Preamble

The Western Canada Study on Animal and Human Effects Associated with Exposure to Emissions from Oil and Natural Gas Field Facilities is intended to determine if exposure to emissions from oil and gas batteries and other field facility sites impacts animal and human health in western Canada.

The study designs which follow provide details of the research methodology. The study designs have been reviewed, revised and endorsed by a Science Advisory Panel comprised of ten internationally renowned scientists with collective experience in environmental and reproductive epidemiology, animal and human health and toxicology. The research methodology, including the peer review process, is designed to ensure credible scientific findings which will assist in the future development and implementation of recommendations regarding future practices. The research is being conducted by university and private sector researchers under the supervision of the Western Interprovincial Scientific Studies Association (WISSA) Board of Directors and the Science Advisory Panel.

There are five components to the study:

- Beef Cattle Productivity;
- Assessment of the Immune Function in Beef Cattle;
- Assessment of Wildlife Reproduction and Immune Function;
- Exposure Monitoring; and,
- Human Health.

The Human Health component is still under development and as such a study design for this component has not been included in the information which follows. The study design for the human health component will be released at a later date following its review, and endorsement of the Science Advisory Panel.

The objectives of the **Beef Cattle Productivity component (Tab 1)** are to determine whether beef cattle exposed to emissions from oil and gas batteries and other field facility sites are at greater risk of productivity failure than those that are less exposed and secondly, to collect information necessary to describe the health, reproductive performance, and factors affecting these outcomes for cow-calf herds in western Canada. Nested within the Beef Cattle Productivity Study are several other studies as described below. Study designs for these components are included as Appendices to the Beef Cattle Productivity Study.

The Assessment of Immune Function in Beef Cattle component (Tab 2) is designed to determine whether exposure to emissions from oil and gas batteries and other field facility sites has an adverse effect on the immune function in beef cattle.

A wild bird species will be studied in the component, Assessment **of Wildlife Reproduction and Immune Function (TAB 3)**, to determine whether exposure to emissions from oil and gas batteries and other field facility sites has an adverse effect on reproductive success and the immune function in wildlife.

The **Exposure Monitoring** component **(Tab 4)** will provide study exposure information for air quality parameters including sulphur dioxide, volatile organic compounds, particulate matter, and hydrogen sulphide at the study sites. The air quality data will be used to test for the existence of relationships between exposure to emissions and documented observations from other components of the Study.

Data collection for the Study will occur throughout 2001 and 2002, with data analysis and peer review in 2003. A final report is not anticipated until 2004. This timeline is dependent upon receiving funding for project activities through to the conclusion of the study. In the event that funding for the overall project is not secured, the timeline will be subject to change.

Western Canada Beef Productivity Study

A Component of the Western Canada Study on Animal and Human Health Effects Associated with Exposure to Emissions from Oil and Natural Gas Field Facilities

Study Design

November 2001

Department of Large Animal Clinical Science
Western College of Veterinary Medicine
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WESTERN CANADA BEEF PRODUCTIVITY STUDY
STUDY DESIGN – November 2001

Background

The agricultural industry and oil and gas sector drive a substantial portion of the economy in western Canada. The close proximity of oil and gas facilities to agricultural activities has created concern in some areas. One of these concerns is the potential impact of oil and gas activities on livestock health and production.

These concerns and specific suggestions for future research have been documented in a series of public and industry workshops during the 1980's and 1990's. These included, but were not limited to, two workshops on the Effects of Acid Forming Emissions in Alberta (Edmonton AB, May and November 1986), the Acidifying Emissions Symposium (Red Deer AB, April 1996), the Scientists' Advisory Meeting (Edmonton AB, January 1997), and a conference on dealing with the gas flaring problem in the petroleum industry (Calgary AB, April 1999). Key questions identified by the Red Deer workshop examining the potential effects and impacts of acidifying emissions on animal health included:

- Is animal health effected by acid-forming emissions?
- Are there effects on respiratory, immunological and / or reproductive systems?
- Chronic exposures need to be investigated.
- What are the differences between wildlife and domestic animals as receptors?

In July 1996, the Alberta Environmental Centre released a report prepared for the Alberta Cattle Commission titled "Cattle and the Oil and Gas Industry in Alberta: A Literature Review with Recommendations for Environmental Management". One of the recommendations from that report was to study the "possible toxicological effects on cattle from prolonged, low-level, exposure to contaminants… with special attention to the reproductive and immunological systems."

Recent studies completed for the Western College of Veterinary Medicine (WCVM), University of Saskatchewan (Waldner, 2001a; Waldner, 2001b) and the Ontario Veterinary College (OVC), University of Guelph (Scott, 1998) have suggested that there may be an association between emissions from sour gas processing facilities and reproductive outcomes in cattle. A WCVM study uncovered associations between flaring at oil and gas battery sites and an increased risk of some forms of reproductive failure in cattle, especially stillbirths. This study was the first intensive, multi-year study designed to examine the affects of oil and gas activities on beef cattle. However, the study was restricted to a small geographical region of Alberta and hence the study's results my not be applicable to other regions in western Canada. Briefly, three conclusions can be drawn from the WCVM study:

1. The overall health and productivity of beef cattle in one area of intensive oil and gas activity in west-central Alberta was similar to other areas of the province.

2. There was a small, but significant, association between sour flaring at oil and gas battery sites and reproductive performance in beef cattle. Of the six reproductive outcomes examined, the most consistent association was for increased risk of stillbirth.

3. There were also some examples of associations between increasing sulfation deposition and adverse reproductive outcomes in cattle.

The results of these preliminary studies raised enough questions to warrant further investigations and more definitively determine if some oil and gas activities are impacting livestock health and productivity. The studies also provide valuable information on the types of activities and outcomes that should be the focus of further investigations and the study methodology that should be followed.

The four western provinces determined that there were sufficient data, as well as public concern, to warrant further investigation of the affects of oil and gas industry on beef cattle health and productivity. In the spring of 2000, a committee of representatives from the western provinces approached the Western College of Veterinary Medicine to assist in conducting a multi-disciplinary study of the oil and gas industry. Faculty from various departments within the WCVM were asked to design a study to examine cow-calf productivity in western Canada and its potential association with exposure to emissions from the upstream oil and gas industry.

The Western College of Veterinary Medicine was directed to:
1. To determine whether previous findings could be generalized across the major oil and gas producing areas of western Canada.
2. Consider issues of public concern not previously evaluated.

There will be substantial **"value added"** data generated from the Western Canada Beef Productivity Study (WCBPS) that will provide insight beyond that gained from previous research.

1. The study will determine whether the results from previous studies can be generalized to (or replicated across) the major oil and gas producing areas of western Canada.

    - The only study to date to examine the potential reproductive effects of flaring emissions in beef herds was conducted in a very small, localized area of west-central Alberta (WCVM). The second study did not examine the association between flaring and reproductive outcomes in beef herds (OVC).
    - Previous studies suggested that there may be a potential association between herd location in high sulfur deposition areas and increased risk of reproductive failure. The current study will re-evaluate this association in more detail.

2. Exposure assessment has been a major limiting factor in all previous work to date.

    - The Western Canada Beef Productivity Study (WCBPS) will include exposure markers for both sweet gas emissions as well as sour gas sources. Passive monitors will measure volatile organic carbons (VOCs) (including BTEX – benzene, toluene, ethylbenzene, xylene as well as 22 other compounds of interest) – as surrogates for hydrocarbon (or sweet gas) emissions.
    - The WCBPS will use a new generation of field tested monitors to measure marker compounds for sour gas emissions including SO_2 and H_2S. Initial data suggest that this new generation of monitors is superior to those used in previous studies.
    - Monitoring for $PM_{1.0}$ and polycyclic aromatic hydrocarbons has been investigated and should be conducted in a subset of study herds.
    - The earlier WCVM study provided extensive experience in tracking individual cow location and movements. This experience will be applied in the WCBPS on a scale sufficient to address many of the questions not adequately examined in the previous reports.

3. The Western Canada Beef Productivity Study (WCBPS) will provide opportunities to **link** the study of domestic animals with plans to address human health concerns.

    - Data from the cattle study could potentially be used as a model or sentinel system for aspects of the human health investigation. The information collected from these **sentinel** herds could suggest potential locations for planned human studies. If this sentinel system is to be effective, sufficient numbers of herds are necessary to achieve broad geographic coverage.

4. We have an opportunity to accurately describe health and productivity in cow-calf herds. During the initial studies of this question and ongoing investigations of herd owner concerns, the absence of accurate information describing normal productivity or background levels of disease was apparent.

    - For example, what proportion of cows abort each year in western Canadian beef herds? What is the expected rate of loss at the time of birth and from birth to weaning? What are the causes of these losses? The currently available information is restricted to that collected in a series of mail out questionnaires.
    - This study will be used to develop an accurate baseline for reproductive performance and disease occurrence in western Canadian cow-calf herds.

Objectives

This study will determine whether beef cattle that are exposed to emissions from oil and gas batteries and other field facility sites are at greater risk of productivity failure than those that are less exposed.

Primary question and outcome variables:

The study will focus on assessing whether increasing levels of exposure to oil and gas facilities adversely affect the risk of five important reproductive outcomes in beef cattle. These five outcomes include the individual animal risk of:

1. non-pregnancy
2. abortion
3. stillbirth
4. calf mortality
5. length of calving-to-calving interval.

Other outcomes of interest:

Using nested projects within the larger study, we will also examine several important secondary outcome measures, including effects of exposure to emissions from oil and gas facilities on:

1. Disease-specific mortality (especially respiratory related losses) in cattle,
2. Immune system structure and function in cattle **(Appendix A, Tab 2),**
3. Neuropathology in cattle, and
4.

It will be necessary to collect very detailed and accurate information on other risk factors known to affect cattle productivity and health, including management, nutrition, infectious disease exposure, and numerous environmental factors. This information will be used to address other important productivity and health issues of interest to the Canadian cattle industry that are not necessarily directly related to exposure to oil and gas field facilities.

General Conduct of the Study

An observational study design was chosen to answer the questions outlined above. As there is limited data on ground level exposures to emissions from oil and gas facilities under field conditions in western Canada, it would be extremely difficult to examine these questions within a laboratory setting with currently available information. Information is not available on the concentration, composition, or timing of exposure necessary to reproduce conditions in the laboratory that are expected near representative oil and gas facilities in western Canada. Finally, to measure the small relative reproductive loss in exposed herds suggested by previous studies, large numbers of cattle would be required in any laboratory-based study that might attempt to duplicate conditions found in the field.

The overall design of this observational study utilizes elements of the techniques applied in the Ontario-based Benchmark Study (McDermott et. al., 1991) and two previous, Alberta-based, studies of this question (Scott, 1998; Waldner, 2001a; Waldner, 2001b). The study will compare the risks of reproductive and productivity losses across beef cattle that are exposed to different sources, types and concentration of oil and gas field facilities, and located at varying distances from these facilities. Herds will be selected based on apparent exposure status, location, quality of records, herd size, and adequacy of cattle handling facilities. Final assessment of exposure status for data analysis will be determined by the results from the passive air monitoring network.

Exposure will be defined initially (for the purposes of herd selection) using currently available information. Data used to classify herds for the initial selection procedure include estimates of proximity of cattle to emissions sources, flare type/volume, and, where available, air quality data. Exposure measurements used in the final analysis will be derived from a comprehensive passive air-monitoring program applied to all investigation herds for selected marker compounds including SO_2, H_2S, and various VOCs.

