

Analysis of Ergot (*Claviceps purpurea*) Alkaloids in Grain and Effect on Cow-Calf Performance

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ABSTRACT

Ergot contamination of cereal crops and grasses has been of increasing concern for both grain and animal producers. The overall objective was to examine ergot contamination from an analytical, regulatory and biological perspective.

The objectives of the first study were to determine the correlation between number of ergot sclerotia and weight compared to the total ergot alkaloid concentration, to evaluate the effect of particle size (PS) on ergot alkaloid analysis and to determine the impact of sample volume on analytical variability. This study demonstrated that correlations existed between both ergot sclerotia count and weight compared to the total alkaloid concentration (P 's < 0.001) but did not exist for either, at ergot alkaloid concentrations below 350 $\mu\text{g}/\text{kg}$ ($P = 0.956$ and 0.769 respectively). This study also determined that a finer grind (PS = 192 μm) produced a lower variability ($P = 0.041$) than a coarser grind (PS = 516 μm). The coefficient of variation (CV) was also numerically reduced as sample volume increased (97% CV for 75 mL to 64% CV for 1000 mL; mean of all concentrations) but increased as sample concentration declined (17% CV for 81678 $\mu\text{g}/\text{kg}$ to 284% for 35 $\mu\text{g}/\text{kg}$; mean of all sample volumes). An analytical approach with fine grinding of a minimum sample volume of 1000 mL (if not the entire sample available) should be used to assess ergot contamination to reduce variability.

The objective of the second study was to determine if the percentages of individual ergot alkaloids were similar across different cereal grains collected from across Western Canada over different years. Ergocristine was the predominant alkaloid accounting for half of the total alkaloids in all grain types. Ergocornine ($6\% \pm 1$; $P = 0.201$), ergocristine ($48\% \pm 2$; $P = 0.939$), ergocryptine ($17\% \pm 2$; $P = 0.302$) and ergosine ($5\% \pm 0.5$; $P = 0.239$) were of similar proportions in barley ($n = 39$), rye ($n = 7$), triticale ($n = 9$) and wheat ($n = 94$). However, small differences were found between grain types for both ergometrine and ergotamine ($P = 0.027$ and 0.011 respectively). There were no yearly alkaloid proportion differences between the six alkaloids in barley and wheat 2015/2016 samples ($P = 0.969, 0.680, 0.572, 0.080, 0.119$ and 0.189 for ergocornine, ergocristine, ergocryptine, ergometrine, ergosine and ergotamine respectively). However, ergocornine was higher in wheat ($P = 0.017$) and ergometrine was higher in barley ($P =$

0.002) when comparing the 2015/2016 barley and wheat samples. With the overall proportions of ergot alkaloids comparable among the four grain types collected across Western Canada a maximum total ergot alkaloid concentration, as opposed to individual ergot alkaloid concentrations, can be considered acceptable.

The objective of the third study was to evaluate the impact of ergot consumption in cow-calf performance. Overall, ergot alkaloid concentrations up to 820 µg/kg for 9 weeks had no effect on cow weight, calf weight, prolactin concentration, rectal temperature, progesterone concentration or timing of first progesterone rise ($P = 0.931, 0.077, 0.298, 0.163, 0.792, 0.376$). There was also no effect on the size of the first and second follicle to ovulate postpartum ($P = 0.403$ and 0.414 respectively) or the number of days until the first and second postpartum corpus luteum appearance ($P = 0.949$ and 0.984 respectively). The maximum size of the corpus luteum was 4 mm larger in the 820 µg/kg ergot treatment group compared to the control treatment ($P = 0.028$) for the first ovulation postpartum, however no differences were observed between the control and 820 µg/kg groups by the second ovulation ($P = 0.113$). A revised ergot tolerance concentration of 820 µg/kg for beef cows is suggested based on the reproduction/hormone endpoints.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
DvsA	During vs. After
µg	micrograms
µL	microliter
µm	micrometer
CFIA	Canadian Food Inspection Agency
CGC	Canadian Grain Commission
CL	Corpus Luteum
cm	centimeter
CV	Coefficient of Variation
EFSA	European Food and Safety Authority
ELISA	Enzyme Linked Immunosorbent Assay
ESI	Electrospray Ionization
FLD	Fluorescence Detection
g	gram
GS	Gas Chromatography
HPLC	High-Performance Liquid Chromatography
IU	International Units
kg	kilogram
L	liter
LC	Liquid Chromatography
LOQ	Limit of Quantification
LSD	Lysergic Acid Diethylamide
mg	milligram
MHz	Mega Hertz
mL	millilitre

mM	millimolar
mm	millimetre
mo.	month
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
<i>n</i>	number
n/a	Not applicable
ng	nanogram
NIR	Near Infrared Spectroscopy
No.	number
<i>P</i>	probability
PDS	Prairie Diagnostic Services
ppb	parts per billion ($\mu\text{g}/\text{kg}$)
ppm	parts per million (mg/kg)
PS	Particle Size
PSA	Particle Size Analyzer
RIA	Radioimmunoassay
SD	Standard Deviation
SE	Standard Error
TLC	Thin Layer Chromatography
Tx	Treatment
wk	week
α	alpha
β	beta

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Ergot is a fungal disease of cereal crops and grasses produced by the fungus *Claviceps (C) purpurea* which infects nearly 600 plant species (Bove, 1970). Ergot contamination is most easily identified by purplish/black structures on the grain or grass replacing the seeds known as sclerotia or ergot bodies. Secondary metabolites produced by the fungus known as ergot alkaloids are toxic to humans and animals. Most countries have strict regulations for human consumption (Lorenz & Hosenev, 1979) which redirects the ergot-contaminated grain to livestock feed. These ergot alkaloids can cause a range of effects including but not limited to, convulsions, gangrene, hyperthermia, agalactia and reduced weight gain and intake (J. L. Klotz, 2015; McMullen & Stoltenow, 2002) following consumption of contaminated feed.

1.2 Ergot (*Claviceps purpurea*)

1.2.1 History

Ergotism epidemics occurred frequently during the middle ages following the consumption of rye bread contaminated with *C. purpurea*. Poor people were more susceptible associated with a higher intake of contaminated rye bread, especially during famines (Gaudet, Menzies, & Burnett, 2000). An epidemic of gangrenous ergotism on a large-scale was first recorded in 'Annales Xantenses' (Germany) 857 A.D. (Fuchs, 1834) whereas the first convulsive ergotism epidemic was described in Paris, France 945 A.D. (Barger, 1931). Ergotism which was originally known as 'ignis sacer' i.e. Holy Fire in the middle ages (Matossian, 1989) is associated with the burning sensations felt in the limbs. Many people suffering from ergotism could identify with the lifelong tortured St. Anthony (Van Dongen & de Groot, 1995) which lead to ergotism to be known as St. Anthony's Fire throughout the remainder of the middle ages. Ergotism began to decline in many areas particularly for peasants as the potato became a staple (Schumann, 2000).

Ergot was originally thought to be sunbaked kernels of grain (Caporaël, 1976) however ergot bodies were demonstrated to be fungal sclerotia by Louis Tuslane and Kühn in the mid 1800s (Menziés & Turkington, 2015). Hallucinogens were attributed to several ergot alkaloids including lysergic acid which are produced by the ergot bodies. When baked with dough, ergot alkaloids which include the lysergic acid structure (Figure 1.1a) may be transformed into lysergic acid diethylamide (LSD), a known hallucinogen (Van Dongen & de Groot, 1995). Many of symptoms associated with the consumption of ergot were associated with “bewitchment” which was likely a factor in the Salem Witchcraft Trials (Caporaël, 1976). Midwives used ergot as an aid of childbirth and abortion (Haarmann, Rolke, Giesbert, & Tudzynski, 2009) which was believed to be a form of witchcraft (Bennett & Bentley, 1999). Ergot alkaloids are still however used for medical purposes such as the treatment of migraines (Haarmann et al., 2009) and Parkinson’s disease (Crews, 2015).

In most developed countries, human ergot poisoning has been eliminated due to the strict regulatory guidelines and our understanding of the cause of ergotism. However, ergot alkaloid contamination related to agriculture represents a major problem and requires attention and control measures (Krska & Crews, 2008) particularly for animal consumption.

1.2.2 Ergot Biology

Ergot was named, based on its appearance, after an old French word ‘argot’ meaning the cock’s spur (Van Dongen & de Groot, 1995). Ergot contamination is most easily identified by purplish/black structures on grass or grain replacing the healthy seeds known as sclerotia or ergot bodies. Sclerotia are typically cylindrical with round ends, straight to curved and tapered at the distal end (Alderman et al., 1999). Size and shape differ depending on the host plant. In some grains, sclerotia are up to four times larger than the grain kernels, whereas in other grains, such as wheat, the sclerotia are of a similar size (Seaman, 1980). The rinds of the sclerotia are hard, therefore halves or thirds may be found in grain but generally the sclerotia remain unbroken. The interior of the sclerotia are grayish white giving a distinguishing feature between broken sclerotia and other debris (Seaman, 1980).

