigens than somatic antigens found in the crude antigen preparation. The objective of this study was to determine the agreement between ELISA tests conducted using 3 O. ostertagia antigens: crude adult worm, larval stage 4 (L4) (ES) and adult ES. Temporal distributions of ODR values obtained using the 3 antigens (between May and September) were also evaluated. Composite milk samples were collected from 289 Holstein cows from 5 herds in Prince Edward Island and 1 herd in Nova Scotia. Each milk sample was tested with 3 ELISA tests, with each test using a different O. ostertagi antigen. Results showed that ODR values for ES antigens were generally higher than those for the adult crude antigen. Temporal ODR distributions showed that there was a slight increase in ODR values in warmer months, when both adult antigens were used. However, there was a more pronounced increase in ODR between May and September when the L4 ES antigen was used. Increases in ODR coincided with periods when there were increases in the
emergence of adult from the L4 stage (after hypobiosis in spring and at the beginning of the fall). The results of the study showed that the agreement between tests performed using both ES and the crude Ag was low. The highest Pearson correlation coefficient was obtained between ODR values from the adult ES and crude adult antigen (reflection of a common source of antigen). The highest CCC was observed between tests done using both ES antigens. The highest ODR values were obtained in tests where the L4 ES antigen was used. Generally, the study confirmed that ES antigens may elicit a stronger antibody response compared to the crude adult worm antigen.

8.3 General discussion

8.3.1 Study design and statistical attributes

Considering that this was a large multi-site clinical trial and that a limited number of visits to participating herds were made, the present study may be considered to be successful. Only 3 out of 68 herds, dropped out of the study and after attrition, the targeted sample size was achieved. Site coordinators did a good job in encouraging compliance among producers. Collection of treatment data and samples (milk and fecal) from producers, and production data from DHI, proceeded without any problems. The major challenge was to verify and validate the data. For example, the formatting of dates on treatment forms was inconsistent even on treatment forms form one herd. Cow identification was also inconsistent in some cases, with one farm using both DHI and farm identification of cows. Cow identification inconsistencies provided a challenge
mainly in the process of merging production and treatment data. In chapter 4, reproduction data from herds in Ontario and Quebec was collected from the veterinary clinics that provided them routine herd health service. Data from Quebec was in spreadsheet format while that from Ontario came in the form of a text file obtained from a herd health management software called VetCheck. Converting the text file into Stata format was a major challenge, especially since each cow had its own folder, however, this conversion was successful.

The multi-site nature of the study ensured the extrapolation of results to a wider population in the northern temperate regions. Random allocation of treatment and blinding are very essential attributes in clinical trials so as to avoid bias. Both of these qualities were successfully implemented in this study, as indicated by the equality in the distribution of factors (e.g. parity groups) between treated and placebo cows. Accounting for potential confounders in the analyses ensured that any possible bias was eliminated. Monthly phone calls and progress reports that were distributed to participating herds ensured a high level of compliance. All these steps taken ensured that the study had a good internal and external validity. Statistical analyses that took into account the hierarchical nature of the datasets were used to account for the clustering of observations (for example, the linear fixed effects model used in Chapter 2).

The CCC, Bland-Altman plots and reproducibility were used to determine agreement. The CCC is robust and is better than the Pearson correlation since it takes into account the scale of the measurements. The strength of using reproducibility (obtained from variance components of a random effects model) is that it takes into account the magnitude of the agreement using the scale of the variable. Use of both the
CCC and reproducibility is recommended since it gives a complete picture of the agreement between 2 variables.

The calculated sample size of the clinical trial was based on the milk production study. This may have been a limitation on other studies, especially the reproduction study that may have required a larger sample size. Since the sample size was determined at cow level, the study was not powerful enough to detect an interaction between the herd level variable, ODR and treatment (Chapter 2).

8.3.2 Diagnosis of infection with GIT nematodes in adult cattle

There are 3 main reasons that can be given for using anthelmintic treatment. These are: therapeutic, production-based and preventive. Because adult dairy cows are less prone to clinical disease, anthelmintic administration for therapeutic reasons is not common. As a result of the importance of subclinical infection in adult dairy cows that has been documented in temperate climatic regions (1;2), the decision to treat is based on the negative effects that GIT parasites have on production. The level of parasitism in adult dairy cows does not always warrant anthelmintic treatment. Therefore, in order to prevent indiscriminate administration of anthelmintics, it is necessary to come-up with a treatment threshold. The ability of various diagnostic tests to identify herds/cows that would benefit from treatment has been investigated.