Measurements of pasture proximity to various emissions sources will be used as a second method to quantify exposure. **The most consistent association, observed to date, between reproductive effects and exposure was based on a proximity analysis to sour gas flares (Waldner, 2001b).**

Some consideration of proximity and facility type is necessary to account for fugitive emissions in very close proximity to oil and gas field facilities. Passive monitors placed to measure stack

There is no single basic unit of observation or analysis within this study. Beef herds in western Canada are normally split up into several groups during the breeding season but are often wintered in relatively close proximity to each other either as a single group or in a smaller number of groups. The initially obvious unit of analysis is the management group within the herd; however, the management group in many of these herds is a highly fluid unit. Animals are frequently moved between groups during the summer, in the fall, and again during relocation from calving to nursery pastures. The actual effective unit of analysis will depend on the time frame over which exposure is being considered. For example, if we are looking at exposure during the period immediately preceding calving, the unit of analysis might be the herd as there would be very little within herd variation in exposure because of the close proximity of animals to each other. If we were looking at exposure over the breeding period, the unit of analysis would be the breeding or bull management group. If we are looking at exposure over a large part of the cow-calf cycle, then the unit of analysis is the individual animal because of the high degree of within herd variation in exposure associated with shifting of animals between management groups and variation in exposure among these groups.

Descriptive statistics will include summaries of exposure data, beef cattle productivity and health data, and the occurrence of other important risk factors. Statistical analyses will also include examination of the associations between exposure and outcomes, using appropriate statistical methods and current software developments available at the time the analyses are completed. (Currently available options include: SAS Proc GENMOD, SAS Proc MIXED, SAS GLIMMIX macro, and MLwiN). Both individual animal and herd-level factors will be considered in the analysis. Statistical methods that account for clustering of data at the herd level and management group are required. A more detailed analytical design has been developed for review by the Science Advisory Panel. The resulting analysis will provide estimates of the association between exposure to oil and gas emissions and beef cattle productivity indices.

Action Plan and Duration of the Study

1. Draft of working study plan completed by October 2000 with initial review by the Scientific Advisory Committee in November 2000. The University of Saskatchewan Animal Care Committee has granted approval for the study.
2. Final selection of herds was completed by March 31, 2001. Herd owners have signed a contract with study personnel outlining both the obligations of all participants and a confidentiality agreement protecting individual herd data (completed March 31, 2001).
3. Collection of herd data began in January 2001 with the run-in period (opportunity to pilot some data collection tools) extending through calving season 2001.
4. Formal exposure analysis (collection of passive monitoring data) began in April 2001.
5. Collection of health and productivity information from all cattle began with the start of breeding season: May 2001.

6. Collection of field data will end when the resulting calf crop is weaned and pregnancy testing is completed in the fall of 2002.
7. Analysis of data should be completed by the fall of 2003.
8. Reporting of results for initial peer review should be expected in the first quarter of 2004.

Study Management

The study will be managed by a team of faculty from the Western College of Veterinary Medicine (WCVM) at the University of Saskatchewan with expertise in environmental epidemiology, beef herd medicine and management, theriogenology, veterinary and wildlife toxicology, behaviour, nutrition, infectious disease, immunology, pathology, wildlife health, environmental risk assessment, and biostatistics (College of Medicine). A study administrator will be responsible for coordination of the management group, staff and financial administration, and day-to-day questions arising from study operations.

A group of "Project Veterinarians" has been contracted to collect and enter data (one veterinarian per approximately 40 herds, depending on location and herd size. Data collection and analyses will be assisted by graduate students at the WCVM and the University of Saskatchewan Toxicology Centre. Exposure measurement data will be collected by an independent environmental monitoring contractor (RWDI West Inc.) working in collaboration with the WCVM team, but under the supervision of the overall project manager located in Calgary.

Facilities

The Western College of Veterinary Medicine provides an independent base of operation for the study. The college is recognized for its expertise in beef-herd field epidemiology and depth of experience in supporting sciences. The Toxicology Centre at the University of Saskatchewan will oversee the immunotoxicology aspects of the cattle study, as well as study the effect of exposure on wildlife health and reproduction. Prairie Diagnostic Services in Saskatoon will provide histopathological services and infectious disease screening.

The Project Veterinarians working on this study (not including U of S faculty) have previous research experience including a PhD and Master of Veterinary Science. Three members of this group (again in addition to university faculty) have advanced training in statistics and data base management and extensive experience in field investigations.

Study Plan

Sample Size Assumptions and Estimates:

Previous research has suggested that the risk of stillbirth may be higher in cattle exposed to sour gas flaring (Waldner, 2001b). Sample size has been estimated to re-examine this finding. Exposure may be analyzed as both a continuous and categorical outcome. For this initial crude sample size estimation, herds will be considered as varying from highly exposed, through moderately exposed, or to unexposed to sour gas flares. We cannot readily estimate sample size for the change in risk in stillbirth across the actual range of exposures anticipated from the passive monitors, because there are no data available on the expected range of cumulative $S0_2$ and VOC results using the monitors planned for this study. We also are unsure of what the range of cumulative exposure to the presence of sour flares and other facilities will be. Approximately 66 herds in each group of herds would be required to detect a difference between stillbirth risks of 3% and 6% across the range of exposures with 95% confidence and 80% power if we assume there are approximately 165 cows per herd and that the within herd correlation of response to exposure is approximately 0.4 (rho). This sample size for the highest and least exposed herds would allow us to include up to 12 covariates in our model with an average correlation of 0.25 with exposure. By including an equal number of moderately exposed herds, the effective study power should be increased.

Low or no exposure herds will be selected from areas near the exposed herds as well as areas where there is no potential for exposure. All "project veterinarians" working out of Alberta, Saskatchewan and northern British Columbia have attempted to recruit low or no exposure herds within their area, wherever possible. Fifteen to twenty herds will also be selected from central areas of Saskatchewan with no potential for exposure.

Selection of Study Veterinarians

In addition to the contract researchers, the study requires the participation of veterinarians in western Canada. The veterinarians were involved in the identification and selection of the herds and have an on-going role in the collection of herd reproduction and necropsy data.

Multi-person large or mixed practice veterinary clinics were chosen from the listings of the Alberta and Saskatchewan Veterinary Medicine Associations. Practices in areas of high oil and gas activity identified by the university employed study veterinarians as having a strong interest in cow-calf herd medicine were approached first to increase the probability of finding herds fitting the study criteria and maximizing the highest identifiable exposures. Practices were selected across Saskatchewan, Alberta, and the north-eastern part of British Columbia to achieve adequate geographic coverage of the major oil and gas producing areas of all provinces. Geographically similar adjacent areas were also selected to ensure the inclusion of herds with no identifiable local exposures in the study.

Where two or more practices with an established cow-calf clientele were located in the same town, the largest practice was approached first. If the first practice declined, subsequent practices were contacted until an adequate number of interested herd owners were identified in all major oil and gas producing areas. Some practice owners expressed interest in the study, but indicated they were too busy to increase their existing caseload.

During the period of participant recruitment, additional veterinarians became aware of the study through media releases, communication with peers and professional meetings in the later months of 2000 and early months of 2001. Some of these veterinarians contacted the study-employed veterinarians directly to indicate interest in participating. Clients from all veterinarians who contacted the study and asked to be included were added to the list of potential study participants. Study veterinarians continued contacting veterinarians until the planned number and distribution of herds fitting the study criteria had been identified. Greater than one third of all eligible practices in the geographic areas of interest were eventually recruited for participation in the study.

***Herd Selection*:**

Random selection of study herds would have required an accessible complete listing of all cow-calf operations in western Canada containing information on herd size and location. There was no complete selection framework containing the required information and, therefore, random herd selection was not possible. Herds were recruited by contacting veterinary clinics from cattle producing areas in Alberta, Saskatchewan, and northern British Columbia.

Study sites were chosen to achieve adequate geographic coverage and optimum climactic and herd size comparability between exposed and non-exposed herds. The power of the study to find an association, if one is present, was maximized by working to identify the highest exposed herds across western Canada meeting the study criteria and comparing them to herds with no or very low exposure. Details of the herd selection process and criteria follow.

Herds were recruited by contacting veterinary clinics from cattle producing areas in Alberta, Saskatchewan, and northern British Columbia. Veterinary practices in areas with a high concentration of oil and gas production facilities were contacted first and asked to identify producers with herds that were highly exposed to continuous solution gas flares (both sweet and sour) or other sources of gas and oil processing emissions, and who might have an interest in participating in the project. These practices were initially selected to maximize the potential for exposure based on maps of oil and gas facilities and flaring volume provided by provincial regulatory agencies.

The veterinarians were asked to contact the owners of herds pastured within 1 mile (1.6 Km) of a facility with a short flare stack or within 5 miles (8 Km) of a gas plant with a large flare stack or a

processing plant with an incinerator stack. These distances were selected after consultation with environmental consultants and were based on expected dispersion distances from the most common stack heights seen in the field. Some herds were also recruited outside the 8 km distance where existing air monitoring data has verified that the cattle are in high deposition areas for plumes from large sour gas processing plants.

This group of veterinarians and veterinarians in areas with no oil or gas activity were also contacted and asked to identify producers with little or no exposure who may be interested in participating in the project.

The criteria for a "no identifiable exposure" herd was considered to be no oil or gas facilities within 30 miles (50 Km) of any pasture. Many of the unexposed herds selected were considerably farther than 50 km from all oil and gas facilities.

Herds with no small facilities within at least 2-3 miles and no large gas processing facilities within at least 10 to 15 miles were designated as low exposure in areas where it was not possible to identify no exposure herds. The size of selected herds was restricted to maximize similarity of herd size and management practices across exposure levels and major geographic zones. Producers invited to participate in the study had to meet the following selection criteria.

The Selection Criteria for Optimum Study Herds:

1. Herd size, where possible, should fall between 50 and 250 breeding females.
    - Very small herds often have radically different management practices than the larger herds and make up a very small proportion of the total cow herd in western Canada. The inclusion of very small herds (< 40 breeding females) would introduce and important and not easily controlled variable into the analysis and decrease study power.
2. All animals must be individually identified with a readily visible plastic ear tag.
3. All calf births should have been recorded during the previous calving season.
4. The herd owner must have access to facilities suitable for pregnancy testing, bull evaluation, and blood sample collection.
5. Cows and heifers should have been pregnancy tested by a licensed veterinarian following the last breeding season.
6. A defined spring - summer breeding season must be used.
    - Fall calving or continuous calving would introduce an additional variable that will decrease the power of the study.
7. Bulls should have been evaluated for use by a licensed veterinarian prior to use in the previous breeding season.
8. The herd owner must have an established, working relationship with a local veterinary clinic.

9. The herd owner (and their veterinarian) must be interested in participating in the study, and must commit to completing the study design.

Process of Herd Selection

The veterinary clinic made initial contact with the producers to see if they had an interest in the project. A list of potentially interested candidates was supplied to a university employed project veterinarian.

Interested producers were visited by a university employed project veterinarian, to assess whether each producer suggested by the veterinary clinic met the selection criteria. The project veterinarian completed a herd profile form on each producer visited. Based on the producer interviews, producers were ranked based on the degree of exposure and type of exposure.