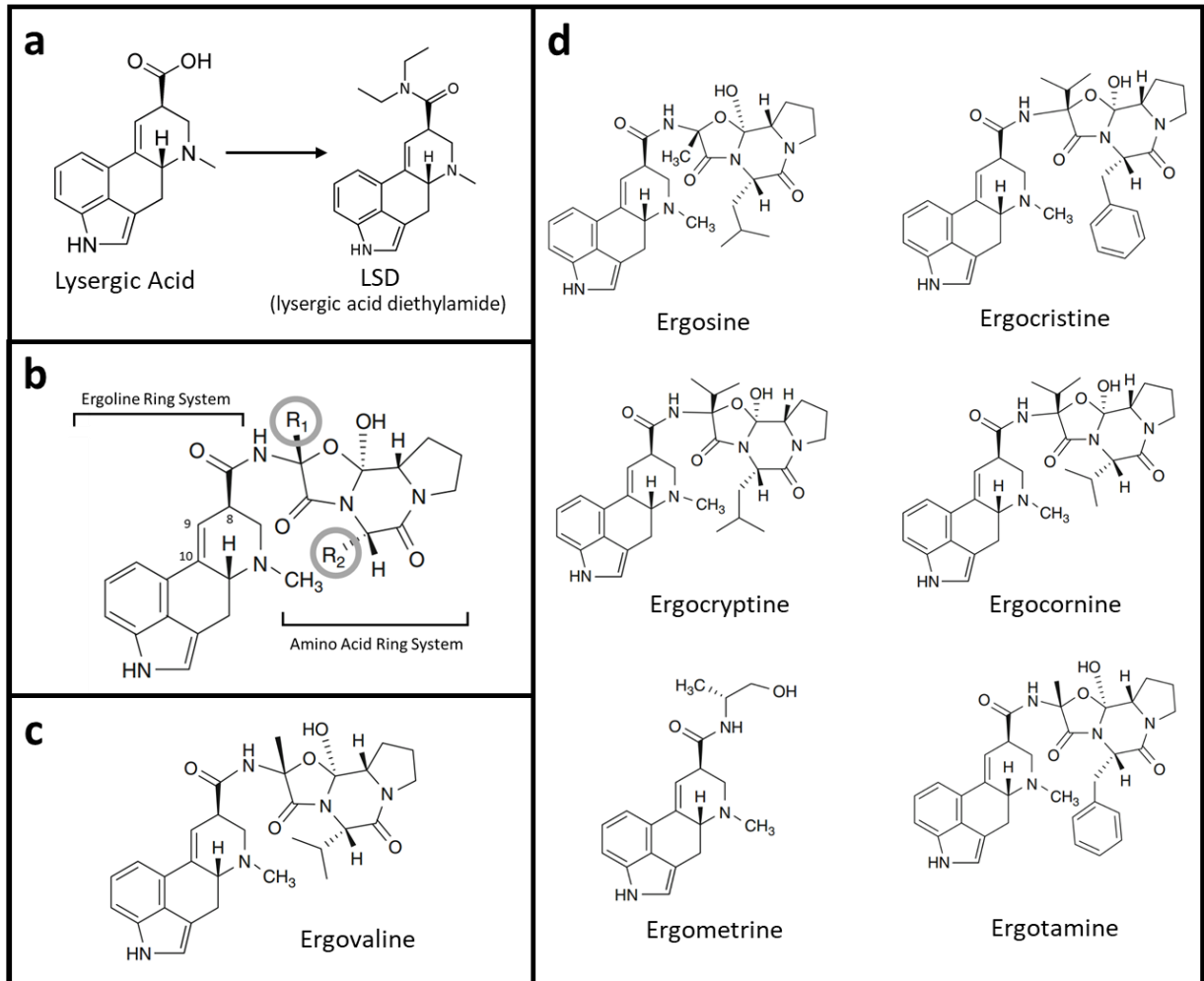


Figure 1. 1 Conversion of lysergic acid to hallucinogen LSD (a); Basic ergot alkaloid structure (b); Structure of the predominant alkaloid found in tall fescue (c); Structures of the six ergot alkaloids found in Canadian grains (d)

All ergot alkaloids contain an ergoline ring structure with a methylated N-6 nitrogen and a functional group on the C-8 (Flieger, Wurst, & Shelby, 1997)(Figure 1.1b). Most ergot alkaloids also feature a C-8, C-9 or C-9, C-10 double bond (Flieger et al., 1997). The most prominent ergot alkaloids found in contaminated grains across Canada are: ergosine, ergocristine, ergocryptine, ergocornine, ergometrine and ergotamine (Figure 1.1d) with ergocristine and ergotamine being found in the highest concentrations (Grusie, Cowan, Singh, Blakley, & McKinnon, 2015). The 8- α -isomers, identified by names ending in -inine, are not of toxicologic concern (Berde & Stürmer, 1978; Pierri, Pitman, Rae, Winkler, & Andrews, 1982), however, the 8- β -isomers are biologically active (Burrows & Tyrl, 2012) thus, the focus will be placed on them.

1.2.3 The Lifecycle

The ergot lifecycle consists of two stages, a germination stage followed by a honey dew stage as seen in Figure 1.2. Ergot bodies can be present in the soil from a previous cereal crop (Schumann, 2000), infected grasses along roadside or neighbouring pastures or from planting contaminated seed (Seaman, 1980).

Activation of germination typically begins after 4 to 8 weeks of 0-10°C temperatures (Mitchell & Cooke, 1968). Therefore, germination is seen in spring when the ergot bodies produce tiny “drumstick” shaped structures (Bailey, Gossen, Gugel, & Morrall, 2003). When adequate soil moisture or rainfall has been achieved ascospores eject into the air from these structures (Kren & Cvak, 1999; Menzies & Turkington, 2015). The spores are carried by wind until they attach to the stigmatic hairs of the ovary of flowering plants within 24 hours (Mai & Li, 2013), replacing the healthy kernel (Kren & Cvak, 1999).

Production of a yellow-white, sweet, sticky fluid known as honeydew (McMullen & Stoltenow, 2002) appears between 7 to 10 days depending on the crop after the infection occurs (W. Campbell, 1957). The honeydew contains conidia (Belser-Ehrlich, Harper, Hussey, & Hallock, 2013) which begin to germinate once diluted by dew or rain (Schwartzing & Hiner, 1945). A secondary infection occurs by rain splash or insects, who are attracted to the honeydew, spreading the conidia (Bailey et al., 2003). The conidia can continue to spread for as long as flowering occurs. Cool, wet and/or cloudy weather delay pollination which increases the duration

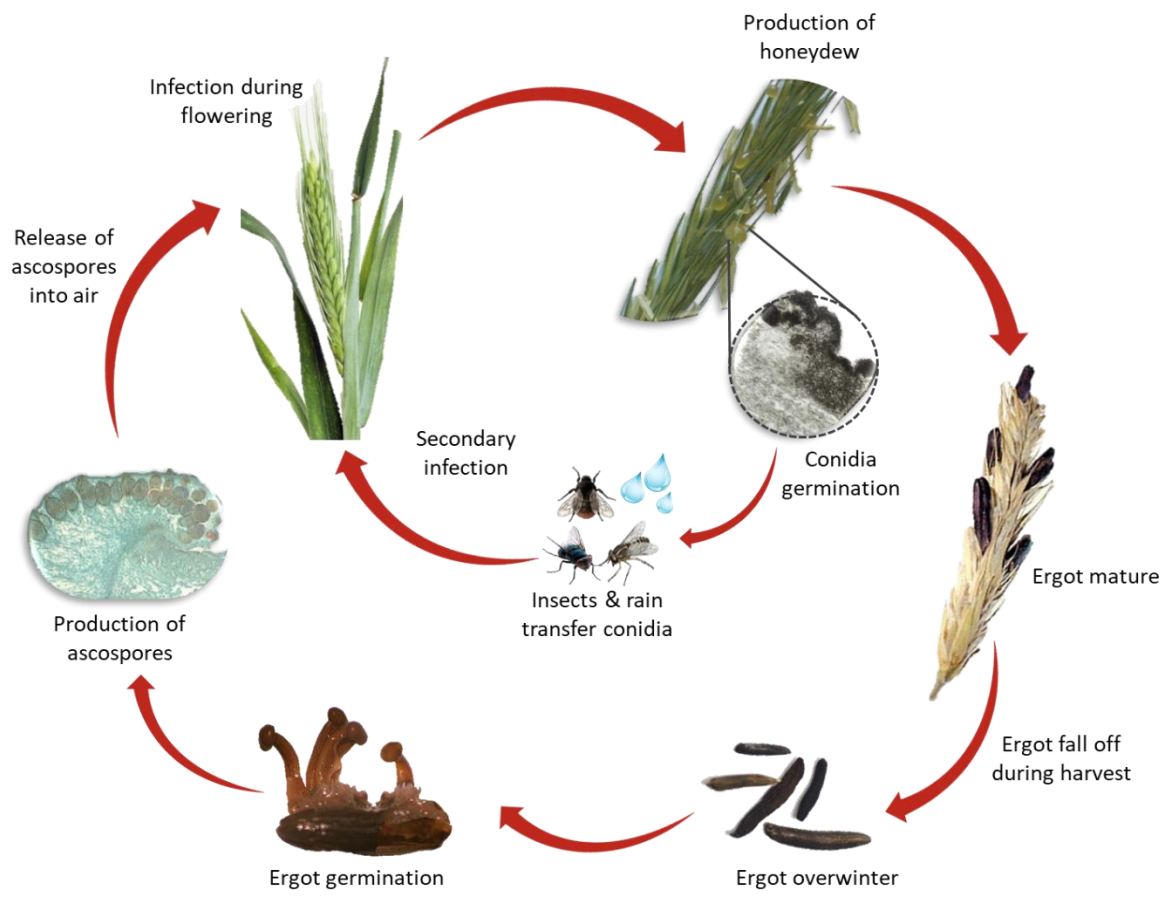


Figure 1. 2 Life cycle of *Claviceps purpurea*. Re-drawn from Bailey et al. (2003) Images provided by (Armstrong, 2001; Schwartz, 2008; Wong, 2011)

of flowering and thus increases the period of susceptibility (Bailey et al., 2003; Seaman, 1980). Production of honeydew stops after approximately two weeks (Mai & Li, 2013).

The infected ovary becomes enlarged and a sclerotium is formed as a resting structure of the fungus (Mai & Li, 2013) taking approximately 5 weeks to mature (Belser-Ehrlich et al., 2013). The sclerotium protects the fungus during low temperatures in winter (Mai & Li, 2013). They will remain inactive until spring (Van Dongen & de Groot, 1995). Sclerotia have been shown to survive in or on the soil or in contaminated grain for 1 (Mitchell & Cooke, 1968) to 3 years (Rapilly, 1968).