The determination of a threshold level for treatment of dairy herds is hindered by the rather low infection levels in adult cows, the limited sensitivity of most diagnostic
techniques and by the high variability of milk production, which is influenced by a number of factors other than gastrointestinal nematode infection (3).

Faecal egg output in cows is generally low and faecal egg counts are considered to be a poor indicator of infection level in adult cattle (1). The ability of FEC to predict a treatment response is poor (4). Similarly, no correlation was found between the effect of anthelmintic treatment on milk production and *Ostertagia* infection levels, as estimated by serum pepsinogen levels (5). Pepsinogen levels in adult cattle may overestimate the actual adult worm burden, possibly due to a hypersensitivity reaction to ingested L3 larvae, which do not necessarily develop to adult worms (6).

Antibody levels against *Ostertagia* have been considered as a more useful parameter. Using the crude antigen ELISA developed by Keus et al. (7), Ploeger et al. (5) found a significant between-herd variation in serum antibody titres against *Ostertagia* and *Cooperia*, suggesting that an estimation of the infection level in an adult dairy herd is possible by examining serum antibody titres. Subsequently, the relationship between serum and milk antibody levels was investigated, because for routine screening purposes milk samples from bulk milk in the cooling tank are less costly than serum samples. A good correlation was demonstrated between individual and herd mean serum antibody titres and milk antibody titres, and between the herd mean of individual milk antibody titres and the mean of two bulk milk samples from the refrigerating tank (8). Individual milk samples had the same ability to discriminate between herds as serum antibody titres (8). Increasing levels of Bulk milk antibodies (as determined by ELISA) was associated with a drop in milk production in the summer and fall in Prince Edward Island (9). Guitián et al. (10) found a positive correlation between some management
practices known to be associated with infection levels and bulk milk antibodies against *Ostertagia*, and suggested that bulk milk antibody levels are a reasonable measure of parasite infection levels in a dairy herd. There was a negative relationship between antibody levels and the level of milk production during the summer in herds exposed to grass. The authors suggested that herds with high milk antibody levels may benefit from parasite-control programmes in the milking herd (10). Kloosterman et al. (11) used bulk-tank *Ostertagia* antibodies to select 16 and 18 farms as having high and low levels of infection. The response in milk yield to anthelmintic treatment was larger in the high antibody level herds than in the low antibody level herds, but these differences lacked statistical significance.

In conclusion, *Ostertagia* antibody levels in milk are the most promising parameter to monitor gastrointestinal nematode burdens in dairy herds, but a threshold for anthelmintic treatment still needs to be determined. Other diagnostic methods for adult cattle, such as a copro-antigen detection test are still in an experimental phase (12).

8.3.3 Study results

Overall, the results of chapters 2 and 3 showed that there was no production benefit of deworming cows kept under no or limited outdoor exposure. However chapter 2 showed that the bulk tank ELISA is better than FEC at identifying herds that would benefit from anthelmintic treatment. These results are consistent with results of other studies (1; 4) that have exposed the weaknesses of FEC in adult dairy cows. Chapter 2 showed that there was a positive treatment response in high ODR herds whereas none
was found in low ODR herds. This was reflected by a treatment by ODR interaction term which was marginally significant (P = 0.15). These results are consistent with those of Kloosterman et al. (11). A cut-point of 0.5 for high and low ODR was used in this study. This may not apply in other regions where the worm burden and Ostertagia ostertagi epidemiology is different. Levels of antibodies (hence ELISA ODR) reflect both protective immunity and immunity as a result of current infection levels. Since levels of antibodies due to protective immunity are bound to differ from region to region, so will be the ELISA ODR. For this reason, the ELISA ODR threshold for treatment will differ. In Chapter 2 a negative association between milk production and bulk milk ELISA ODR was demonstrated in placebo-treated cows. The question still remains on the whether there is a causal relationship between antibodies against Ostertagia ostertagi in milk and milk production. Other factors that may cause a negative relationship are increasing milk production (8) and mastitis. A significant interaction between ELISA ODR and treatment will demonstrate a causal relationship between milk antibodies and milk production.