Exposure Rank:	**1**	High exposure, flares, batteries, pump jacks in the pasture, small acid gas plants
	2	Exposed, batteries, pump jacks, gas plants with sulphur recovery, sweet gas and oil, occasional flaring
	3	No exposure, no exposure within 50 Km (30 miles)

Exposure Type:	**1**	Solution Gas - oil batteries
	2	Large Gas Plant
	3	Small Gas Plant
	4	Compressor Station
	5	Any combination of above

The number of veterinary clinics involved was maximized to ensure broad geographic coverage. This practice also ensured that individual clinics were not overloaded and, therefore, should minimize the chance of compliance problems with study designs. Having a large number of participating clinics should also decrease the potential for a substantial "local veterinary clinic effect" in the final effect estimation.

The final herd selection was made by the university employed project veterinarians within each of their operating zones based on estimated exposure rankings. Each of these project veterinarians visited and evaluated both exposed and unexposed herds. More exposed herds than unexposed or lower exposure herds were selected initially because some of the flares and other emission sources identified during the planning process could be phased out or shut-in during the course of the project. Some of the herds initially considered as unexposed herds had new wells drilled on their land prior to the start of the study design and are, therefore, now considered as recently exposed herds.

Final selections were made to maximize, wherever possible, the differences in exposure across study herds within geographic regions and by selecting participants most likely to comply with the study design.

All of the most highly exposed herds that met all the study criteria and wished to participate in the study were included. Some of these herds have reported previous herd health problems they associated with exposure to the oil and gas industry.

Producer interest in the study was high. Given this high level of interest in conjunction with the parameters of the study design, it was necessary at times to exclude some interested producers even though they may have met many of the selection criteria. For example, the location of producers relative to recognized emission sources was a key criteria for the selection of herds. A key objective of herd selection was to maximize the range of exposures included in the study to optimise the power
of the study to identify any potential exposure outcomes associations. The study design specified a distribution of herds across the continuum of no exposure to highly exposed. Some herds which were > 5 miles from an emission source but within 30 miles from an emission source were included in the study design as low exposure herds i.e. herds which have no small facilities within at least 2 -3 miles and no large gas processing within at least 15 miles. A greater number of producers with moderate to low potential levels of exposure expressed interest than could be accommodated in the study design.

A second criteria for herd selection was size of herd. The costs of monitoring very large herds (some with substantially greater than 250 head with extensive grazing areas) compared with the potential benefits of an increase in study power, would be significant. Data quality from these very large herds would also be substantially more difficult to assess. In order to justify the additional costs and data management challenges, producers with greater than 250 head needed to demonstrate exceptionally good records and a strong interest in participating. Only a few producers with a herd size in excess of 250 head were selected. These herd owners will be compensated to a maximum of 250 head for their participation in the study. Also, producers from some of the larger operations had both purebred and commercial herds within a single unit. The producer and project veterinarian in many of these cases decided to include only the purebred herd because the cows were tattooed, calving history was available and they were often kept as a separate management group.

Drought conditions in southeast Alberta also became a minor factor in the selection of participating herds. Producers in areas affected by drought conditions were uncertain of the number of cattle they would be able to pasture in 2001 because of lack of grass and water. Given the relatively high cattle prices at the time of herd selection, some producers indicated they were uncertain as to the number of cattle that they would maintain throughout the duration of the study. In some cases, producers suggested they were considering selling out of the business while cattle prices are high.

The power of the study to find all potential associations between exposure and outcome was further optimised by minimizing the number of management and environmental risk factors that must be corrected for in the final analysis. For example, herds located such that they would have exposure to H_2S and SO_2 emissions from significant sources other than oil and gas field facilities such as swine barns or pulp and paper operations, were excluded from the study.

Similarly, producers who calved during the fall were excluded because the different management factors influencing fall calving herds would decrease the efficiency of the analysis. Complete calving records were also necessary for participating herds. Producers who had inadequate calving records i.e. had calving records for the first part of the calving season but no records for the last calves born were also excluded, given the study's requirement for complete records in order to ensure the integrity of the data.

A few additional herds were excluded because there was no veterinary clinic within a 2 to 3 hour driving radius of their farm. This lack of access to veterinary services would make pregnancy testing, bull evaluations, and timely necropsy exams very difficult.

The final study sites were chosen to achieve adequate geographic coverage and optimum climactic and herd size comparability between exposed and non-exposed herds. The power of the study to find an association, if one is present, was maximized by working to identify the highest exposed herds across western Canada meeting the study criteria and comparing them to herds with no or very low exposure.

Withdrawal of participants from the study prior to completion is expected to be relatively low, because of the criteria used to select herds, as well as the relatively short duration of the data collection phase. The study team has had previous experience in maintaining long-term (greater than 5 years) compliance with field data collection designs in beef herds.

Producers will receive compensation for submitting required herd records:

- The study will pay the cost of semen evaluating all herd bulls. Local veterinarians were asked to invoice the study directly.
- The study will pay all cost associated with post-mortem examinations. Local veterinarians were asked to invoice the study directly.
- The study will cover the cost of pregnancy checking individual cows (or very small groups) culled prior to herd pregnancy checking where necessary.
- The study will provide metal ear tags for calves born in 2002 and as supplemental identification for cows throughout the study
- The study will pay the producer $15.00 per cow each year for his completed individual cow records. No payments will be made until the records are received.
    - $15.00 after all records are received in the fall of 2001

  - $ 7.50 after calving records are received in the spring of 2002
  - $ 7.50 after treatment, disposal and pregnancy checking records are received in the fall of 2002
- An additional $ 10.00 will be paid for each blood sample collected.
- An incentive of $50.00 will be paid to producers for delivering calves (within 24 hours of finding the calf) to their local veterinary clinic or provincial veterinary diagnostic laboratory for post-mortem examination. The producer must identify the calf so that it can be traced back to its dam.

***Confidentiality Agreements*:**

A confidentiality agreement will be signed by the project veterinarians, graduate students, and technical staff with any access to herd data. The identity of study participants will not be released by study personnel to anyone not directly involved in the day-to-day work of the study, including the sponsoring agency and associated committees.

Exposure Measurement

The details of the passive air sampling program is described in the Exposure Monitoring Study design as provided by RWDI West Inc. **(Appendix C, Tab 4).**

Exposure Monitoring:
The passive air monitoring network for the cattle study was established before the start of the breeding season in each herd. SO_2 and VOCs (volatile organic carbons) monitors were placed on each pasture used by the cattle beginning in April 2001 and H_2S monitors were placed beginning August 2001.

There is only one passive sampler validated for SO_2 monitoring (Maxxam Passive Ambient Sampling System - detection limit 0.01 ppb). This monitor is currently being used in other areas of the province and has been lab and field evaluated under extreme weather conditions. The Alberta Research Council has also examined a passive sampler for VOCs (3M Model 3500 Organic Vapor Monitors). Additional information on the samplers is available from RWDI West Inc.

A passive air monitoring system was established for all study herds, with cumulative air measurements for selected marker compounds recorded for each month of the study period, for each individual pasture used by cattle on the project. Passive air monitors will measure sulphur dioxide (SO_2), hydrogen sulphide (H_2S), and volatile organic carbon compounds (VOCs) as surrogates for total exposure. Field staff have received intensive training to ensure the consistency with which the air monitors will be located with respect to monitor height, active emission sources and roadways and other potential non-oil and gas related sources of emissions. The placement of these monitors will be verified by the project veterinarians and RWDI West Inc. management.

The data will be entered into a series of Microsoft Access database forms and tables designed to summarize and integrate the data from different sources. All study personnel have received formal training on Microsoft Access and will receive ongoing training and support on the database. All herd data will be entered under the supervision of the university employed project veterinarians. Regular backups are scheduled and copies of all electronic and hard copy data are held by study veterinarians, the information services manager, and the central study office.

The study manager will review all data collection procedures with study personnel before each new section of the study design is initiated. Regular group meetings are held to allow open discussion of all database procedures and questions. All questions or comments regarding the database are routinely copied to all study veterinarians to further maximize consistency in data entry procedures.

Participating herd owners will continue to use their regular herd veterinarian for necropsies, bull evaluations, and pregnancy examinations to minimize any potential for any perceived bias by study personnel. All pregnancy testing must be done by a licensed veterinarian. Cooperating herd owners will be paid a token amount for each cow that is pregnancy checked in the fall of 2001 and 2002 to compensate them for the time involved with data collection and record keeping for the project. The study manager will make payments to each herd owner following receipt, review and entry of herd records by the project veterinarian for each herd. The project veterinarians will verify that all required data have been received.

Initial Herd Visit and Body Condition Scoring:
The project veterinarians began on-farm data collection for all identified project herds, early in 2001, to establish an initial herd inventory. The period from January 1, 2001 through the start of breeding season provided an opportunity for the herd owners to become familiar with the study design. The project veterinarians had some time to become familiar with the management of each herd, and to find practical solutions for any herd specific challenges to collecting complete and accurate data.

The project veterinarian visited many of the herds prior to calving season 2001. They recorded all pastures to be used by the herd during the study on a series of maps to aid in the placement of exposure monitors. A complete list was made of all cows due to calve. Cow breed and age (year of birth) actual or estimated was recorded. Cows were body condition scored by palpation and/or visual inspection using a 1 to 9 system. The project veterinarians contacted all selected herds during calving season to document calving management and nutrition, and to answer any questions about the study design. Immediately prior to the start of breeding season, the project veterinarian visited the herd to verify the herd inventory, and body condition score of all breeding females and the herd bulls. Formal exposure assessment began with the beginning of breeding season (either April 1, 2001 or May 1, 2001 depending on the start of breeding in each herd).

Bull Breeding Soundness Evaluation:
Prior to breeding season, herd bulls were evaluated for breeding soundness, by a veterinarian chosen by the herd owner, using the criteria established by the Western Canadian Association of Bovine Practitioners. A written record of the bull evaluations for each herd will be collected by the project veterinarian. Only those evaluations for bulls destined to become herd sires during the summer of 2001 were paid for by the project. The breeds of bulls used in each herd were to be recorded on the forms completed during the breeding soundness evaluation.

The project veterinarian will also obtain the following information during the breeding season:

- a record of all cows and heifers exposed to a bull during the breeding season;
- the legal land location and size of each breeding pasture;
- the identity of the bull or bulls used on each pasture;
- the identity of the cows or heifers on each pasture;
- the date the cows were moved onto each pasture;
- the date bulls were put out onto the pasture; and,
- the date bulls were pulled from the pasture.

The project veterinarian will contact each herd owner approximately once per month during the study to confirm the location of the cattle and record any changes in location or movement of animals between management groups.

Breeding and Pregnancy Checks:
Background information will be collected for each herd concerning management factors known or suspected to affect risk of non-pregnancy, including a record of vaccinations used and dates administered. The types of ration fed and/or pasture conditions during the six weeks prior to breeding, and in the first nine weeks of the breeding season, will also be recorded. The project veterinarians will visit the breeding pastures during the early part of the breeding season, to verify that exposure monitors are in place, confirm locations of oil and gas facilities, record pasture conditions (see documentation for pasture scoring procedure), and observe bull activity, where possible.

The condition of each pasture will also be assessed for each month of the summer pasture season during 2001 by the air monitoring technicians during their monthly visits to service the air monitors. This pasture monitoring assessment will follow a standard design. Grass clippings were collected from 60 project pastures during the summer of 2001 to assess the validity of the system with respect to both pasture quality and quantity. Intra- and inter-observer reliability will also be assessed.