1.2.4 The Prevalence of Ergot Alkaloids of Concern in Canada

Ergot alkaloids found in endophyte-infected (*Neotyphodium coenophialum*) tall fescue (*Lolium arundinaceum*) have been studied for more than 50 years (Coufal-Majewski et al., 2016). It is estimated to cost the beef industry more than \$600 million annually in the United States due to decreased productivity (Hoveland, 1993). The alkaloids found in fescue, which are commonly found in the States, differ from those found in grain infected by *C. purpurea* (Canty et al., 2014), although the clinical signs and effects of ergotism and fescue toxicosis are similar (Yates, Plattner, & Garner, 1985). Ergotamine (Figure 1.1d) is predominantly associated with ergotism where as ergovaline (Figure 1.1c) is predominantly associated with fescue toxicosis (Canty et al., 2014). It has also been noted that lower alkaloid concentrations are found in infected tall fescue compared to sclerotia of *C. purpurea* which may account for any clinical differences (Shelby, 1999).

Prevalence and severity of ergot contamination depends heavily on the weather (Bailey et al., 2003). Ergot prevails in the spring and early summer when moisture is available at the soil surface (Seaman, 1980). Therefore, depending on the geographical region and host plant, the amount and pattern of ergot alkaloids will vary (Krska & Crews, 2008). Ergot is also more prevalent when rain persists during the flowing stage of grasses and grains (Seaman, 1980). Concentrations of ergot in Canadian grains are likely to increase as a result of increased precipitation predicted by climate change models (Coufal-Majewski et al., 2016). The impact of the weather on the presence of ergot, accounts the sporadic nature of ergot from year to year as seen in Canadian Western Red Spring wheat samples between 2002 and 2013 (Figure 1.3). This

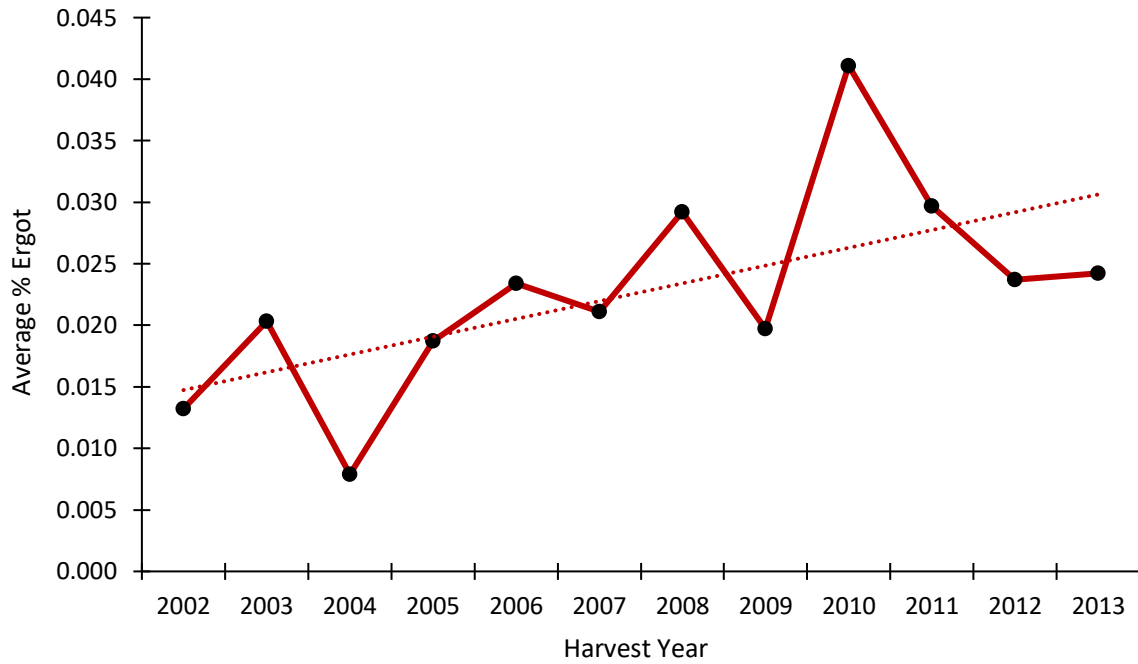


Figure 1. 3 Occurrence of ergot in Canadian Western Red Spring wheat samples submitted to the Canadian Grain Commission Harvest Sample Program. Dotted line represents trend line. (Data provided by Sheryl Tittlemier (2015))

sporadic occurrence has probably influenced why minimal research has been conducted on ergot (Menzies & Turkington, 2015), particularly in Canada.

1.2.5 Management Techniques

Prevention of ergot contamination is the ideal management strategy however once ergot is present other management techniques should be applied. The focus of modern ergot management is to limit the presence of ergot sclerotia in cereal grain (Tittlemier et al., 2015). The primary concern with the presence of ergot sclerotia is the various toxic alkaloids produced, while yield loss is usually minor and of secondary importance (Menzies & Turkington, 2015). Unfortunately, there is not much a farmer can do to control ergot in the field, however, the following management techniques can be used to help reduce the presence of ergot sclerotia.

1.2.5.1 Sanitation

In most years, ergot can be found in grasses of head lands and along roadsides of prairie provinces (Seaman, 1980). It has been shown that all indigenous and forage grasses constitute a reservoir of ergot inoculum for cereal crops (W. Campbell, 1957). The sclerotia produced by the grasses survive over winter and can provide an initial inoculum in the next season (Menzies & Turkington, 2015). The timing of flowering of the grain in relation to these nearby grasses is important as cereal crops bloom at the time the inoculum is dispersed (Bailey et al., 2003; W. Campbell, 1957). The different flowering periods of different grass species also create a larger susceptible time frame for the cereal crop infestation (Menzies & Turkington, 2015). To minimize the spread of ergot, grasses along headlands, roadsides and fences should be eradicated or mowed to prevent flowering (McMullen & Stoltenow, 2002; Schumann, 2000) so they cannot serve as a host for the first stage of the disease cycle.

1.2.5.2 Harvesting Techniques

Typically, ergot contamination is greater around the edges of a field surrounded by grasses ditches or headlands due to the proximity of the crop to host grasses as mentioned previously. The field should be scouted prior to harvest and areas where ergot contamination is high ought to be harvested independently (Bailey et al., 2003). These heavily contaminated

sections should be binned and taken to the elevator separately as mixing the infested seed with the remaining crop can result in extra charges for special handling along with potential downgrading or rejection of the entire lot (Seaman, 1980). If the contamination is extremely high, the shipment of grain may be best destroyed. The amount of ergot in harvested grain can be reduced by delaying swathing or harvest as the ergot bodies fall out of the head with wind (Menzies & Turkington, 2015), however, the ergot will remain in the field becoming a source of infection for the following year.

1.2.5.3 Tillage

As farming practices have been evolving, many cereal crops are now grown using a “no-till” or “low-till” policy to reduce the amount of soil erosion (Schumann, 2000). There has been an almost 20% increase between 2002 and 2008 on the percentage of no-tillage area (Nagy & Gray, 2012). By not tilling, crops are seeded directly into the stubble of the previous years crop which if infected can re-infect the newly planted cereal crop. To prevent this from happening the sclerotia should be covered by 5-8 cm of soil which is generally a sufficient depth to prevent the discharge of ascospores from reaching the surface (Bretag & Merriman, 1981; Schumann, 2000; Seaman, 1980). If the no tillage policy is utilized, burning the stubble will reduce the amount of viable sclerotia, however, it can be difficult to achieve a sufficiently elevated temperature to destroy an entire field contaminated with ergot (Bretag, 1985; Johnston, Golob, Sitton, & Schultz, 1996).

1.2.5.4 Seed Cleaning to Improve Grain Quality

Reduction of grain grade quality accounts for the majority of economic loss for grain farmers (Coufal-Majewski et al., 2016). Increasing ergot reports contamination has been accompanied with increased downgrading of wheat across the prairie region (Menzies & Turkington, 2015). To reduce the downgrading of large lots of grain, commercial cleaning using gravity or color sorters can be used to remove sclerotia (Bailey et al., 2003). These sorting methods can be costly. However, if available, it is likely economically beneficial to save a grade at the elevator. For smaller lots of grain, the floatation method can be used in which the grain is soaked in a 20% salt solution. The sclerotia will rise and can be skimmed off the top, the remaining

seed can be rinsed and dried. If the grain is heavily infested and cannot be adequately cleaned, the grain should be disposed of insuring there is no access to livestock (Menzies & Turkington, 2015).

1.2.5.5 Plant Clean Seed

In order to reduce the spread of ergot, sclerotia-free seed should be planted, especially if the field is considered ergot-free. If, however, clean seed is not available the seed should be planted a minimum of 5 cm below the soil surface to prevent emergence of the ergot stromata and discharge of ascospores (Schumann, 2000). Holding the seed for approximately 2-3 years before seeding can also reduce the spread of ergot by decreasing the viability of the sclerotia to germinate (Seaman, 1980). It is important to note that even though the sclerotia may be non-viable after 2-3 years, the toxic ergot alkaloids are still present.

1.2.5.6 Crop Choice

Resistant varieties and chemical seed treatments to control ergot are currently not available (Bailey et al., 2003; Seaman, 1980). Therefore, crop choice is an important consideration for ergot management. Rye and triticale, a grain developed from rye and wheat, are considered the most susceptible to ergot contamination (Menzies & Turkington, 2015; Seaman, 1980). Rye is an open pollinator making it more susceptible to ergot as open, unpollinated flowers easily allow the fungus to enter (Bailey et al., 2003; Coufal-Majewski et al., 2016). If rye is the crop of choice, winter rye should be used as opposed to spring rye since flowering occurs earlier, minimizing the ergot spread from late-flowering grasses (Bailey et al., 2003). Wheat and barley are considered less susceptible to ergot infection as they are self-pollinators with a shorter flowering period (Coufal-Majewski et al., 2016; Schumann, 2000). Oats are the least susceptible and rarely infected (Menzies & Turkington, 2015). In general cereal crops that flower early, self-fertilize before flowering or have a short flowering period have a lower chance of becoming infected (Menzies & Turkington, 2015). Cereal crops with longer flowering periods should be avoided if ergot contamination is expected to be high as they are more frequently infected (McMullen & Stoltenow, 2002).