Individual treatment of cows, as was done in this study, may result in placebo treated cows being in contact with eprinomectin through licking. Licking is part of social behaviour in cows. It can be self-licking or allo-licking (cows licking each other). About 58 to 80 % of the pour-on dose of ivermectin is ingested through licking, while 10 % is absorbed percutaneously (13). About 72 % of the ingested ivermectin remains in the feces and is not absorbed into the bloodstream (13). Licking behaviour could potentially reduce the power of the study to detect a difference in milk production between eprinomectin and placebo treated cows. Whilst this was a possibility in this
study, the fact that the cow density was relatively low and that a few cows were treated at any one time, may have reduced this possibility. Randomization of treatment by herd would prevent this problem from occurring.

The problem with treatment at calving is that it does not take into account the epidemiology of the parasite. The effect of treatment on milk production is bound to be higher during periods when the cow is exposed to highest worm challenge. Typically this would be early summer and fall in temperate climatic regions. We evaluated whether the effect of treatment on milk production depended on the treatment season but there was no interaction between treatment and calving season. Therefore, in this study the absence of a treatment effect was most likely not due to treatment at calving. There was evidence (Chapter 5) that parasite burden (as determined by ELISA ODR) had a detrimental effect on energy balance around the time of calving. Energy status around calving determines the cows overall production for that lactation as well as susceptibility to metabolic diseases. Increased negative energy balance around calving reduces the cow’s milk production peaking potential and delays its return to estrus. Therefore, mitigating the negative effects of parasites by treatment at calving, should improve the energy status and hence increase milk production and reduce susceptibility to metabolic disease. The study in chapter 5 did not find an association between deworming and energy status. A study conducted in pastured or semi-confined herds may be able to demonstrate a treatment effect and solidify the association between worm burden and energy status. A significant association between deworming around calving and energy status will provide further justification for treatment at calving. Assuming a positive treatment effect, the main advantage of deworming around calving (as opposed to group...
treatment at strategic seasons of the year) will be the prevention of metabolic diseases that arise as a result of peri-parturient negative energy balance. The overall production and economic benefit (milk production, reproduction, reduction of metabolic diseases) of calving treatment versus group treatment (at particular seasons) will need to be evaluated.

The results of Chapter 7 suggested that the ES antigens may be more immunogenic than the adult crude antigen because of the high ODR values observed using the ES antigens. It should be noted that ES antigens are also a complex mixture of epitopes, maybe to a lesser extent than the crude antigen. It was also found that the temporal distribution of ODR values obtained using the L4 ES follows a more distinct pattern related to L3 intake, than the adult antigens. This suggests that the L4 ES antigen is more sensitive in detecting L3 intake. This may put the use of the L4 ES antigen in predicting treatment effects at a disadvantage since the adult antigens are more stable over time and may reflect the average herd worm burden better at any given time. However, the predictive abilities of the L4 ES antigen still have to be compared to that of the adult antigen. The disadvantage of the use of these complex antigens is that they may show cross reaction with other species like *Fasciola hepatica* and *Dictyocaulus viviparulus* which are all not gastro-intestinal nematodes.

In Europe, there is a lot of consumer pressure to reduce or do away with the use of chemotherapy in food producing animals, including anthelmintics. This may force producers to use management practices that reduce parasite transmission. These practices include pasture rotation, mowing the grass and late turnout to pasture. All these practices reduce L3 intake. Prevention of L3 intake means that there are less
worms that develop into adults and therefore a reduction in egg production leading to reduced egg pasture contamination.

A study in Northeastern USA showed that 20% of dairy producers make a decision to deworm based on productivity (14). The milk production study in this thesis has shown that herds with limited or no outdoor exposure may not benefit from deworming their cows. Indiscriminate use of anthelmintics needs to be discouraged since this leads to the problem of resistance. At the moment, this problem is not as bad in cattle as it is in sheep. Anthelmintics should be used in combination with management interventions, to prevent resistance. Research on the use of vaccines against parasites is slowly emerging, but it will be long before it replaces anthelmintic use as the main method of parasite control.