The pregnancy status of individual cows will be determined by rectal palpation by the herd veterinarian in the fall of each year. Pregnancy testing must be at least 90 days after first bull exposure or 40 days after last bull contact to be considered an accurate assessment of fall pregnancy status.

- *Risk of non-pregnancy (%) will be determined as the number of females found non-pregnant divided by the number of females examined for pregnancy in the fall of the year (x 100).*

Pregnancy checking is usually done between September and November, when the cows are between two and six months of gestation. Thus, there is some risk of undetected fetal loss occurring between conception and the time of pregnancy checking. Earlier pregnancy checking is not usually practical, because of the difficulty in accessing cattle during the summer pasture period. Individual cows or small groups culled prior to the herd pregnancy check will also be pregnancy tested wherever possible.

The herd owner will be asked to have the cull cows pregnancy checked before sale and the study will cover the cost, if necessary. Data collection forms were provided to local veterinary clinics to facilitate data collection from early pregnancy tests and increase the probability that all cows and heifers will have complete body condition scoring and pregnancy status records.

The herd owner will be required to maintain a record of all herd treatments including:

- vaccinations;
- internal/external parasite control; and,
- vitamin/mineral injections.

The project veterinarian will regularly contact the herd owner to verify these records and update them, if necessary.

Disposal Records:
The identification, date of removal from inventory, and reason will be recorded for all cows lost from the herd because of:

- culling;
- sale of breeding stock;
- slaughter; or,
- death.

Regular phone contacts with the herd owner by the project veterinarian will also be used to ensure these records are kept current and complete.

Abortions:
An abortion is defined as:

- an observed premature calving, judged to be at least one month prior to full term; and/or,
- a cow that was found to be pregnant by a veterinarian but which subsequently failed to calve.

All herd owners and local veterinarians will be provided with information for estimating fetal age. (There is a guide in the back of each calving book).

- *The abortion risk will be defined as the number of females aborting expressed as a percentage of the number of pregnant females retained after fall pregnancy testing for spring calving*.

Herd owners will be asked to record the cow identification and date (in the calving book) for every animal known to have or suspected to have aborted. The herd owner will also be asked to make every effort to locate the fetus and placenta, and to have the herd veterinarian examine the fetus and placenta. The fetus will be tagged for identification and if only placenta is recovered, it will receive a tag so its identity can be tracked. Tissue samples will be collected and submitted to Prairie Diagnostic Services following the necropsy design for fetuses. Cows suspected to have aborted will be pregnancy tested by either the local or project veterinarian before culling wherever practical.

The date, identification, breed, and source of any new animal introductions to the herd should be recorded using herd inventory forms. The herd inventory will be verified and the cows will be body condition scored before calving by the project veterinarian.

Calving, Calf Mortality and Disease Status:
The project veterinarian will also visit the herd during calving to collect data on management and risk factors for neonatal disease. The size of the calving and nursery areas will be recorded. Individual herd protocols for routine calf processing, dystocia management, and movement of cows and cow-calf pairs between pens will be recorded.

Detailed calving records at the individual animal level will be maintained by the herd owner. All calving events are to be recorded in the calving record book provided by the study. The date (as accurately as possible) and circumstance of all neonatal losses should be recorded. All losses regardless of circumstance are to be identified with an ear tag and necropsied as soon as possible.

Calving-to-calving Interval:
The calving-to-calving interval is defined as:

- The number of days from calving in 2001 until the subsequent calving in 2002.

Stillbirths:
A stillbirth is defined as:

- a calf dead at or within one hour of birth.

Stillbirth risk is defined as the number of calves dead at or within one hour of birth as a proportion of the number of calves born (dead or alive) during the period that appeared to be within one month of full-term gestation. All stillborn calves are to be tagged and recorded in the calving book so they can be tracked through the diagnostic laboratory.

Early Calf Mortality:
An early calf mortality is defined as:

- a calf dead between one hour of birth and 28 days.

Early calf mortality risk is the number of calves dead from one hour of age to 28 days, as a percentage of the number of calves alive at one hour of age.

Later Calf Mortality:
A late calf mortality is defined as:

- a calf dead between 28 days of birth and weaning.

Later calf mortality risk is the number of calves dead from 28 days of age to weaning, as a percentage of the number of calves alive at one hour of age.

Spring Herd Processing Design:
The herd owner will be asked to record all routine herd treatments prior to breeding season including:

- vaccinations, for both cows and calves,
- parasite treatments, including fly tags,
- and, vitamin/mineral injections.

The Project Veterinarian will body condition score the breeding herd (cows and replacement heifers) before first bull contact. Producers with larger herds often have a round-up in May or June to brand, castrate and vaccinate the calves. All herd treatments will be recorded as they occur.

Summer Pasture Season:
The producer will maintain records of the date on which cattle are turned into a pasture, the location of the pasture, the date when the cattle were removed from the pasture, and the identity and number of cattle on the pasture. Animal location information will be verified by monthly telephone interviews during the summer pasture season and checked by herd visit, if necessary. Calf inventory will be verified prior to sale, where possible, in the fall of 2001 and 2002.

Treatment Records:
Herd owners will maintain records of the diagnosis and treatment of all sick animals. The treatment record book will be provided by the study. A list of common conditions and case-definitions will be provided to participating producers and local veterinarians. Any perceived herd-level problem should be reported by the herd owner or local veterinarian as soon as possible to the project veterinarian to permit necessary documentation and investigation. The decision on what defines a herd problem will be made by project personnel together with the local herd veterinarian. Documentation of any disease outbreaks would be part of the descriptive function of this study. Questions about recent treatments and disease problems by the project veterinarian during monthly telephone updates should increase the completeness of the treatment records.

Post-mortem Examinations:

Background

Necropsies are required to describe the physical condition of the animal, ascertain the probable immediate cause of death (where possible), and collect tissue samples. A complete histopathological assessment is also very important for all necropsies. Some previous anecdotal reports have suggested lesions in:

- the trachea and lungs (tracheitis, and pneumonia, proliferation of submucosal glands, discharged goblet cells, squamous metaplasia of the tracheal mucosal, submucosal perivascular lymphoid infiltration, and severe intraalveolar edema);
- lymphoid tissue (clinical reports of poor treatment response and histopathological reports of lymphoid hypoplasia of the thymus, spleen, and lymph nodes); and,
- the nervous system (clinical descriptions of proprioceptive difficulties, unusual behavior in mother cows and calves, and histopathological reports of axonal swelling and demyelination in one or more animals).

We will collect tissues necessary to evaluate the prevalence of the major histological lesions in this study population and to complete the gross post-mortem diagnosis.

All local veterinarians will be provided with training materials describing the collection and preparation of tissues for submission using a standard format during the spring and summer of 2001. This should improve the consistency and quality of submissions. The university employed project veterinarians will be making frequent contacts with the local clinics to ensure study designs are being followed, that forms are being filled out in a timely fashion and there are no concerns about sample submissions. These project veterinarians are also available to do post-mortem examinations if necessary.

The pathologist examining the tissues will be blind to the location from which the samples were collected. (He will have only the sample number and will not know if the samples come from a potentially exposed area.) The pathologist will record his findings using a standard form and will be evaluating the tissues for the presence or absence of a list of common histological lesions suggested by previous laboratory work and field observations. The form will consist of a series of tick boxes outlining the potential lesion descriptions for each tissue examined.

- Infectious agent present
- Anomalous
- Necrosis
- Inflammation
- Degeneration
- Proliferation
- Neoplasia
- Autolysis

The risk of occurrence of these lesions will be compared across exposure levels. The major morphological diagnosis will also be summarized for descriptive purposes.

Immunohistochemistry is necessary to address the potential contribution of infectious disease in lesions that may be observed during post-mortem examinations and histopathology. For example, the potential role of BVDV must be examined in cases where immune compromise is suspected.

We have requested immunohistochemistry for BVDV on all submitted samples from calves less than 6 months of age. Immunohistochemistry will also be available to the pathologist as needed to confirm etiological diagnosis where required.

Procedures

Gross Post-mortem:

Gross post-mortem examinations will be done by the herd owner's local veterinarian. If the local veterinarian is unavailable to do the necropsy, the producer will call the project veterinarian to make alternate arrangements for the post-mortem examination. Alternate arrangements will include delivering the animal to a local diagnostic laboratory (where available) or the project veterinarian conducting the post-mortem examination. A standard training manual and necropsy video have been provided for all local veterinarians to encourage consistency of gross post-mortem examinations, sample collection procedures, and reporting. The project pathologist will review the tissue collection process with project personnel. University employed project veterinarians will then review sample collection techniques with the participating private, local veterinarians.

Prairie Diagnostic Services (PDS) Submissions:

Only fixed samples will be sent to Prairie Diagnostic Services. Fixed samples are to be no more than one cm thick. All histological samples for the study will be examined by the same board certified veterinary pathologist within PDS. Findings for standard tissues will be recorded on a series of tissue specific data collection forms.

Post-mortem Protocol for Abortions:

A standard set of tissues are to be submitted for histopathological diagnosis for abortions. *Only formalin fixed tissues are to be submitted (with one exception). (The head of the femur should be collected and submitted frozen to PDS for all fetuses and calves less than 3 weeks of age.)* The study will supply the shipping kits for submitting tissue samples.

The tissue sampling design for fetal tissue submissions:

- Lung
- Liver
- Kidney
- Spleen
- Heart (3-4 sections)
- Thymus
- Thyroid
- Bronchial and mesenteric lymph nodes
- Ileum (Peyer's patches)
- Skeletal muscle
- Eyelid
- Brain
- Placenta
- Femur
- Any other tissues considered appropriate based on the gross pathology examination.

Post-mortem submission forms are to be completely filled out for each case. The history should include cow age, estimation of fetal age, date of abortion, duration of illness (if noted), condition of fetus, major morphological diagnosis, and any relevant gross post-mortem findings.

Post-mortem Protocol for Routine Post-mortems:

A slightly expanded set of tissues are to be submitted for routine histopathological diagnosis. *Only formalin fixed tissues are to be submitted.* The study will supply the kits for submitting tissue samples.

The tissue sampling design for routine post-mortems:

- Trachea
- Lung
- Liver
- Kidney
- Spleen
- Heart (3-4 sections)
- Bronchial and mesenteric lymph nodes
- Skin
- Duodenum
- Jejunum
- Ileum (Peyer's patches)
- Spiral colon
- Skeletal muscle
- Brain
- Spinal cord
- Sciatic nerve
- Endometrium or testes
- Any other tissues considered appropriate based on the gross pathology examination.

A report of the gross post-mortem examination should be completed within 48 hours of the examination, and include history, duration of illness, treatments administered, date of death, animal identification, carcass condition, major morphological diagnosis, estimated or reported age, sex, and estimated weight.

Blinding Procedure:
The pathologist will be blind to the exposure status of the submission. Each veterinarian will be provided with a series of postmortem kits. An identification label with a code number matching pre-labeled postmortem forms will be attached to a one liter jar containing 10% buffered formalin. The tissue samples will be submitted to the central study office by overnight courier. The producer's name, location, and veterinary clinic identification will be covered and the form photocopied to produce a "blinded copy" for submission to PDS. Only the form code number will identify the tissue samples. Laboratory findings will be recorded onto a database and sent to the studies central office, the code will be broken and the results faxed to the submitting veterinary clinic. Once the laboratory results have been finalized on the PDS database they can not be altered.