1.2.5.7 Crop Rotation/Placement

Ergot sclerotia can survive in the soil over winter, therefore, it is important to partake in proper crop rotation practices. Cereal grains or grasses should not be planted after an ergot-infected crop (Seaman, 1980), instead a non-cereal crop can be planted such as canola or legumes. Planting fall sown crops are typically less infected than spring sown crops (Menzies & Turkington, 2015). If a grain must be planted after an ergot-contaminated crop a less susceptible plant species such as oats should be used (Seaman, 1980). Spring-sown cereals should not be planted near winter rye (Bailey et al., 2003) and early-maturing crops should not be planted near late-maturing crops to prevent the spread of ergot (Seaman, 1980).

1.2.5.8 Ensure Uniform Standards

The goal to ensure uniform standards is to reduce the flowering period since flowers produced by late tillers have an increased chance of becoming infected by ergot (Bailey et al., 2003; Seaman, 1980). Seed with good germination, planted at a consistent depth and balanced soil fertilizer will increase the uniformity of the cereal crop and thus decrease its susceptibility to ergot. Soil nutrients are heavily involved in ergot susceptibility as infection has been found to be more severe on copper- and boron-deficient crops (R. D. Graham, 1983). These deficiencies lead to the development of smaller anthers and pollen sterility (R. Graham, 1975) which causes the flowers to open and stay open longer remaining susceptible until they are fertilized (Menzies & Turkington, 2015; Schumann, 2000). Excessive or untimely use of herbicides on cereal crops can result in plant injury (Seaman, 1980) which can also cause the flowers to remain open for longer periods of time.

1.2.5.9 Sprays

Herbicides when used at the proper time and rate can reduce flower sterility (Bailey et al., 2003). It is important to apply the herbicide at the label recommended growth stage and only use the minimum rate needed to control problem weeds to prevent plant injury (Seaman, 1980). Chemical control with fungicides on the other hand is not practical (Bailey et al., 2003). Sterol-inhibiting fungicides have been used effectively in grass seed production, however on cereal crops these treatments are not usually economical (Schumann, 2000). Fungicide seed treatments

can make ergot incidence worse as it can delay decomposition of sclerotia which may allow them to persist for longer than a year (Shaw, 1988). It is also noteworthy that fungicides used for this purpose are not registered for use in Canada or for use to control ergot in Canada (Menzies & Turkington, 2015).

No single management technique will be successful in controlling ergot individually. Control measures used in combination will be more successful therefore, ergot management should be approached as an integrated process (Menzies & Turkington, 2015).

1.3 Detection

1.3.1 Methods of Quantification

There are three methods currently being used to quantify ergot contamination levels: count, weight and analytical.

1.3.1.1 Count

Ergot bodies/sclerotia in a working sample are counted. This value is recorded as number of ergot bodies per either mass of grain (#/kg), volume of grain (#/L) or number of grain kernels (#/1000 Kernels).

1.3.1.2 Weight

Ergot bodies/sclerotia in a working sample are weighed. This measurement is presented as the percentage of the net weight of the working sample. This is typically used if kernel count is excessive.

1.3.1.3 Analytical

There are a variety of techniques available to determine the alkaloid concentration of a working sample. The techniques are described below. Some are rapid, but are only able to determine total alkaloid concentration while other techniques involve an extraction prior to measurement, but can usually determine individual alkaloid concentrations. Results are presented as parts per million (ppm – mg/kg) or parts per billion (ppb - µg/kg) (dry weight). An overview of the methods available can be found in section 1.3.3.

While all three methods are currently being used, the count and weight measurement techniques might not be appropriate. A study by Blaney et al. (2009) on ergot in rye noted, ergot concentrations were not related to the sclerotia size or source region the grain was obtained. The European Food and Safety Authority (EFSA, 2005) has also mentioned that alkaloid concentrations are extremely variable and a consistent relationship between the amount of ergot bodies and the total ergot alkaloid concentration cannot be established.

1.3.2 Current Canadian Standards

In Canada, allowable concentrations of ergot in grain can be found using all three methods of measurements.

The Canadian Seeds Regulations found on the Government of Canada Justice Laws Website reports the maximum number of ergot bodies per kilogram of grain. For wheat, durum, barley, rye, triticale and oats at various No. 1 and 2 grades the regulation is anywhere from 1-15 ergot sclerotia depending on grade and grain type (Government of Canada, 2015) (Table 1.1). Saskatchewan provincial specialists state toxic concentrations of ergot to livestock are 10 ergot bodies per liter or one ergot body per 1000 kernels (Ergot monitoring and management.2011).

Canadian Grain Commission (CGC) uses the weight of ergot as a percentage of the net weight of the sample as a grading factor in the official grain guide. The minimum representative sample is 500 g and the optimum is 1000 g. According to this guide toxic concentrations to livestock are 0.1% ergot material or lower for higher grades (CGC, 2016) (Table 1.2).

The Canadian Food Inspection Agency (CFIA) recommended tolerance concentrations of ergot for cattle as a maximum alkaloid content in feed requires an analytical measurement approach. The CFIA recommends 2-3 mg/kg or 2000 to 3000 parts per billion total maximum ergot alkaloid content in feed (dry weight) (CFIA, 2015).

There are clearly multiple approaches by the Government of Canada to establish Canadian ergot standards. There is however no evidence to support a correlation between each of the different measurement methods to show these standards are comparable. Tittlemier et al. (2015) has suggested there is a relationship between ergot alkaloid concentration and percent

Table 1. 1 The maximum number of ergot bodies allowed in different grades of grain from different grain types in Canada (Summarized from the Justice of Laws Website – Seeds Regulations, February 2015)

Grade Name	Maximum Number of Ergot Bodies per kg					
	Wheat	Durum	Barley	Rye	Triticale	Oats
Canadian Foundation No. 1	1	1	1	2	2	1
Canadian Foundation No. 2	8	8	8	10	10	8
Canadian Registered No. 1	1	1	1	2	2	1
Canadian Registered No. 2	8	8	8	10	10	8
Canada Certified No. 1	2	2	2	4	4	2
Canada Certified No. 2	8	8	8	15	15	8
Common No.1	2	2	2	4	4	2
Common No.2	8	8	8	15	15	8

Table 1. 2 Percent weight of ergot allowed in Canadian grain (summarized from the Canadian Grain Commission – Official Grain Guide, August 2015)

Wheat, Rye, Oats, Triticale, Barley	Threshold ergot levels (% net weight)					
	Grade					
	No.1	No. 2	No.3	No.4	No.5	Feed
CWRS ¹	0.04	0.04	0.04			0.1
CWHWS ²	0.04	0.04	0.04			0.1
CWAD ³	0.02	0.02	0.04	0.04	0.1	
CWRW ⁴	0.04	0.04	0.04			0.1
CWSWS ⁵	0.04	0.04	0.04			0.1
CWES ⁶	0.04	0.04				0.1
CPSW ⁷	0.04	0.04				0.1
CPSR ⁸	0.04	0.04				0.1
CWGP ⁹	0.1	0.1				
CER ¹⁰	0.04	0.04	0.04			0.1
CERS ¹¹	0.04	0.04	0.04			0.1
CEHRW ¹²	0.04	0.04	0.04			0.1
CESRW ¹³	0.04	0.04	0.04			0.1
CEAD ¹⁴	0.02	0.02	0.04			0.1
CEHWW ¹⁵	0.04	0.04	0.04			0.1
CEWW ¹⁶	0.04	0.04	0.04			0.1
CESWS ¹⁷	0.04	0.04	0.04			0.1
CEHWS ¹⁸	0.04	0.04	0.04			0.1
Canadian Western Rye	0.05	0.2	0.33			
Canadian Western Oats	Nil	0.025	0.025	0.05		
Canadian Eastern Oats	Nil	0.05	0.05	0.1		
Triticale	0.025	0.05	0.1			
General Purpose Barley	0.05	0.1				
Select Malting Barley	Covered, Hulless, 2- & 6-row: 0.025					
Select Food Barley	Covered, Hulless, 2- & 6-row: 0.02					
Western Mixed Grain	0.1					
Eastern Mixed Grain	0.25					

¹Canada Western Spring, ²Canada Western Hard White, ³Canada Western Amber Durum, ⁴Canada Western Red Winter, ⁵Canada Western Soft White, ⁶Canada Western Extra Spring, ⁷Canada Prairie Spring White, ⁸Canada Prairie Spring Red, ⁹Canada Western General Purpose, ¹⁰Canada Eastern Red, ¹¹Canada Eastern Red Spring, ¹²Canada Eastern Hard Red Winter, ¹³Canada Eastern Soft Red Winter, ¹⁴Canada Eastern Amber Durum, ¹⁵Canada Eastern Hard White Winter, ¹⁶Canada Eastern White Winter, ¹⁷Canada Eastern Soft White Spring, ¹⁸Canada Eastern Hard White Spring

of ergot by mass however, this relationship might be more analytically relevant than biologically relevant.

1.3.3 Analytical Techniques

1.3.3.1 Enzyme Linked Immunosorbent Assay

An Enzyme Linked Immunosorbent Assay (ELISA) is a plate based assay which uses antibodies and a color change to detect and quantify substances. The ELISA is an attractive option as it is a rapid technique which does not require expensive equipment (Crews, 2015). This makes the ELISA easy to use and adaptable to non-laboratory environments (Flieger et al., 1997). On the down side, most ELISA techniques are only able to detect certain ergot alkaloids (Mai & Li, 2013) where the specificity for ergot alkaloids varies depending on the antibody used (Scott, 2007). The ELISA methods are also not specific for individual ergot alkaloids resulting in cross reactivity and a of lack specificity (Crews, 2015; J. Strickland et al., 2011). This is associated with the common ring system of all ergot alkaloids (Flieger et al., 1997).

While the ELISA is rapid and inexpensive it is less specific and less quantitative than other analytical methods where knowledge of individual alkaloid toxicity is required. Therefore, commercially available ELISA kits would be better suited for the screening of ergot alkaloids in agricultural crops, grain flour and feedstuffs (Scott, 2007; J. Strickland et al., 2011).