8.4 General conclusions

1. Overall, there was no indication that the milk production and reproduction of cows with limited pasture exposure, benefits from eprinomectin treatment around calving.

2. The low level of parasite burdens did not allow for a conclusive investigation on the ability of the bulk milk ELISA to identify herds whose cows' milk production and calving to conception intervals would benefit from eprinomectin treatment. However, there was some slight evidence that the difference between treated and non-treated cows was higher in high-ODR than in low-ODR herds.
There was no evidence that the effect of treatment depended on whether the herd was low-FEC or high-FEC.

3. The studies demonstrated significant associations between bulk milk ELISA ODR and milk production as well as ODR and energy. Cows in low-ODR herds that received the placebo produced 2.8 kg of milk more than cows in high-ODR herds that received the placebo. There was evidence that BHB levels increased when ODR values in the previous lactation were greater than 0.40. These associations provide a means of validating the ELISA test.

4. Adoption of the ELISA to wide scale testing has been made possible since there are blocking agents (Svanova and Stableguard) that enable plates to be stored dry at room temperature without any adverse effect on the ELISA, for at least a year. The use of a monoclonal antibody conjugate is worthy of consideration.

5. The antibody response to ES antigens (especially the L4) is stronger than to the crude adult worm antigen. It is not known whether ES antigens are better at predicting a treatment effect.

6. DHI reproduction recording is insufficient, except for calving dates. Therefore, only the calving to conception interval derived from subtracting the gestation period from the inter-calving interval can be sufficiently made from DHI data.

8.5 Future research

Research presented in this thesis is part of a larger study that has an ultimate aim of producing a commercial ELISA test that can be routinely used in a herd health
management program to identify dairy herds whose production would benefit from endectocide treatment. This study has shown that blanket endectocide treatment of cows from confined and semi-confined herds is not warranted. The ELISA test has shown a lot of promise in being used as a tool to identify herds/cows that would benefit from eprinomectin treatment. Whether bulk tank or individual cow late lactation milk samples are most suitable is an outstanding question. The exact ELISA threshold for treatment also still has to be determined. A larger study conducted in semi-confined and fully pastured herds is needed to address these questions.

Results of this thesis as well as previous work on determining the predictive ability of the ELISA has been done using the crude adult worm antigen ELISA. There is a need to evaluate the predictive abilities of the ES antigen ELISA compared to the crude antigen one.
8.6 References


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Appendix A – Recording of herd data at first farm visit (Chapters 2 and 3)

Eprinex 2

First Farm Visit - Data Collection Sheet

<table>
<thead>
<tr>
<th>Name:</th>
<th>Date:</th>
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<tbody>
<tr>
<td>Herd Study #:</td>
<td>Bottles Delivered:</td>
</tr>
<tr>
<td>Current Herd # cows milking:</td>
<td># cows dry:</td>
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</table>

Reproductive (breeding) records on computer (Y/N):

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<tr>
<th>if Yes</th>
<th>Dairy Comp:</th>
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<tbody>
<tr>
<td></td>
<td>DHI:</td>
</tr>
<tr>
<td></td>
<td>Other (specify):</td>
</tr>
<tr>
<td></td>
<td>Willing to provide? (Y/N)</td>
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Anthelmintic Treatments - 2001

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<th>Approx. date(s) (if applicable)</th>
<th>Product (if applicable)</th>
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<tbody>
<tr>
<td>none</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>group treatment</td>
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<td></td>
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<table>
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<tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>ind. tx. at calving</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
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<th>none</th>
<th>group treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ind. tx. at calving</td>
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<table>
<thead>
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<th>group treatment</th>
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</thead>
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Indoor Housing - Winter 2001 / 2002

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<th>Loose Housing</th>
</tr>
</thead>
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<tr>
<td>Heifers &lt; 15 mo</td>
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<td></td>
</tr>
<tr>
<td>Bred heifers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactating cows</td>
<td>Dry cows</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>----------</td>
<td></td>
</tr>
</tbody>
</table>