Infectious Disease Screening 2001:
Blood samples will be collected from 20 herds reporting a pregnancy rate greater than 90% and from all herds where the pregnancy rate is less than 90%. In these herds, all the open cows, and enough pregnant cows will be sampled until 40 samples have been drawn. These samples will be analysed for antibodies to:

- *Neospora caninum;*
- Infectious bovine rhinotracheitis virus (IBR);
- Bovine viral diarrhea virus (BVD); and,
- *Mycobacterium avium* subspecies *paratuberculosis*

Random sampling of herds with normal pregnancy rates will be used as a base line for antibodies to the listed diseases. Samples from herds with lower than expected pregnancy rates will be collected in an attempt to examine the contribution of common infectious diseases to reduced herd fertility, increased culling, and decreased productivity. Serum will be analysed at Prairie Diagnostic Services using commercially available ELISA tests. Project veterinarians will collect the blood samples while the cows are being worked through the chute. The serum will be drawn off the clotted sample and submitted with ice packs. Submissions are to be identified by the producer's code number and the samples are to be numbered sequentially. The blood sampling form will be used to record the cow identity and pregnancy status. A copy of the blood sample collection forms are to be sent to the studies central office, laboratory results will also be sent to the central office.

As a subset to the larger study, the association between exposure and pregnancy status of cows from which blood samples were collected will be evaluated after examining the role of serological status for each infectious agent in the analysis. Any potential interactions between serological status and exposure with the occurrence of non-pregnancy will also be examined.

Infectious Disease Screening 2002:
Blood samples will be collected from the calves that are part of the immunological study. These samples will be analysed for antibodies to:

- *Neospora caninum*

- Infectious bovine rhinotracheitis virus (IBR)
- Bovine viral diarrhea virus (BVDV)
- The samples will also be tested for BVD virus.

The object of collecting these blood samples is to examine the recent exposure history of the herd. These calves will be approximately 5 to 8 months old when the blood samples are collected. With this information we can determine if target diseases of interest have been recently active in the herd. High antibody levels should indicate either prenatal infection after attainment of immune competence or postnatal infection. (This group of animals would not likely have yet been vaccinated for the diseases of interest and should no longer have colostral antibodies at this age. Herd vaccination records will be checked to verify vaccination status.)

If the disease has recently been active in the herd, there could potentially be an interaction between titres for diseases, neonatal mortality and exposure status. For example, a very high incidence of BVDV titres in 6-month old heifers would suggest the recent presence of a persistently infected animal in the herd. Alternately, we might identify several persistently infected animals during this process. The presence of active BVDV in the herd must be examined in relation to abortion and neonatal losses during the previous calving season. Any obvious sources of persistently infected calves must be evaluated for each herd including the potential role of recently purchased animals. These diseases are capable of causing substantial reproductive and productivity losses in the absence of exposure and must be considered as potential contributors to the herd losses. The data from the infectious disease screening design will be used to describe the occurrence and pattern of infectious disease in these herds.

The potential for interaction with exposure can also be evaluated if immunological function assays suggest that there is evidence of biologically important compromise of the immune system in exposed animals. Results from studies on the immune system are necessary to determine how to evaluate the potential for interaction between exposure and infectious disease in the analysis.

If there is no effect of exposure on the immune system and we do not correct for the effect of infectious disease on reproductive efficiency, then the data could suggest an apparent exposure effect where it does not exist. For example, there could be more BVD in the exposed herds because of regional variations in biosecurity and vaccination practices. If there is an important effect of exposure on the immune system then we should not adjust for the presence of infectious disease in the herd using traditional regression approaches. Exposed herds would perhaps be more likely to have BVDV and have more severe immunocompromise in the face of a BVDV infection than unexposed herds. More complex modeling techniques that would consider infectious disease as potentially a confounder, effect modified, and an intermediate variable would be considered (Robins JM, 1989 and others).

Other Environmental and Meteorological Data

Daily temperature data will be obtained for January 1, 2001 through October 31, 2002, from the nearest meteorological station to each of the study herds. Wind-speed, direction, and precipitation data will also be obtained where available. A map of agro-ecological zones will be constructed from information obtained from provincial environment departments. Other vegetation and soil type data will also be integrated if the information is available.

Strategies for Minimizing Bias in Herd Selection, Data Collection and Analysis:

Work to date on herd documentation suggests that the herd selection strategy should have provided an adequate number of "highly" exposed herds, "moderately" exposed herds, and herds with no identifiable exposure to oil and gas facility emissions. This assumption will be examined by reviewing the first six months of air quality data collected from the passive air monitors placed in study herds and data from facility maps provided by the provincial regulatory authorities. The active recruitment of the most highly exposed herds has been emphasized in order to maximize the range of exposures examined and increase the power of this study to detect any potential association with productivity.

The potential for herd selection bias during recruiting can be examined by looking at the differences between selected and non-selected herds and herd owners. Some descriptive data were collected from all herd owners that were interviewed during the recruitment process. These data can be compared between those herd owners that are enrolled and those that were not be included in the study due to lack of interest or failure to meet study criteria. Using this comparison, we can estimate the potential for selection bias.

Standard operating procedures and quality assurance checks will be developed for all data collection procedures to minimize bias due to misclassification. The potential for information bias will be minimized with objective outcomes that can be independently verified by on-farm observations from both the participating local veterinarians and project veterinarians. For example, herd inventories can be cross checked by the project veterinarian during body condition scoring when the cattle are handled prior to calving, before breeding, and at pregnancy testing. Necropsy of all recoverable deaths by local veterinarians will verify the occurrence and timing of herd losses. Assessment of more subjective outcome measures, such as the histopathological description and interpretation, will be made blind to the location of the herd and exposure status. Misclassification during exposure analysis will be minimized by the use of a comprehensive network of passive air monitors in addition to data on facility proximity. Records of individual animal location will minimize errors in exposure classification due to variation within herd.

The collection of herd management information and data on other known risk factors for productivity loss will permit adjustment for potential confounders during data analysis at both the individual animal and herd level. Variation in vegetation types and meteorological conditions across

the study area should also be considered as a potential confounder. Meteorological data will be collected and used in the analysis of calf mortality. Both exposed herds and unexposed herds will be selected where possible in the different agro-ecological zones from both the southern and northern parts of the study area. Additional pasture quality measurements during the summer grazing season will provide information to adjust for differences across study regions.

Statistical Analysis

All data will be collected and stored until final publication on a hard copy record system. Most data will be held in duplicate (one copy at the central study office and one copy with the field veterinarian). Regular computer backups will be scheduled and reviewed by a dedicated technician.

A GIS program (ArcView) will be used to link exposure information (air monitor location, battery and processing facility location, flaring and venting data), agro-ecological zone, and meteorological data to individual herd records and animal location information. Individual herd records will be maintained on a commercial software packages (Microsoft Access 2000, Microsoft Corporation) for summary and reporting purposes. Each herd owner will be provided with the opportunity to review computer printouts of the data summary and analysis of their herd records for accuracy and completeness. The field veterinarian will correct any errors he or she identifies during this review.

Appropriate descriptive statistics and graphical representations of the data will be generated to both aid in checking for data entry errors and guide appropriate statistical analysis. All prevalence estimates will be reported with appropriate 95% confidence limits adjusted for clustering at the herd level.

The primary hypotheses to be tested will be whether cows and heifers that have higher cumulative exposures to SO_2, H_2S, the seven marker VOCs, or proximity to field facilities (less than 1 mile) and flaring will have an increased risk of nonpregnancy, abortion, stillbirth, neonatal calf losses, or increased calving-to-calving interval.

Methods for statistical modelling will be developed together with a biostatician. It is likely that mixed effects models will be used for continuous outcomes and random effects logistic regression for binary and proportion data. The methods chosen must be able to examine individual animal risk while being able to account for variable levels of clustering within the herd and management group. Modelling strategies including procedures for dealing with confounding, potential intermediate variables, interaction, and missing data will be outlined in detail in the standard operating procedures (SOPs).

Data reporting will include effect estimates and appropriate 95% confidence intervals. Strategies for model checking will also be outlined in the SOPs.

Final Reporting

Final assessment and analysis of the data should be complete by late 2003 with a report available for peer review during the first quarter of 2004. Only summary data and analyses will be released by study personnel. All identified, individual herd data is strictly confidential and will be accessible only to study personnel and the herd owner to which the data apply. All final computer records will contain only a code number to identify the herd. The names of participating herd owners and veterinarians will not be released by study personnel.

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Appendix A

Assessment of Immune Function in Beef Cattle

A Component of the Western Canada Study on Animal and Human Health Effects Associated with Exposure to Emissions from Oil and Natural Gas Field Facilities

Study Design

November 2001

Toxicology Centre and Department of Veterinary Biomedical Sciences
University of Saskatchewan
Saskatoon, SK, S7N 5B3

ASSESSMENT OF IMMUNE FUNCTION IN BEEF CATTLE
STUDY DESIGN – November 2001

Background

There are numerous anecdotal reports from western Canada that implicate proximity to oil and gas facilities with increased incidence of various infectious diseases in livestock. Most commonly reported are increased occurrences of respiratory disease, increased incidence of sick calves with poor response to treatment, and elevated death losses in neonatal calves associated with increased virulence of opportunistic bacteria. These reports have led to speculation that chronic exposure to emissions from oil and gas facilities may have an immunosuppressive effect, but no data are available to address these concerns. Several classes of chemical contaminants reported in natural gas flare emissions (e.g., halogenated and non-halogenated aromatic hydrocarbons, various heavy metals, and SO_2) are known immunotoxicants in laboratory animals. However, the effect of these compounds, when presented as a complex mixture, on the immune system of beef cattle is unknown. As a result of this concern and the lack of data to answer it, a study of immune function in beef cattle was added to the investigation of the affects of oil and gas industry activities in western Canada on livestock health and productivity.

Objectives

The overall objective of this study, which is nested within the Western Canada Beef Cattle Productivity Study, is to determine whether exposure to emissions from oil and gas batteries and other field facility sites has an adverse effect on immune function in beef cattle.

Outcome Variables

The study will focus on assessing whether increasing levels of chronic exposure to oil and gas facilities adversely affects the immune system of individual animals as determined by changes in five important immune endpoints:

1. Humoral immune system response to vaccination
2. Cell-mediated immune system response to antigenic challenge
3. Peripheral lymphocyte subpopulation enumeration
4. Differential and total circulating white blood cell count
5. Histopathologic examination of immune system tissues

The project employs one full time technician at the Toxicology Centre and WCVM with expertise in veterinary immunology.

ASSESSMENT OF IMMUNE FUNCTION IN BEEF CATTLE
STUDY DESIGN – November 2001

Herd Selection

A subset of 40 herds will be identified from the total group of about 200 herds currently involved in the larger beef cattle productivity study. This subset will include about 15 herds with high, continuous exposure potential, 15 herds classified as very low/no exposure, and about 10 herds with intermediate exposure. Eligible herds (as determined by selection criteria outlined below) will be categorized as high, intermediate, or very low/no exposure based on a scoring system derived from information collected from passive monitors, and by considering the presence of field facilities within a 1-mile radius of the pastures used by cattle during the study period. These data will be used to place eligible herds within exposure quartiles for air concentrations of SO_2, benzene, and hexane, and for proximity to active flares. Herds will be assigned a score based on their exposure quartile for each of these contaminants, and for flare proximity, with a score of 4 assigned to the highest quartile, and 1 for the lowest quartile. Herds with a total score of 4-8 will be classified as having low exposure, while herds with a total score of 12-16 will be classified as high exposure.