1.3.3.2 Near Infrared Spectroscopy

Near Infrared Spectroscopy (NIR) can be used as a screening tool for ergot bodies in grain samples. Ergot sclerotia can be detected and quantified in cereals based on differences in fat, starch or crude protein content between the ergot and healthy grain (Crews, 2015). Vermeulen et al. (2013) was able to detect and quantify ergot contamination with an ergot net weight concentration of 0.02%. A few advantages have been noted by Vermeulen et al. (2013) and Crews (2015) with using NIR: 1- A higher volume of samples can be analyzed in a shorter period of time; 2- NIR can distinguish sclerotia from other visually similar shaped and colored plant structures and debris; 3- This method does not require secondary or tertiary sampling before analysis which immensely reduces sampling error, a downfall with most analytical methods. On a down side,

NIR typically determines a percent weight concentration of ergot rather than an alkaloid concentration. However, Roberts et al. (2005) was able to determine total alkaloid content of tall fescue by calibrating using immunoassay. Overall NIR could be a useful analytical screening tool to detect samples positive for ergot which could be further analyzed by methods to determine the alkaloid concentration.

1.3.3.3 Separation

Separation of the ergot alkaloids allows one to determine alkaloid concentrations of individual alkaloids rather than only a total ergot alkaloid concentration. Different methods have been and can be used to separate ergot alkaloids which are described below.

1.3.3.3.1 Thin Layer Chromatography

Thin Layer Chromatography (TLC) uses glass plates covered with a layer of silica gel and a mobile solvent system to separate compounds in a mixture. Components with varying in solubility will migrate and be present at different locations on the plate. The TLC separation method was the major technique used in the 1960's and 1970's and was able to separate most of the ergot alkaloids (EFSA, 2005). While TLC has been used for identification of *Claviceps* ergot alkaloids, it requires relatively high concentrations of alkaloid (mg/kg (ppm)) for detection (McLaughlin, Goyan, & Paul, 1964). It also has limited separation ability (Mohamed, Gremaud, Tabet, & Guy, 2006). Due to these limitations TLC may be useful as a qualitative identification of alkaloids, but not quantitative assessment. In order to quantify ergot alkaloids, TLC must be paired with a detection technique which are described in section 1.3.3.4. It has been suggested however that TLC could be used as a technique in developing countries (Scott, 2007).

1.3.3.3.2 Gas Chromatography

Gas Chromatography (GS) employs a gas as the mobile carrier medium to separate compounds. Gas Chromatography alone or in combination with a detection technique is rarely used for ergot analysis (Flieger et al., 1997). Several drawbacks are associated with GC including: limited molecular weight range, the need for compounds to be volatile or amenable to derivatization, a low loading capacity and instability of some compounds towards heat

(Mohamed et al., 2006). Therefore, the disadvantages of using GS separation on ergot is that ergopeptides have a high molar mass, are non-volatile, have low vapor tension and are susceptible to heat causing them to decompose in a hot injector (Crews, 2015; Flieger et al., 1997; Scott, 2007).

1.3.3.3.3 High-Performance Liquid Chromatography

High-Performance Liquid Chromatography (HPLC) or sometimes referred to as Liquid Chromatography (LC) in short uses pumps to move a pressurized liquid solvent containing the sample mixture through a column where each solute flows through at a different rate producing separation of the components. This method has mostly replaced TLC screening/separation (EFSA, 2005; Krska & Crews, 2008). While HPLC is slower than the ELISA method, it has the advantage of providing selectivity because it separates the compounds before going through a detection system (J. Strickland et al., 2011). Reverse phase chromatography is always used for ergot alkaloids which allows for the separation of the six major alkaloids found in Canada (Crews, 2015). To further enhance LC, ultra-high performance LC can be used which has been shown to have a run time as short as 4.5 minutes (Kokkonen & Jestoi, 2010). High performance LC is currently the best option to separate ergot alkaloids prior to detection. It has proven to be the most useful instrumental analytical method for determination of ergot alkaloids (Mai & Li, 2013).

1.3.3.4 Detection:

As stated previously, LC is the preferred method to separate the ergot alkaloids prior to detection. The major methods for ergot analysis consist of LC coupled with either fluorescence detection or mass spectrometry detection (Crews, 2015). Both detection methods are explained below.

1.3.3.4.1 Fluorescence

Fluorescence detection (FLD) involves using a light beam to excite electrons in molecules causing them to emit light of different intensities which can be measured. Ergot peptides are naturally fluorescent, characterized by a bright-blue fluorescence (Komarova & Tolkachev, 2001), which allows the use of FLD. All six of the major alkaloids found in Canada can be detected using

FLD providing sufficient chromatographic resolution is provided (Crews, 2015). Using HPLC-FLD for analysis of ergot alkaloids Craig et al. (2015) was able to achieve a limit of detection of 11-14 ppb and a limit of quantification of 39-50 ppb depending on the specific alkaloid.

1.3.3.4.2 Mass Spectrometry

Mass Spectrometry (MS) measures the masses and specific fragmentation pattern of compounds within a sample to identify, quantify and confirm the component(s) of interest. Mass spectrometry can be used on its own or in tandem, however, HPLC coupled with tandem mass spectrometry (LC-MS/MS) has become a standard approach for trace quantification and identification of ergot alkaloids (Crews, 2015). The MS/MS uses an electrospray ionization operated in the positive mode ESI (+) for identification of fragments and unknown ergot alkaloids (Crews, 2015; Krska & Crews, 2008). Due to its ability to identify product ions present in classes of compounds HPLC-MS/MS is a powerful analytical tool to identify unknown natural compounds and metabolites generated during biotransformation processes (J. Strickland et al., 2011). Krska et al. (2008) obtained limit of quantifications (LOQ) between 0.17 and 2.78 µg/kg for six ergot alkaloids depending on the alkaloid. Burk et al. (2006) determined LOQ's between 0.1 and 1.0 µg/kg for five ergot alkaloids depending on the alkaloid using HPLC-MS/MS. Coupling tandem MS to an ultra HPLC, Kokkonen et al. (2010) obtained low LOQs ranging from 0.01 – 1.0 µg/kg for wheat. Strickland et al. (2011) has indicated that limits of detection ≤ 0.005 pmol on the column, with chromatographic runs of ten minutes, are possible. Tandem MS can also be used without any LC separation utilizing one stage of the MS to isolate the compound of interest from the matrix and a second stage of MS for analysis (Plattner, Yates, & Porter, 1983). This technique can identify ergot alkaloids directly from extracts of ergot, ergot-contaminated pelleted or ground feed, or flour but there is no individual identification of the alkaloids (Scott, 2007).

1.3.3.5 Prairie Diagnostic Services Equipment

Prairie Diagnostic services (PDS) uses an HPLC-MS/MS for detection of the six ergot alkaloids ergotamine, ergometrine, ergocornine, ergocristine, ergocryptine and ergosine. This analytical equipment has provided an unequivocal identification of the ergot alkaloids (Krska et

al., 2008). All ergot alkaloids are determined using an electrospray ionization operated in the positive mode. The LOQ in PDS is 1.25 ppb ($\mu\text{g}/\text{kg}$) for each of the individual alkaloids.

1.4 Effect of Ergot in Cattle

1.4.1 General Mode of Action and Influencing Factors

The manifestations of ergot alkaloids are related to the structural similarity of the ergoline ring structure to serotonin, dopamine, epinephrine and norepinephrine (Figure 1.4). The similarity to these biogenic amines allows the ergot alkaloids to bind and interact with a number of different receptors (Berde & Stürmer, 1978; Canty et al., 2014). Ergot alkaloids have the ability to act as agonists, partial agonists and antagonists to produce their effects (Berde & Stürmer, 1978).

Limited studies have investigated the pharmacology and kinetics of ergot alkaloids produced by *Claviceps purpurea*, however, significantly more research has been done on endophyte infected tall fescue which produces similar alkaloids to those of *Claviceps purpurea*. Fescue toxicosis and ergotism operate by the same mode of action and are often clinically indistinguishable from each other (Canty et al., 2014; T. Evans, Rottinghaus, & Casteel, 2004). Any clinical differences seen between the two toxicities are most likely related to higher alkaloid concentrations in ergot as compared to endophyte infected tall fescue and the tendency for generally a longer duration of exposure to infected fescue (Burrows & Tyrl, 2012; T. J. Evans, 2011; T. Evans et al., 2004).

Ergotism has been divided into four disease syndromes known as, convulsive, gangrenous, hyperthermic and reproductive (or in general decreased animal productivity) (T. Evans et al., 2004). The form and symptoms an animal exhibits will depend on type and location of the receptor, amount of alkaloid bound to the receptor along with other environmental factors and stressors (J. L. Klotz, 2015). Structural differences between the ergot alkaloids can also influence the degree of receptor binding (J. Klotz, Kirch, Aiken, Bush, & Strickland, 2010). Various strains of *Claviceps purpurea* have different capacities for synthesizing various alkaloids (Barger, 1931), which permits the concentration of each of the ergot alkaloids contained in an ergot body