**Outdoor Exposure - Summer 2001**

<table>
<thead>
<tr>
<th>Pasture (grazing)</th>
<th>Paddock</th>
<th>gravel / dirt / paved yard</th>
<th>indoors</th>
</tr>
</thead>
<tbody>
<tr>
<td>total acres</td>
<td># cows</td>
<td>total acres</td>
<td># cows</td>
</tr>
</tbody>
</table>

Heifers < 15 mo
Bred heifers
Lactating cows
Dry cows

<table>
<thead>
<tr>
<th>Mange Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td># of cows:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Tape Weights - 10 cows systematically selected (e.g. every 8th cow in an 80 cow herd)**

Instructions

- Name: producer name and herd study number
- Date: date of visit
- Bottles delivered: record number range of bottles left on farm
- Current Herd: record current # of milking and dry cows
- Reproductive records:
  - determine if reproductive records are recorded electronically
  - what form they are in, and
  - is the producer willing to provide access to them

- Anthelmintic treatments in 2001: for each age group of animals, determine if any treatments with *anthelmintics* or *ecto-parasiticides* were applied.
  - Tick of "none" if none were applied
  - Room for two group treatments has been left. If there were more than that, just squeeze it in somehow.
  - For group treatments record the approximate date (month will be fine).
  - For all treatments, record the product used (trade name)

- Indoor housing (winter): tick off the type of housing for each age group

- Outdoor Exposure (summer 2001)
  - pasture means deriving some of their nutrition from the pasture
- record total # of acres grazed by that age group
- record total # of animals in the age group
- Opaddock means a grassed lot but not expecting nutrition from it
- record total acres and # of animals
- Oyard or indoors - just tick off
- Onote: if pasture is used by more than 1 group (e.g. young heifers and bred heifers) and they are grazed together, record the total acreage under each group and the respective # of animals (i.e. don’t divide the total acreage between them)

mange score: record the # of animals (lactating herd only) with each of the mange scores
Appendix B – Treatment record form (Chapters 2 and 3)

Eprinex Study
Data Recording Form

Farm:

<table>
<thead>
<tr>
<th>Cow Name or Number</th>
<th>Treatment Date</th>
<th>Calving Date</th>
<th>Bottle Number</th>
<th>Mange Score (0 - 3)</th>
</tr>
</thead>
<tbody>
<tr>
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</tbody>
</table>
Appendix C – Data collection during the second, third and fourth farm visits

Eprinex 2

Second Farm Visit - Data Collection Sheet

<table>
<thead>
<tr>
<th>Name:</th>
<th>Date:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herd Study #:</td>
<td>Bottles Delivered:</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Anthelmintic treatments other than study treatments</th>
<th>Jan 2002 - this visit</th>
</tr>
</thead>
<tbody>
<tr>
<td>check all that apply</td>
<td>Approx. date(s) (if applicable)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Heifers &lt; 15 months</th>
<th>none</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>group treatment</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bred Heifers</th>
<th>none</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>group treatment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ind. tx. at calving</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lactating Cows</th>
<th>none</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>group treatment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ind. tx. at calving</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dry Cows</th>
<th>none</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>group treatment</td>
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</tbody>
</table>
### Plans for Summer Housing - 2002
(only necessary if different from 2001)

<table>
<thead>
<tr>
<th>Pasture (grazing)</th>
<th>Paddock</th>
<th>gravel / dirt / paved yard</th>
<th>indoors</th>
</tr>
</thead>
<tbody>
<tr>
<td>total acres</td>
<td># cows</td>
<td>total acres</td>
<td># cows</td>
</tr>
</tbody>
</table>

- **Heifers < 15 mo**
- **Bred heifers**
- **Lactating cows**
- **Dry cows**

If lactating or dry cows are going to pasture (instead of just a paddock), please specifically ask the composition or quantity of stored feeds fed to the group will be changed once the cows go out to pasture.

Yes - composition/quantity will change  No - will not change

<table>
<thead>
<tr>
<th>Mange Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td># of cows:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Identification of 8 cows fecal sampled (please record ID on sample too)
Appendix D – Publication status of thesis chapters


Chapter 7. F. Sithole, I. Dohoo, F. Markham, J. Sanchez. Assessing the agreement between Ostertagia ostertagi ELISA tests performed using the crude adult antigen and the adult and larval stage 4 excretory/secretory antigens. Submitted to Veterinary Parasitology.

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