Criteria used to select the subset of herds for participation in this study include: 1) a commitment by the producer to retain an adequate number of calves for up to three months after weaning in the fall of 2002 (30-40 calves from each herd will be used in the immune system assessments); 2) availability of adequate animal handling facilities in close proximity to the test animals; 3) no previous history of herd vaccination against rabies (the commercial vaccine used to assess humoral immunity); and 4) a high level of producer interest and commitment to the project. The latter criterion is necessary because this study requires additional intervention (in the form of cattle handling and environmental sample collection) beyond that required in the cattle health and productivity study. In addition, we are attempting to select herds from similar ecological zones, to minimise potential variability introduced by differences in soil type (and thus trace mineral nutrition status) or climatic regimes.

Immune Endpoints Selected

Humoral Immune Response
The humoral antibody response will be assessed by vaccinating calves with a killed, commercial rabies vaccine (IMRAB Bovine Plus®, Merial Canada). This product was selected because background titres
to the rabies virus antigen will be very low in calves, enabling detection of a vaccine-specific response. The systemic antibody response to vaccination will be measured using a standardized ELISA.

Lymphocyte Phenotyping
Identification and enumeration of important subpopulations of circulating lymphocytes (phenotyping) is based on flow cytometric analysis of cell-type specific surface markers using monoclonal antibodies against bovine lymphocyte surface marker antigens, and fluorochrome-conjugated secondary antibodies (Howard and Morrison, 1994; Davis et al., 1995). Immunologically important lymphocyte subpopulations to be identified in this study include $CD4^+$ and $CD8^+$ T-lymphocytes (T-helper cells and cytotoxic T-cells, respectively) (Goddeeris, 1998), B-lymphocytes (antibody-producing cells), and Natural Killer cells and/or ($*^+$ T-lymphocytes (important in respiratory tract immunity in cattle) (Davis et al., 1996; Cohen et al., 2001). Quantification of systemic antibody response and (especially) lymphocyte subpopulation enumeration have the highest individual predictive values for immunosuppression of commonly used immune function assays (Luster et al., 1992, 1993).

Cell-Mediated Immune Response
Cell-mediated immune response will be assessed using an *in vivo* delayed type hypersensitivity reaction (DTH) to the antigen keyhole limpet hemocyanin (KLH). This is a dermal reaction, equivalent to the tuberculin skin test, and has been previously used successfully to study immune competence in cattle (Pollock et al., 1991; MacKenzie et al., 1997; Lonneux et al., 1998).

Differential WBC and Immunopathology
Both differential and complete white blood cell counts and immunopathology are commonly employed in Tier I (screening) panels for detecting immunosuppression following chemical exposure. They are usually considered to be less sensitive endpoints compared with the three tests described above (Dean et al., 1998). However, in the context of the present study, they are relatively easy to accomplish, and will contribute to our total assessment of immune function in exposed cattle. See Western Canada Beef Cattle Productivity Study – Study Design **(Tab 1)** for details of immunopathology assessments.

Protocol

Pilot Studies
Small-scale pilot studies are currently underway at the Western College of Veterinary Medicine's livestock research facilities. These studies involve up to 20 heifers. The objectives of these preliminary studies include: 1) to provide information to optimise dose and vaccination protocols,

and confirm reproducibility of measurement techniques for the KLH DTH assay; 2) to establish an optimal panel of leukocyte markers, and identify the best commercially available combinations and dilutions of primary and secondary antibody reagents; and 3) to optimise sample handling and processing procedures for the lymphocyte phenotyping assay under field conditions.

Field Studies

The field component of this project will be conducted around weaning time in 2002. Because we are only able to include a maximum of 40 herds in this study, it is essential that we select herds with the appropriate (i.e., maximum possible) range of contaminant exposures. Several months of passive monitor data are required to make these determinations. As a result, no data can be collected from the 2001 calf crop. In addition, immune function information will be most valuable if coupled with exposure data that include mean monthly concentrations of important PAHs and metals, as well as $PM_{1.0}$ values, since these contaminants are potentially immunotoxic. Particulate samplers, which will provide these exposure data, are being installed late in 2001, so conducting field sampling in the fall of 2002 (rather than the fall of 2001) will enable us to make full use of this exposure information.

Calves

The principal population sampled to assess immune function will be the calf crop born in the spring of 2002. We will have complete exposure histories for these animals, including exposure of the dam during gestation, and complete information on any health problems, treatments, or vaccinations.

At or just prior to weaning in the fall of 2002, blood samples will be collected from 30-40 calves in each herd. These initial samples will be used to determine differential and complete white blood cell counts (as well as total plasma protein and hematocrit), for lymphocyte subpopulation enumeration, to determine baseline (pre-vaccination) antibody titres to the rabies virus antigen, and to assess trace mineral nutrition status. In addition to blood sampling, the calves will be vaccinated with the IMRAB Bovine Plus® rabies vaccine, and receive the initial exposure (sensitisation) to the KLH antigen (both by subcutaneous injection).

Three weeks after vaccination, the calves will be bled again, and the systemic antibody response to the vaccine quantified by ELISA (no booster is needed with this vaccine). At the same time, calves will receive an intradermal injection of KLH in the skin of the tail fold. The magnitude of the dermal response (swelling) will be measured with a digital micrometer at 48 hours after the intradermal challenge. The overall response is determined by comparison with the pre-challenge skin measurements.

Bred Heifers

In addition to the assessment of immune function in calves, a smaller number (10-15 per herd) of bred heifers born in the spring of 2001 will be bled one time during pregnancy checking in the fall of

2002. These blood samples will be used for lymphocyte phenotyping, and to determine differential and total white blood cell counts, in order to obtain some information on the immune status of older animals in the same herds.

Assessment of Soil Contamination

In addition to air monitoring, samples of topsoil (0-5 cm) will be collected during the summer of 2002 from each of the 40 sites used in the immunotoxicology study. This sampling effort will focus on summer pastures used most heavily by the calves and their dams. These samples will be analysed for a selected group of potential contaminants, especially PAHs, since they are the most likely class of major contaminants to be deposited on pastures and to persist in soil. Standard operating procedures and quality assurance/quality control protocols will be developed for the collection of these samples.

Plant uptake from soil is not expected to be a significant cattle exposure pathway for these contaminants. However, incidental ingestion of soil during grazing is common in large herbivores (Mayland et al., 1977). Therefore, results of the soil analyses will be used to characterize cattle exposure to selected contaminants of concern via the soil ingestion route.

Statistical Analysis

All data will be collected and stored until final publication on a hard copy record system. A GIS program (ArcView) will be used to link exposure information (air monitor location, battery and processing facility location, flaring and venting data), agro-ecological zone, and meteorological data to individual herd records and animal location information. Individual herd records will be maintained on a commercial software packages (Microsoft Access 2000, Microsoft Corporation) for summary and reporting purposes.

Appropriate descriptive statistics and graphical representations of the data will be generated to both aid in checking for data entry errors and guide appropriate statistical analysis. All prevalence estimates will be reported with appropriate 95% confidence limits adjusted for clustering at the herd level.

The primary hypotheses to be tested will be whether calves and heifers that have higher cumulative exposures to SO_2, H_2S, the seven marker VOCs, or proximity to field facilities (less than 1 mile) and flaring will have an increased risk of adverse effects on immune system function.

Methods for statistical modelling will be developed together with a biostatician. It is likely that mixed effects models will be used for continuous outcomes and random effects logistic regression for binary and proportion data. The methods chosen must be able to examine individual animal risk while being able to account for variable levels of clustering within the herd and management group.

Modelling strategies including procedures for dealing with confounding, potential intermediate variables, interaction, and missing data will be outlined in detail in the standard operating procedures (SOPs). Data reporting will include effect estimates and appropriate 95% confidence intervals. Strategies for model checking will also be outlined in the SOPs.

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Appendix B

Assessment of Wildlife Reproduction and Immune Function

A Component of the Western Canada Study on Animal and Human Health Effects Associated with Exposure to Emissions from Oil and Natural Gas Field Facilities

Study Design

November 2001

Toxicology Centre and Department of Veterinary Biomedical Sciences
University of Saskatchewan
Saskatoon, SK, S7N 5B3

ASSESSMENT OF WILDLIFE REPRODUCTION AND IMMUNE FUNCTION
STUDY DESIGN – November 2001

Background

The size of the environmental footprint of the oil and gas industry in western Canada is enormous, yet the potential impact of the industry and the chemical emissions associated with it on terrestrial wildlife populations is largely unknown. When a study was initiated to investigate the health, productivity, and immune competence of domestic livestock chronically exposed to emissions from oil and gas batteries and associated field facilities in the western provinces, an opportunity arose to conduct a parallel study of representative wildlife species, in order to gain some insight into the potential impact of the industry on ecosystem integrity. The wildlife study will be nested within the larger cattle health and productivity study, in order to take advantage of the robust environmental exposure data being collected.

The assessment of wildlife health as a component of the overall project is essential in order to address concerns of many stakeholder groups in western Canada. Some individuals contend that flare emissions and associated processes may be adversely impacting environmental quality in ways that may not be evident from studies of domestic animals. Cattle on fenced pastures sample their environment in a somewhat limited fashion, which does not include all possible exposure pathways of concern for human and wildlife health. Studies of wildlife populations may also increase our insight into potential toxicological mechanisms operating in cattle and humans, and may lead to the identification of specific biological indicators of flare emission exposure that could be used in future research.

Objectives

The overall objective of this study is to determine whether exposure to emissions from oil and gas batteries and other field facility sites has an adverse effect on reproductive success and immune function in a representative wild bird species.

Outcome Variables
The study will focus on assessing whether increasing levels of chronic exposure to oil and gas facilities adversely affects the reproductive performance and/or immune system function of European starlings (*Sturnus vulgaris*) as determined by changes in the following endpoints:

<u>Reproductive outcomes</u>
1. Clutch initiation date (laying date)
2. Clutch size
3. Mean egg mass
4. Mean egg volume
5. Mean nestling body weight
6. Mean nestling size
7. Hatchability (number of egg hatching/number of eggs laid)

8. Fledging success (number of nestlings alive at 20 days post hatch/number hatching)

Immune function outcomes (in nestlings)

1. Humoral immune system response to vaccination (systemic IgG production)
2. Cell-mediated immune system response to a mitogen *in vivo* (PHA test)
3. Local respiratory/mucosal immune response (mucosal IgA production)
4. Differential and total circulating white blood cell count
5. Histopathological examination of immune system tissues

Selection of Wildlife Species

European starlings have been selected as the representative species to address the question of effects of emissions from the oil and gas industry on wildlife reproduction and immune function. Starlings are an appropriate wildlife indicator species for this study because they:

- are widely distributed across the entire geographic range of the study. They are especially abundant around livestock and other agricultural facilities, and are not easily disturbed by intensive human activity - even at oil and gas sites.

- are gregarious and tolerant of conspecifics, so they will nest relatively close together. High nest box occupancy rates and larger clutch size (5-7 eggs) compared to those expected with kestrels will improve the statistical power of the study.

- are cavity nesters, so they readily choose to nest in artificial boxes. They are tolerant of nest box disturbance during most phases of incubation and rearing, allowing access to eggs and nestlings for observation and repeated sample collection.