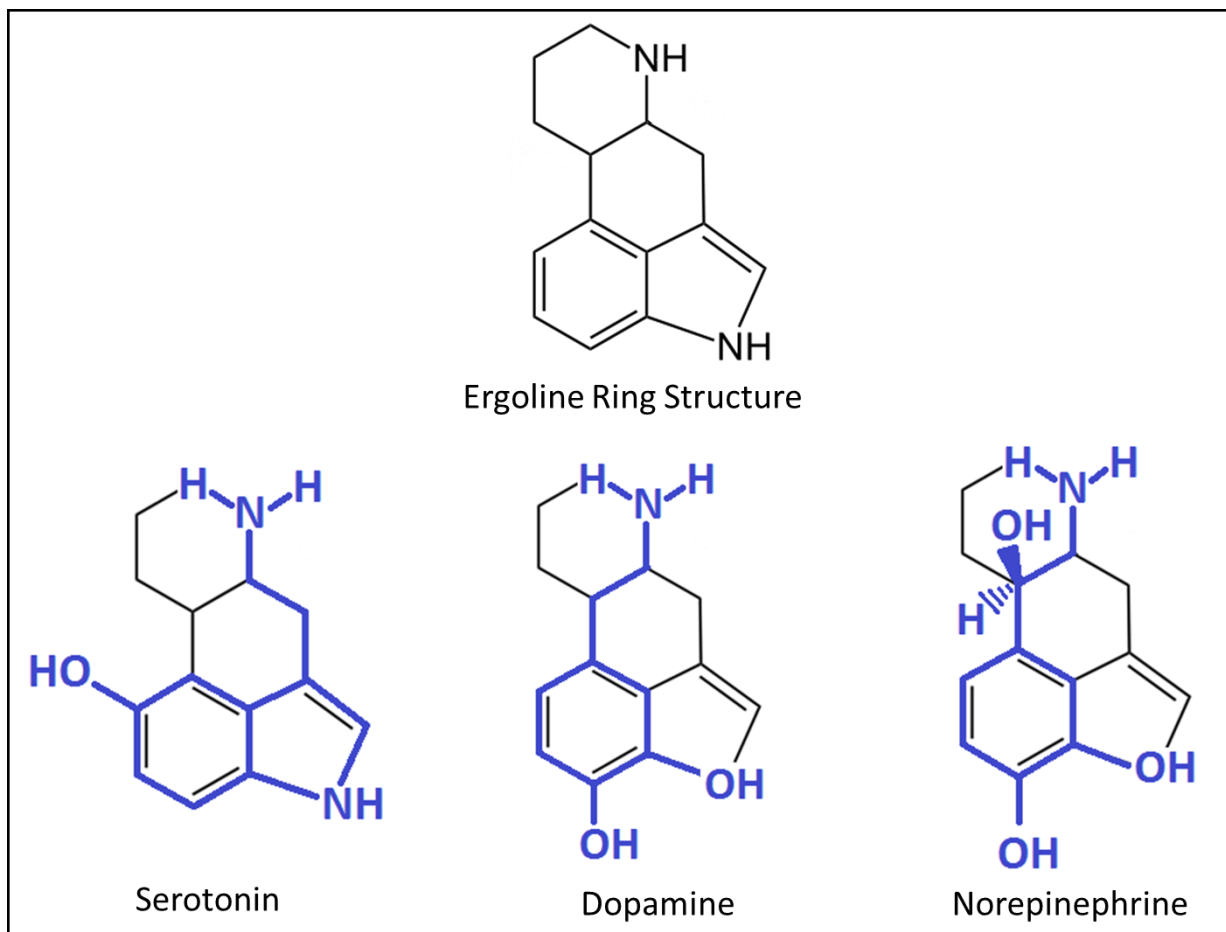


Figure 1. 4 The structural similarities between the ergoline ring and biogenic amines serotonin, dopamine and norepinephrine (in blue). Re-drawn from Berde et al. (1978)

to fluctuate considerably (Belser-Ehrlich et al., 2013; T. Evans et al., 2004).

Overall the extent of pharmacological effects and which form of ergotism will depend on the type of ergot consumed and the ratio of major toxic alkaloids present in the ergot. Risk factors including environmental extremes, animal physiological state and ergot alkaloid dose also play a role in the development of clinical signs of ergotism (Mostrom & Jacobsen, 2011).

1.4.2 Convulsive Form

The word convulsive in terms of ergotism has been used not to denote an underlying epileptic basis, but rather in a descriptive sense (Eadie, 2003). Convulsive ergotism is also sometimes referred to as nervous ergotism and includes the symptoms of writhing, tremors, twisted neck or head tilt, confusion, hallucinations, tingling sensation underneath the skin, sweating, fever lasting for several weeks and death in a variety of mammalian species (Belser-Ehrlich et al., 2013; Eadie, 2003). Ergot alkaloids have the ability to overstimulate the central nervous system as they are serotonin agonists (Eadie, 2003). Vasoconstriction and cerebral ischemia have been considered responsible for some neurologic signs (Carson, 1977). Evans et al. (2011; 2004) has advocated that convulsive ergotism may actually be confused with a toxicosis associated with tremorgens produced by *Claviceps paspali* rather than then ergot from *Claviceps purpurea*.

Overall, this acute convulsive form of ergotism is seldom observed in a variety of species (Bourke, 2000; Lorgue, Lechenet, & Rivière, 1987) and is more likely to occur in sheep, horses or carnivores (Shelby, 1999) rather than in bovine species if this form does prevail. Many of the clinical manifestations are dose dependent and vary with species susceptibility.

1.4.3 Gangrenous Form

Gangrenous ergotism, also referred to as cutaneous ergotism, is the most common form of ergotism in cattle (Burrows & Tyrl, 2012; Shelby, 1999), and is identical to fescue foot (T. Evans et al., 2004). This form of ergotism is caused by vasoconstriction of the peripherals and produces lameness followed by the loss of ears, several centimeters of the tail and hooves (Burrows & Tyrl, 2012; J. L. Klotz, 2015; Mantle, 1969; Mostrom & Jacobsen, 2011; Seaman, 1980; Shelby, 1999).

Initial symptoms usually appear as a result of diminished blood flow to the extremities and manifest as pain and lameness (Carson, 1977; Mostrom & Jacobsen, 2011). Cattle affected may be found standing in ponds or mud wallows in an attempt to relieve the intense burning pain (J. L. Klotz, 2015). Burrows et al. (2012) describes the progression of the condition: the feet become numb, next skin dies and a crack forms over a joint causing the skin to eventually slough finally the tendons to rupture. In severe cases an entire limb, particularly in the pelvic region, may be lost (Burrows & Tyrl, 2012; Shelby, 1999).

Gangrenous ergotism is associated with winter months and tends to be more pronounced in colder climates, when capillary circulation in the extremities is further restricted (Belser-Ehrlich et al., 2013; J. L. Klotz, 2015; Shelby, 1999). Craig et al. (2015) discuss a number of ergot toxicosis cases involving cattle and the effects in weather conditions considered cold ($\leq 1^{\circ}\text{C}$) (Table 1.3). In particular, one case in Canada, during the month of February where temperature was -20°C , tail loss occurred at 473 ppb total ergot alkaloid concentration, dry weight in feed, which is much lower than the current Canadian standard for ergot in feed (Craig et al., 2015).

Early signs of ergotism vascular effects can be reversible if the cattle are removed from ergoty feed (T. Evans et al., 2004). However, if cattle are not removed immediately from the ergoty feed, the animals are at risk of losing the affected tissue which can lead to the loss of the animal or require euthanasia (J. L. Klotz, 2015; Mostrom & Jacobsen, 2011).

The underlying mechanism of gangrenous ergotism is the ability of the ergot alkaloids to cause general blood vessel vasoconstriction thereby diminishing blood circulation and supply (Seaman, 1980; Shelby, 1999; J. R. Strickland, Aiken, & Klotz, 2009). Binding of ergot alkaloids to biogenic amine receptors causes a disruption to the regulatory mechanisms that control and regulate blood flow causing these vasoconstrictive effects (J. L. Klotz, 2015; J. Strickland et al., 2011). This vasoconstriction caused by the ergot alkaloids is associated with D_1 -dopaminergic receptor inhibition and partial agonism of α_1 -adrenergic and serotonin receptors (Canty et al., 2014; T. Evans et al., 2004; Mostrom & Jacobsen, 2011; J. Strickland et al., 2012). Vascular tissues contain these receptors which act as on/off switches to control blood pressure and flow (J. R. Strickland et al., 2009).

Table 1. 3 Clinical Evaluation of Ergot Toxicosis in Cattle (adapted from Craig et al. (2015))

Total Ergot ¹ level observed ² (ppb) ³	Month	Temperature (°C)	Location	Clinical Signs observed
473	February	-20	Canada	Tail loss
1,500	December	1	Oregon	Moderate lameness
2,909	January	-2	Idaho	Decreased feed intake
2,555	February	-5	Oregon	Early term abortions, low milk yield
5,999	January	-5	Idaho	No feed consumption
11,538	April	-4	Canada	Sloughing of hooves
54,916	January	1	Oregon	Early term abortions
62,245	January	-1	Idaho	Hooves sloughing completely off

¹In feed on a dry weight basis

²Includes ergometrine, ergosine, ergotamine, ergocornine, ergocryptine, and ergocristine

³ppb = parts per billion (µg/kg)

Smooth muscle cells are targeted as they are contractile cells around blood vessels which can open or close when activated (J. R. Strickland et al., 2009). There is also *in vitro* evidence found by Strickland et al. (1996) demonstrating that ergot alkaloids may cause excessive smooth muscle growth which would result in decreased diameter in blood vessels and a further decrease in blood flow. Ergometrine and ergocryptine were the two main alkaloids found to cause this growth effect. The decreased blood flow can also result in damage to the blood vessels endothelial lining causing swelling, edema, thrombosis and degeneration development in affected vascular beds (Carson, 1977; Vuong & Berry, 2002). The combination of the smooth muscle and endothelial effects causing changes in blood vessel diameter and subsequent blood flow together result in pain, lameness and eventually gangrene in effected animals (Carson, 1977; J. R. Strickland et al., 2009).

A few studies have examined the duration of these vascular effects. Solomons et al. (1989) noted a persistent contractile response in the bovine dorsal pedal vein in response to ergotamine. Pesqueira et al. (2014) studied the effect of ergopeptine alkaloids, including ergotamine, ergocristine, ergocryptine and ergocornine, on the bovine lateral saphenous veins and found all the alkaloids had persistent contractile response long after the alkaloid was removed from the tissue bath. They noted the ability of these alkaloids to cause vasoconstriction and their possibility to accumulate and potentially delay an animal's recovery. While no studies have been conducted looking at the vascular recovery time of *C. purpurea* alkaloids, Aiken et al. (2013) mentions that in tall fescue, alkaloid-induced vasoconstriction may take greater than 30 days to return to normal.

The amount of ergot required to cause symptoms is unclear and varies depending on the source. The following dietary levels have all been reportedly associated with gangrenous ergotism: 0.3% to 1% sclerotia (Carson, 1977), 0.2% total alkaloid (Burfening, 1994), 1.6 ppm total alkaloid (T. Evans et al., 2004), 10 g ergot/kg (EFSA, 2005) and 473 ppb total alkaloid (Craig et al., 2015) (Table 1.3).