- are early nesters, and aggressive in their selection of nest sites, such that they often out compete other cavity nesters, leading to high nest box occupancy rates.

- are omnivores, eating a wide variety of plant and insect material. They will therefore be exposed to environmental contaminants by ingestion of vegetation (and incidental soil) and insect prey, as well as through the major route of inhalation. The selection of a model species that feeds in the middle of the food chain, rather than a top-level consumer, is appropriate because the contaminants of concern are not biomagnifiable in terrestrial food chains. They do not increase in concentration to any significant degree between trophic levels (unlike, e.g., organochlorine pesticides or methyl mercury) because they are readily metabolized and excreted. Therefore, top consumers are not necessarily more highly exposed, even with regard to the more lipid soluble PAHs.

- have relatively small home ranges, so that samples obtained from nestlings prior to fledging are representative of local habitat contamination. We are not sampling adult birds, so potential effects of exposure on wintering grounds will be minimized. There is some potential for egg transfer of lipid soluble chemical residues to nestlings, however, there is no reason to assume that starlings nesting near oil and gas facilities would be any more or less likely to be affected by exposure on the wintering grounds than starlings nesting on the control sites.

- have been used successfully in previous studies of environmental contaminants (Wolfe and Kendall, 1998; Fryday et al., 1996; Fryday et al., 1995; Trust et al., 1994; Hart 1993; Meyers et al., 1992; Rattner and Grue 1990; Robinson et al., 1988; Grue et al., 1984; Grue et al., 1982).

- have been thoroughly studied with regard to their breeding biology and behaviour (Christians and Williams, 2001; Reid et al., 2000; Christians and Williams, 1999; Meijer et al., 1999; Smith et al., 1995; Ohlsson and Smith, 1994; Kallander and Karlsson, 1993; Pinxten et al., 1993; Williams and Dawson, 1987; Kessel, 1957).

- the research team has considerable experience with the use of various avian species as indicators of potential effects of environmental contaminants on wildlife. That experience is readily transferable to field and laboratory studies with starlings, and we have already begun that process with the completion of the first field season. In addition to the present investigation, we are currently using starlings as environmental monitors with emphasis on reproductive and immunological endpoints in a pesticide study in southern Ontario.

- the status of these birds as an introduced species and agricultural pest minimizes the ethical concerns associated with sacrificing some chicks in order to obtain biological samples.

- birds in general are an appropriate model for this study of (primarily) airborne contaminants because their high metabolic and respiratory rates, and distinct respiratory tract anatomy and physiology, make them typically more sensitive to inhaled toxicants than mammals.

Study Site Selection

For the first field season (April-August, 2001), a subset of 30 cattle ranches was identified from the total group of about 200 currently involved in the beef cattle productivity study. Ten additional ranches within the same geographic area will be added during the second field season, for a total of 40 study sites. The subset of ranches selected for the wildlife reproduction and immunotoxicology study is intended to include herds with the entire range of exposure potential; from high, continuous flaring scenarios to sites with background/no exposure to oil and gas facilities. These sites were

selected based on proximity to known emission sources, since no passive air monitor data were available at the beginning of the first field season.

Other criteria used to select the subset of herds for participation in this study include: 1) a commitment by the producer to allow access to the pastures used by cattle during two successive starling breeding seasons (summer, 2001 and 2002), and to permit the construction of artificial nest boxes on those pastures; 2) a high level of producer interest in the project; and 3) as much as is possible, location within geographically and ecologically homogeneous zones, in order to minimize potential effects of variable habitat type or quality, or climatic conditions, on reproductive success or measurements of immune function. All of the 30 study sites used in the first field season were located at a similar latitude in a belt across central Alberta, with a small number of control sites in central Saskatchewan (there are no zero exposure sites within our geographic areas in Alberta).

Selection of Productivity and Immune Function Endpoints

Immune Endpoints

Immune competence in starling nestlings exposed to oil and gas facilities will be assessed using endpoints which are well established in the avian immunotoxicology risk assessment literature as having high predictive value in the identification of immunotoxicants, but which are also relatively non-invasive and can be done reliably under field conditions. Outcomes selected for assessment include: 1) quantification of systemic IgG antibody response to antigenic challenge by hemagglutination assay (year 1) and ELISA (year 2); 2) quantification of local respiratory mucosal immune response by ELISA (year 2); 3) measurement of cell-mediated immune response, as evidenced by T-cell migration and dermal accumulation in response to a mitogen *in vivo* (years 1 and 2); 4) determination of differential and total circulating white blood cell counts (years 1 and 2); and 5) histopathological examination of selected immune system organs (spleen, thymus, and Bursa of Fabricius) from sacrificed nestlings (years 1 and 2).

Humoral Immune Response
The humoral antibody response will be assessed by vaccinating nestlings with either sheep red blood cells (SRBC) (first field season) or keyhole limpet hemocyanin-dinitrophenol conjugate (DNP-KLH) (second field season). The systemic IgG mediated immune response to the SRBC antigen used during the first field season will be measured using a standard hemagglutination assay (Lochmiller et al., 1993; Trust et al., 1994; Smits and Williams, 1999). Antibody response to the KLH-DNP antigen used during the second field season will be quantified by ELISA developed specifically for starling IgG (Smits and Bortolotti, 2001).

Mucosal Immune Response
The mucosal epithelial lining of the eye is anatomically continuous with the mucosal lining of the respiratory tract. As a consequence, the concentration of mucosal IgA in ocular fluid may be used as a none-invasive method to assess the immunological defence of the respiratory tract in birds (Davelaar et al., 1982; Baba et al., 1988). For example, specific ocular IgA concentrations are well correlated with resistance to respiratory viral infections in domestic fowl (Toro and Fernandez, 1994; Toro et al., 1997).

Toxicant-induced damage to the Harderian gland (a paraocular gland containing large numbers of IgA-secreting plasma cells) could be expected to negatively impact mucosal defence mechanisms. In this assay, production of ocular mucosal IgA by the Harderian gland in response to a local antigenic challenge (SRBCs instilled into the eye) will be used to assess respiratory mucosal immunity in starling nestlings. The magnitude of the mucosal immune response will be quantified by ELISA developed for starling IgA.

Cell-Mediated Immune Response
Cell-mediated immune response in starling nestlings will be assessed using an *in vivo* response to mitogenic stimulation. The lectin phytohemagglutinin (PHA) is injected subcutaneously into the wing web to produce a measurable thickening at the injection site, reflecting local T-lymphocyte proliferation and accumulation, plus a minor local innate immune response (Goto et al., 1978; Edelman et al., 1986). Skin thickness is measured with a digital micrometer, and the overall response is determined by comparison with the pre-challenge measurement. All measurements are made in triplicate, and the mean value is used in future analyses. This test is a well-recognized measure of cell-mediated immune competence that is applicable to field investigations with wild birds (Smits et al., 2000; Smits et al., 1999).

Differential WBC and Immunopathology
Both differential and complete white blood cell counts and immunopathology are commonly employed in Tier I (screening) panels for detecting immunosuppression following chemical exposure, and in field studies of environmental contamination (Llacuna et al., 1996; Trust et al., 1994). They are usually considered to be less sensitive endpoints in the identification of immunotoxicants (Dean et al., 1998). However, in the context of the present study, they are relatively easy to accomplish, and will contribute to our total assessment of immune function in exposed birds.

Productivity Endpoints

Reproductive success in breeding starlings exposed to oil and gas facilities will be assessed using endpoints which are well established in the avian ecology and breeding biology literature, and which can be done reliably under field conditions. Outcomes selected for assessment include:

1. Clutch initiation date. Laying date is associated with fledging success (Kallander and Karlsson, 1993).

2. Clutch size reflects the reproductive input of the parents as well as potential hatchability of the eggs (Reid et al., 2000).

3. Mean egg mass (Christians and Williams, 1999) and egg size (Smith et al., 1995) are useful indicators of reproductive success in starlings, as is egg volume, derived from egg width and breadth (Wiebe and Bortolotti, 1995; Bortolotti and Wiebe, 1995).

4. Mean nestling mass and size are commonly used as indicators of quality of the young. Combining measurements of mass and size (e.g., tarsus length or length of 10^{th} primary feather) to create an overall index of nesting condition is a recent approach that has been applied to starlings (Reid et al., 2000; Christians and Williams 2001).

5. Hatchability (number of egg hatching/number of eggs laid) (Reid et al., 2000)

6. Fledging success (number of nestlings alive at 20 days post hatch/number hatching) (Christians and Williams, 1999)

Protocol

Pilot Studies
Small-scale pilot studies involving wild-caught adult European starlings are currently underway at the Western College of Veterinary Medicine's captive bird facilities. The objectives of these studies include: 1) to produce a polyclonal rabbit anti-starling IgG antibody that will be used to develop an ELISA to quantify the humoral immune response of nestlings to DNP-KLH vaccination during the second field season; 2) to produce a monoclonal mouse anti-starling IgA antibody that will be used to develop an ELISA to quantify the mucosal immune response of nestlings to ocular SRBC challenge during the second field season; and 3) to test the effectiveness of both newly developed ELISAs in captive starlings prior to applying them in the field study.

Field Study
This study will be conducted over two successive breeding seasons (approximately April through August, depending on latitude and habitat, of 2001 and 2002), in order to minimize potential effects of bad weather or other environmental factors on reproductive success or immune endpoints. Ten artificial nest boxes will be erected on each study site prior to the breeding season, to attract pairs of starlings. Boxes will be placed at 3 to 4 m height on trees or poles, and spaced at least 200 m apart in and around the same pastures used by beef cattle.

Reproductive Success

Nest boxes will be monitored every second day throughout breeding, egg laying (about 4-6 days for the entire clutch), incubation (12-14 days), and nestling periods, until nestlings are successfully fledged at about 20 days post-hatch (Kessel, 1957). Data will be gathered on the productivity outcomes described above, including laying date, clutch size, egg mass, size, and volume, nestling mass and size, hatching success, and fledging success. In addition, general health status, growth rate, and occurrence of infectious or parasitic diseases in the nestlings will be noted.

Immune Function

Immune function will be evaluated in starling nestlings using the panel of assays outlined above. Blood samples will be collected when nestlings are 13 days of age. These initial samples will be used to determine differential and total white blood cell counts (as well as total plasma protein and hematocrit), and to determine baseline (pre-vaccination) antibody titres to the SRBC antigen (year 1) or DNP-KLH antigen (year 2). In addition to blood sampling, nestlings will be vaccinated with either SRBCs (i.p., year 1) or DNP-KLH (IM, year 2) at the same time.

Five days later, when nestlings are 18 days of age, a post-vaccination blood sample will be collected, and the systemic antibody response to the vaccine quantified by hemagglutination assay (SRBC) or ELISA (DNP-KLH). Haematological measurements will also be repeated on the samples collected at this time. During this same visit, nestlings will be challenged with a subcutaneous injection of PHA in the wing web, in order to assess cell-mediated immunity. The magnitude of the dermal response to PHA will be measured 24 hours after this challenge, as described above.

Two randomly selected nestlings from every nest will be euthanized on day 19, immediately after obtaining the post-challenge PHA measurements. The spleen, thymus, and Bursa of Fabricius will be removed from each bird sacrificed, and placed immediately into 10% neutral buffered formalin. Fixed tissues will be routinely processed and stained with haematoxylin and eosin for examination using morphometric analysis to quantify changes in immune system cell types.