1.4.4 Hyperthermic Form

Hyperthermic ergotism is produced by the same mechanism as gangrenous ergotism with the exception that heat is a factor as opposed to cold. This particular form of ergotism is more related to climate than other forms (J. L. Klotz, 2015), as hyperthermia is associated with poor heat dissipation (Mostrom & Jacobsen, 2011). Hyperthermia in North America is considered a 'typical' form of tall fescue poisoning (Bourke, 2000) and resembles summer slump (T. Evans et al., 2004), however, hyperthermic effects have been noted in livestock fed grain containing ergot produced by *C. purpurea* (Burrows & Tyrl, 2012).

Clinical signs of hyperthermic ergotism include rapid and laboured breathing, elevated body temperature ($>40^{\circ}\text{C}$), open mouth breathing with protruding tongue, excessive salivation and decreased appetite (Burrows & Tyrl, 2012; Carson, 1977; Jessep et al., 1987; Ross, Bryden, Bakau, & Burgess, 1989). These signs are a result of reduced blood flow to peripheral tissues caused by the ergot alkaloids binding to biogenic amine receptors causing vasoconstriction as described in gangrenous ergotism. In cattle, the core body temperature is maintained and regulated by constriction or dilation of vasculature to control blood flow to peripheral tissues to regulate heat dissipation (Aiken & Strickland, 2012). Therefore, animals exposed to ergot alkaloids have a reduced ability to remove body heat in hot climates (Carson, 1977; Rhodes, Paterson, Kerley, Garner, & Laughlin, 1991; Spiers et al., 2012; J. R. Strickland et al., 2009).

While most studies exploring hyperthermic ergotism focus on endophyte-infected tall fescue (Aldrich, Paterson, Tate, & Kerley, 1993; Rhodes et al., 1991; J. Strickland et al., 1996), Ross et al. (1989) confirmed steers had varying degrees of hyperthermia within three days following consumption of ergot from rye infected with *C. purpurea* added to their diet. The cattle were fed a diet of 3.75 g/kg ergot of total diet and exposed to an ambient temperature of 37°C for 8 hours daily. It was reported that the most severely affected animal had extremely high temperatures (up to 41.75°C), consistent panting, protruding tongue and copious drooling. A feeding trial in Manitoba demonstrated that cattle receiving as little as 0.1% ergot by weight in high-protein rations are also affected by heat stress compared to cattle fed ergot-free grain (Seaman, 1980).

Despite the fact that hyperthermic ergotism is not an immediate concern in Canada, the possibility for it still exists and should be valued as a potential concern during our hot summer weather.

1.4.5 Decreased Animal Productivity & Performance

Animal productivity and performance is important for livestock farmers in order to maximize economic returns. Therefore, any reduction in cattle productivity or performance is of great concern. The vasoconstrictive effects of the ergot alkaloids, and reduced blood flow may effect hormonal control involving reproduction, digestion and the central nervous system as well as nutrient delivery and metabolism (J. Strickland et al., 2012). In addition to the reproductive effects, cattle may experience a decrease in both weight gain and milk production due to the ergot alkaloids (Burrows & Tyrl, 2012; Carson, 1977).

1.4.5.1 Decreased Weight Gain and Feed Intake

Reduced weight gain has been established as a consequence of ergot alkaloid consumption from endophyte infected tall fescue (Mahmood et al., 1994; Paterson, Forcherio, Larson, Samford, & Kerley, 1995). Cattle have been shown to gain 30% to 100% less when consuming endophyte infected tall fescue (Paterson et al., 1995). Reduced weight gain and feed intake have also been seen in cattle consuming ergot alkaloids produced by *C. purpurea* (Burfening, 1994; Ross et al., 1989). Burfening et al. (1994) noted that average daily gains decreased linearly with ergot consumption from 0 to 12.7 g/day proposing that the ergot alkaloids affect both energy metabolism and feed efficiency negatively.

The loss of normal control of nutrient delivery/metabolism and waste elimination is suggested to partially explain the reduced average daily gain in affected animals (J. Strickland et al., 2012). Most studies exploring the mechanisms causing decreased weight gain and intake have been done using endophyte infected tall fescue, however, the alkaloids produced by *C. purpurea* would be expected to act in a similar fashion. Serotonergic receptors are involved in the regulation of gut motility, therefore, mobility and passage rate in the gut may be negatively affected by the ergot alkaloids interacting with these receptors (J. L. Klotz, 2015). Studies have demonstrated that ergot alkaloids cause a reduction in volatile fatty acid absorption contributing

to decreased growth and performance (Foote et al., 2013; Koontz, Kim, McLeod, Klotz, & Harmon, 2015). Koontz et al. (2015) also noted animals exposed to ergot alkaloids had a lower average ruminal pH, which may be a cause for the reduced absorption, and increased dry matter weight in the ruminal contents. This is most likely related to reduced particulate passage from the rumen. Overall, it was concluded that cattle consuming ergot alkaloids including ergovaline and ergotamine gain less because they eat less.

1.4.5.2 Reproductive Effects

The reproductive form of ergotism includes decreased prolactin and milk production, decreased progesterone concentrations and other reproductive abnormalities including subfertility and birth effects (Burke, Rorie, Piper, & Jackson, 2001; Carson, 1977; Mahmood et al., 1994). Similar to the weight gain and intake evaluations, most studies have been conducted using endophyte infected tall fescue, however, the alkaloids produced by *C. purpurea* would be expected to act in a similar fashion.

1.4.5.2.1 Decreased Prolactin Production

The hormone prolactin is regulated by dopamine acting on a D₂-dopamine receptor to inhibit secretion (Lamberts & Macleod, 1990). Related to structural similarities between the ergot alkaloids and dopamine (Figure 1.4), the alkaloids are able to stimulate D₂-dopamine receptors in the anterior pituitary in an antagonistic manner to reduce prolactin secretion by lactotropes (Berde & Stürmer, 1978; Burrows & Tyrl, 2012; Canty et al., 2014; T. Evans et al., 2004; J. L. Klotz, 2015; Mostrom & Jacobsen, 2011). Prolactin has been functionally linked, together with other mechanisms, to the initiation and maintenance of milk secretion and mammogenesis (Fell, Chandler, & Goding, 1974; Houdebine et al., 1985). Decline in production has been observed in cattle consuming ergot alkaloids (Carson, 1977; Ilha, Loretto, & Barros, 2003; Munkvold, Carson, & Thoreson, 1997; Strahan et al., 1987). It has been reported in most classes of livestock (Carson, 1977).

Decreased circulating prolactin with increasing ergot alkaloid concentrations suggest a subclinical effect (Stamm, DelCurto, Horney, Brandyberry, & Barton, 1994). For this reason,

decreased prolactin is considered a sensitive indicator of exposure and is commonly used for this purpose (J. L. Klotz, 2015).

Prolactin recovery in grazing steers ingesting ergot alkaloids produced by endophyte infected tall fescue was reported by Aiken et al. (2013) to be between 10 to 15 days once the ergot source was removed. Ross et al. (1989) found serum prolactin concentrations rose again within 24 hours of removal from ergot produced by *C. purpurea*. This discrepancy could be associated with the different source of the ergot alkaloids or the total concentration of ergot alkaloids consumed.

1.4.5.2.2 Decreased Progesterone Production

Progesterone is necessary for the establishment and maintenance of pregnancy (J. Strickland et al., 2011). There have been conflicting opinions concerning the impact of ergot on progesterone involving both beef heifers and cows. Some researchers have demonstrated a decrease in progesterone in cattle with the consumption of ergot alkaloids (Jones, King, Griswold, Cazac, & Cross, 2003; Mahmood et al., 1994; Poole et al., 2016; J. Strickland et al., 2011), while other studies found no effect of ergot alkaloids on progesterone (Burke et al., 2001; Schuenemann et al., 2005). Currently the mechanisms thought to cause the observed decrease of progesterone are highly speculative. Strickland et al. (2011) has suggested that progesterone is a cholesterol based hormone. Therefore, the decrease in progesterone could be due to reduced serum cholesterol. This however contradicts the finding of Burke et al. (2001) where concentrations of cholesterol were reduced in cattle consuming ergot alkaloids, although there was no change in the progesterone concentrations. Another theory explaining the reduction in progesterone is related to the vasoconstrictive effects of the ergot alkaloids causing restricted blood flow to the ovary or corpus luteum preventing the release of progesterone into systemic circulation (Jones et al., 2003). More research needs to be done in this field to determine the exact cause.

1.4.5.2.3 Pregnancy and Birth Effects

Ergot alkaloids cause uterine contractions which are associated with the stimulation of α_1 -adrenergic receptors (T. Evans et al., 2004). The frequency and amplitude of uterine

contractions are increased with higher dosages of ergometrine (Burrows & Tyrl, 2012). In ruminants, uterine contractions during late pregnancy are associated with stimulation of these receptors however, evidence for abortion is vague and rarely reported as an effect of ergot consumption (Carson, 1977; Mostrom & Jacobsen, 2011). Mantle (1969) suggests abortions in cattle are unlikely however, Appleyard (1986) found almost one third of cows in late pregnancy grazing heavily ergot contaminated rye aborted between one to two weeks later.

Fertility and pregnancy rates of cows exposed to ergot alkaloids have had mixed reviews as discussed by Strickland et al. (2011). Some researchers claim that fertility and pregnancy rates are negatively affected by exposure to ergot alkaloids, (Burrows & Tyrl, 2012; Carson, 1977; T. J. Evans, 2011) while others claim it is not affected (Burke et al., 2001; Mahmood et al., 1994; Schuenemann et al., 2005). Mahmood et al. (1994) found that although less estrus activity was seen in weanling heifers grazing high endophyte infected tall fescue, the pregnancy rates were similar to weanling heifers grazing low endophyte infected tall fescue. Strickland et al. (2011) has also observed that follicle size and diameters of the corpus lutea in heifers are not affected by the ingestion of ergot alkaloids (Ahmed et al., 1990; Jones et al., 2003; Seals et al., 2005). A more recent study by Poole et al. (2016) has also concluded that no differences were observed in number of follicles or corpus luteum area of heifers exposed to endophyte infected tall fescue.