Finally, during the second field season, the mucosal immune response will be evaluated by sensitizing 13-day-old nestlings to SRBCs by instilling the antigen into the conjunctival sac of one eye. The specific lachrymal IgA response to this antigen will be quantified by ELISA using ocular fluid (“tear”) samples collected immediately before and 6 days after sensitization. **.G 72utinely processe**

maintained on a commercial software package (Microsoft Access 2000, Microsoft Corporation) for summary and reporting purposes.

Appropriate descriptive statistics and graphical representations of the data will be generated to both aid in checking for data entry errors and guide appropriate statistical analysis. All prevalence estimates will be reported with appropriate 95% confidence limits adjusted for clustering at the study site level.

The primary hypotheses to be tested will be whether starling chicks that have higher cumulative exposures to SO_2, H_2S, the seven marker VOCs, or proximity to field facilities (less than 1 mile) and flaring will have an increased risk of adverse reproductive and immune function outcomes.

Methods for statistical modelling will be developed together with a biostatician. It is likely that mixed effects models will be used for continuous outcomes and random effects logistic regression for binary and proportion data. The methods chosen must be able to examine individual animal risk while being able to account for variable levels of clustering. Modelling strategies including procedures for dealing with confounding, potential intermediate variables, interaction, and missing data will be outlined in detail in the standard operating procedures (SOPs). Data reporting will include effect estimates and appropriate 95% confidence intervals. Strategies for model checking will also be outlined in the SOPs.

Proposed Additional Contaminant-Specific Endpoints for Evaluation in the Second Field Season

The protocol employed during the first field season included collection of selected tissues for immunopathology from a proportion of chicks (two per nest). Additional, highly pertinent information on the potential sublethal effects of exposure to these contaminants could be obtained during the second field season by collecting livers, lungs, and bile from the nestlings already being sacrificed.

Samples of liver and lung could be used to determine hepatic and pulmonary microsomal P450 enzyme activity, a biomarker of exposure to many organic compounds. Liver samples could also be used to determine if chronic exposure results in an increase in the formation of DNA adducts, a biomarker of exposure to many genotoxic carcinogens, including PAHs. In addition, bile samples could be analyzed for the presence of PAH metabolites. All of these endpoints are commonly use on petroleum-contaminated sites, and in many other situations where PAH exposure is suspected (Upshall et al., 1993; Trust et al., 1994; Di Giulio et al., 1993; Qu et al., 1997; Hegstad et al., 1999; Billeret et al., 2000; Custer et al., 2000, 2001; Leonard and Hellou, 2001).

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Appendix C

Exposure Monitoring

A Component of the Western Canada Study on Animal and Human Health Effects Associated with Exposure to Emissions from Oil and Natural Gas Field Facilities

Study Design

November 1, 2001

RWDI West Inc.
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EXPOSURE MONITORING
STUDY DESIGN – November 2001

Purpose

The exposure monitoring component of the Western Canada Study on Animal and Human Effects Associated with Exposure to Emissions from Oil and Natural Gas Field Facilities (The Study) has been implemented on behalf of the Western Interprovincial Scientific Studies Association (WISSA) by RWDI West Inc (RWDI), a Calgary-based company that provides a wide variety of services in the field of air quality monitoring, modeling and assessment. The monitoring component provides the Study with air quality data for sites located in British Columbia, Alberta and Saskatchewan.

A sample suite of compounds previously identified with flaring, other oil and gas combustion related sources and fugitive emission products associated with oil and gas operations in the field were targeted to be representative of exposure.

The parameters were selected as follows:

- SO_2 was selected to represent exposures that result from the combustion of sour gas.
- Selected VOC species were selected to represent exposures to intermediate products of combustion from both sour and sweet gas combustion sources.
- H_2S was selected to represent fugitive sour gas sources.
- Selected VOC species were selected to represent fugitive sour and sweet gas sources.
- Particulate matter ($PM_{1.0}$) was selected at a subset of the study sites to represent the recent concerns regarding health effects.

The end result is the use of these exposure measurements as surrogates to identify background, low, medium and high exposures that can be available for correlation to bio-indicators. These data are in the format of parameter, GPS site location, concentration and exact exposure period.

RWDI communicates this exposure information to the researchers conducting the beef productivity, animal health and other study components, who are then able to test for the existence of relationships between air quality exposures and observations they have documented in the course of their study work.

Present Scope of the Monitoring Program

The air quality monitoring program is being conducted at over 200 farms owned by producers participating in the study. The data collected consists of the average monthly concentrations for each air quality parameter. **Table 1** (**following page)** lists the number of samples collected since the sampling program began in April 2001. The monitoring program is scheduled to end in late 2002. At present, about 4,500 samplers are being processed by RWDI each month.

Table 1. Number of Sites and Samples per month, by parameter.

Month	Operational Sites	SO_2 Samples Deployed	VOC Samples Deployed	H_2S Samples Deployed
April	144	310	173	
May	615	1211	691	
June	930	1860	1019	
July	1050	1949	1130	
August	1050	1943	1138	
September	1050	1169[1]	1160[1]	1158[1]

[1] Actual numbers to be verified on receipt of laboratory analyses.

The number of active sites increased from April to July as herds were moved to pasture and as herds were split up because of the unusually dry conditions. The study can handle as many as 1200 operational sites. The number of parameters is greater than the number of sites because of the need for duplicate samples and blanks used to ensure data accuracy and reliability.

The specific VOC compounds being analysed are presented in **Table 2 (following page)**. The monitoring program schedule is presented in detail in **Table 3 (second following page)**, which shows the projected monthly activities from April 2001 to December 2002.

Sample Collection

A team of 15 RWDI field technicians, residents of the areas for which they are responsible, have installed the monitoring sites and perform the monthly sample collection. These technicians have been trained in appropriate site selection, sample storage, handling and shipping procedures. They comply with specific confidentiality requirements to protect the privacy of the producers and strictly adhere to bio-security and cleanliness measures. The technicians collect the exposed samplers and install unexposed samplers over a period of about one week at the beginning of each month. The dates and times of sample collection are recorded so that the exposure periods are accurately known.

The field technicians also record information concerning each site and contribute to detailed chain-of-custody documentation. The site documentation includes digital photographs and GPS co-ordinates that enable the researchers to know precisely where the sites are and what the areas around them look like. The chain-of-custody information is required so that the history of each air quality sampler used in the study is documented, from its arrival at RWDI's offices in Calgary, to its subsequent exposure, to its final destination at an air quality laboratory where the concentration of the sample is determined.

Table 2 PAH, metals and VOC analysis list

PAH's	Metals	VOC Compounds
1,2-Benzanthracene	Al	Dichloromethane
3-Methylchloranthrene	As	n-Hexane
7,12-Dimethylbenz(a)anthracene	Ba	Chloroform
Acenaphthene	Be	1,2-Dichloroethane
Acenaphtylene	Ca	Benzene
Acridine	Cd	Trichloroethylene
Anthracene	Co	Toluene
Benzo(a)anthracene	Cr	Tetrachloroethylene
Benzo(a)pyrene	Cu	Ethylbenzene
Benzo(b,j,k)fluoranthene	Fe	p-Xylene
Benzo(c)phenanthrene	Li	m-Xylene
Benzo(e)pyrene	K	o-Xylene
Benzo(ghi)perylene	Mg	Styrene
Benzo(ghi)fluranthene	Mn	a-Pinene
Chrysene	Mo	1,1,2,2-Tetrachloroethane
Coronene	Ni	n-Decane
Dibenzo(a,h)pyrene	Pb	1,3,5-Trimethylbenzene
Dibenzo(a,I)pyrene	Se	1,2,4-Trimethylbenzene
Dibenzo(a,l)pyrene	Sr	Pentachloroethane
Debenzo(ah)anthracene	Th	d-Limonene
Flouranthene	Ti	p-Cymene
Fluorene	Tl	1,3-Dichlorobenzene
Indeno(1,2,3-cd)pyrene	V	1,4-Dichlorobenzene
Naphthalene	Zn	Hexachloroethane
Perylene		1,2,4-Trichlorobenzene
Phenanthrene		Naphthalene
Pyrene		
Retene		

EXPOSURE MONITORING
STUDY DESIGN – November 1, 2001

RWDI Project Activity Chart

RWDI

Task BA01	Program Inception
Task BA02	Field Staff Training
Task BA03	Co-ordination of Shelter Installation/Removal (1200 Units)
Task BA04	Monthly Operation
Task BA05	Monthly Auditing
Task BA06	Passive Data Review and Interpretation
Task BA18	Particulate Monitoring Staff Training
Task BA19	Particulate Monitoring Installation/Removal
Task BA20	Particulate Monitoring Monthly Operation
Task BA21	Particulate Monitoring Data Review and Analysis
Task BA22	Particulate Monitoring Equipment
Task BA23	Particulate Monitoring Consumables

FIELD STAFF

Task BA07	Field Staff Training - Assuming One (1) Training Program
Task BA08	Field Staff Equipement
Task BA09	Field Staff - Shelter Installation/Removal
Task BA10	Field Staff - 18 Month Monitoring Program

AIRZONE ONE

Task BA11	Field Staff Training - Assuming One (1) Training Program
Task BA12	Staff Support - Implementing Chain of Custody Procedures
Task BA13	Passive Sample Prep. and Analysis
Task BA24	Particulate Monitors
Task BA25	Particulate Monitoring Analysis

MAXXAM

Task BA14	Field Staff Training - Assuming One (1) Training Program
Task BA15	Sampling Shelters
Task BA16	Passive SO2 and H2S Sample Prep. and Analysis

Laboratories

Two air quality laboratories provide analytical services to RWDI. Maxxam Analytics is responsible for the SO_2 and H_2S components; AirZone One is responsible for VOC analysis.

Passive Sampling Technology

The data are collected using what is referred to as passive sampling technology. Passive sampling involves the placement of individual samplers at each site where they are exposed for a period of one month. A filter material in the sampler selectively absorbs the appropriate chemical from the air that passes through it. The sampler is termed passive because the movement of air through the filter is caused solely by the natural movement of air in the vicinity of the site. At the end of each exposure period the samplers are sealed into containers for shipment to RWDI for further documentation, then on to the appropriate laboratory. The samplers are roughly 50mm in diameter and are snapped into place in sampling shelters that protect the samplers from precipitation. The white plastic sampling shelters, about 200 mm in diameter, are mounted about 1.7 metres above ground on steel pipes that are fixed to fence posts.

Passive sampling technology was selected because of its known reliability, simplicity, cost and ability to measure low concentrations as it integrates exposure over a one month period.

Particulate Monitoring

Plans are in place to extend the scope of the study to include additional parameters of particular relevance to health assessment. These parameters would be obtained through the implementation of a particulate matter monitoring program. Such a program would enable the determination of concentrations of $PM_{1.0}$, one-micron diameter particulates and concentrations of various metals and PAH's, of similar interest **(Table 2)** . The particulate monitoring component of the project would be conducted at a sub-set of 40 sites of the current total of over 200. These 40 sites are being selected by using the existing monitoring data to identify sites that are expected to have comparatively high, medium and low exposures, so that a balanced cross-section of exposure will be monitored

Quality Control

Many quality assurance (QA) and quality control (QC) measures have been instituted by RWDI to ensure data integrity. These QA/QC measures involve systems for verifying the identity of each sampler and ensuring that all data entry is checked. All field staff and laboratories subjected to regular documented audits to ensure the reliability of all information used in this study.