Calf birth weight may be reduced following maternal consumption of ergot alkaloids. Watson et al. (2004) found a 15% reduction in the birth weight of calves delivered from cows consuming ergot alkaloids. Klotz et al. (2015) has suggested that related to the vasoactivity of the ergot alkaloids in bovine uterine and umbilical arteries. Blood supply during gestation would be reduced, which would limit nutrient supply to the fetus causing reduced growth during gestation.

Overall, there have been a number of studies and reviews describing the vast range of effects the ergot alkaloids can have on the bovine species, especially in regards to the ergot alkaloids produced by endophyte infected tall fescue. Further research however should be explored in terms of toxicokinetics and effects caused by specifically *C. purpurea* ergot on bovine species as the effects, while assumed to be similar to those of endophyte infected tall fescue, may be different.

1.5 Objectives and Hypotheses

The overall objective of this research was to examine ergot contamination from an analytical, regulatory and biological approach. Three studies were conducted with the following hypotheses and objectives:

Study 1:

- **Hypothesis 1:** The number of ergot sclerotia and weight will be predictive of the ergot alkaloid concentrations in a grain sample
- **Hypothesis 2:** A grinding method that produces finer particles will decrease the measurement variability as compared to a grinding method producing a larger particle size
- **Hypothesis 3:** A 75 mL grain sample will be sufficient to reliably estimate ergot alkaloid concentration

- **Objective 1:** Determine the correlation between ergot sclerotia count and weight as compared to the total ergot alkaloid concentration
- **Objective 2:** Evaluate the effect of the grinding process (i.e., particle size) on ergot alkaloid analysis
- **Objective 3:** Evaluate the impact of sample volume on analytical variability

Study 2:

- **Hypothesis 1:** Ergot alkaloid proportions are similar among different cereal grains and years

- **Objective 1:** Determine the percentage of individual ergot alkaloids across different cereal grains collected in Western Canada over two years

Study 3:

- **Hypothesis 1:** Ergot contamination decreases weight gain in beef cows
- **Hypothesis 2:** Ergot consumption by cows before and after parturition decreases calf weights
- **Hypothesis 3:** Ergot contamination decreases prolactin production in pregnant and lactating beef cows
- **Hypothesis 4:** Ergot contamination increases rectal temperatures in beef cows
- **Hypothesis 5:** Ergot delays the return to cyclicity

- **Objective 1:** Evaluate the impact of ergot consumption in cow-calf performance

CHAPTER 2:
CORRELATION AND VARIABILITY BETWEEN WEIGHING, COUNTING AND
ANALYTICAL METHODS TO DETERMINE ERGOT (*CLAVICEPS PURPUREA*)
CONTAMINATION OF GRAIN

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2.1 Abstract

Ergot alkaloid mycotoxins produced by the fungus *Claviceps purpurea*, are contaminants of cereal crops and grasses. The objectives of this study were to determine the correlation between number of ergot sclerotia and weight compared to the total ergot alkaloid concentration, to evaluate the effect of grinding process (i.e., particle size (PS)) on ergot alkaloid analysis using high performance liquid chromatography – tandem mass spectrometry, and to determine the impact of sample volume on analytical variability. This study demonstrated that correlations exist between both ergot sclerotia count ($R^2 = 0.7242$, $P < 0.001$) and ergot sclerotia weight ($R^2 = 0.9618$, $P < 0.001$) compared to the total alkaloid concentration of 6 ergot alkaloids. However, at ergot alkaloid concentrations below 350 $\mu\text{g}/\text{kg}$ grain, ergot sclerotia count ($R^2 = 0.0002$, $P = 0.956$) and ergot sclerotia weight ($R^2 = 0.0064$, $P = 0.769$) were not correlated to the total alkaloid concentration. A lower variability ($P = 0.041$), defined by coefficient of variation (CV), was observed using a commercial UDY cyclone sample mill (PS = 192 μm , CV = 9 $\mu\text{g}/\text{kg}$) as compared to a household coffee grinder (PS = 516 μm , CV = 66 $\mu\text{g}/\text{kg}$). Total amount and concentration of individual ergot alkaloids varied ($P < 0.05$) among sclerotia of similar weight. For the analytical method, CV was numerically reduced as sample volume increased (97% CV for 75mL to 64% CV for 1000 mL; mean of all concentrations) but increased as sample concentration declined (17% CV for 81678 $\mu\text{g}/\text{kg}$ to 284% for 35 $\mu\text{g}/\text{kg}$; mean of all sample volumes). This implies that analysis of small sample volumes at low ergot alkaloid concentrations may result in highly variable and potentially misleading results. In conclusion, number of ergot sclerotia and weight are unreliable indicators of alkaloid content at ergot concentrations below 350 $\mu\text{g}/\text{kg}$ and particle size influences the variability. An analytical approach with fine grinding (mean PS < 200 μm , 85% particles <400 μm) of a large sample should be used to assess low-level ergot contamination.

Keywords: sclerotia, sample volume, particle size

2.2 Introduction

Ergot is a disease of cereal crops and grasses produced by the fungus *Claviceps purpurea* which infects nearly 600 plant species (Bove, 1970). Ergot contamination is easily identified by purple/black structures (known as sclerotia or ergot bodies) in place of the normal seed head.

Ergot sclerotia are typically shaped cylindrically with round ends, straight to curved and tapered at the distal end (Alderman et al., 1999). Size and shape differ depending on the host plant. In some grains such as rye, sclerotia may be four times larger than the grain kernels, whereas in other grains such as wheat, the sclerotia are similar in size (Seaman, 1980).

The prevalence and severity of ergot contamination depends heavily on the weather (Bailey et al., 2003). Ergot development occurs in the spring and early summer when moisture is available at the soil surface (Seaman, 1980). Secondary metabolites found in the sclerotia produced by the fungus, are known as ergot alkaloids and are toxic to humans and animals. Depending on the geographical region and host plant, the amount and concentration profile of ergot alkaloids will vary (Krska & Crews, 2008; Menzies & Turkington, 2015). The six most commonly found ergot alkaloids produced by *C. purpurea* and tested for in Western Canada are the 8- β -isomers, ergosine, ergocornine, ergocristine, ergocryptine, ergotamine and ergometrine (Krska & Crews, 2008). The 8- α -isomers, identified by names ending in -inine, are not of toxicological concern (Berde & Stürmer, 1978; Pierri et al., 1982) and therefore, are not the major interest in most studies.

There are three methods currently used to assess ergot contamination; count, weight and analytical measurements. The count evaluation is recorded as the number of ergot sclerotia per mass (number/kg), volume (number/L) or per number of grain kernels (number/1000 kernels). The Canadian Seeds Regulations proscribes the maximum number of ergot bodies per kg of Canadian grain. This maximum ranges from 1 to 15 ergot sclerotia per kg depending on the grain type and grade (Government of Canada, 2015). The weight assessment is recorded as the percentage of the net weight of ergot sclerotia as compared to the total sample weight. This method is used as a grading criterion in the official grain guide by the Canadian Grain Commission (CGC, 2016). There are several analytical techniques to determine the ergot alkaloid concentration however, high performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) has provided an unequivocal identification and quantification of the ergot alkaloids (Krska et al., 2008). The Canadian Food Inspection Agency (CFIA, 2015) recommends tolerance concentrations in feed, which varies from 2000 to 9000 $\mu\text{g}/\text{kg}$ depending on livestock species, as a maximum total ergot alkaloid concentration in the sample. All three methods of

evaluation have been used to assess ergot concentrations in Canada, although there has been limited evidence to define a relationship among the three methods.

Regions including the European Union, United Kingdom and the United States have set allowable ergot concentrations in feed as a percent net weight of ergot or “weight concentration”. These concentrations are 0.10%, 0.001% and 0.10-0.30% (depending on grain type) respectively (Coufal-Majewski et al., 2016; Thompson, 2016). Other regions including Australia, New Zealand, Japan and Switzerland do not have recommendations specifically for livestock feed. Most regions do not have regulations relating to the allowable individual ergot alkaloid concentrations, since contamination is always associated with multiple alkaloids.

The objective of this study was to determine the correlation between ergot count and weight as compared to the total alkaloid concentration determination. This study also evaluated the effect of the grinding process (i.e., particle size) on ergot alkaloid analysis using HPLC-MS/MS and the impact of sample volume on analytical variability. The tested hypotheses were 1 – number of ergot sclerotia and weight will be predictive of the ergot alkaloid concentrations in the sample, 2 – a grinding method that produces finer particles will decrease the measurement variability as compared to a grinding method producing a larger particle size and 3 – a 75 mL grain sample will be sufficient to reliably estimate ergot alkaloid concentration in samples. Further, the study design evaluated if the total alkaloid content and the relative amounts of the six alkaloids varied among individual sclerotia.

2.3 Materials and methods

Western Canadian grain samples were obtained from submissions to Prairie Diagnostic Services (PDS; Saskatoon, SK, Canada) and used for sclerotia count, weight and analytical correlation study. Highly ergot-contaminated spring wheat was obtained from a Saskatchewan source. This grain was sieved with a metal strainer with approximately 1 mm² openings to separate the grain dust (i.e., fines) from the grain. The ergot-contaminated fines-free spring wheat was used in the subsequent studies. Ergot-free “clean” (<15 µg/kg total ergot alkaloid contamination) spring wheat was collected from the Canadian Feed Research Center, North Battleford, SK, Canada. Ergot analysis utilized the Krska *et al.* (2008) method. Ergot alkaloid

reference standards for ergosine, ergocornine, ergocristine, ergocryptine, ergotamine and ergometrine were obtained from Biopure/RomerLabs (Union, USA). All analytical reagents used were of HPLC grade.

2.3.1 Sclerotia count, weight and analytical correlation study

2.3.1.1 Sample preparation

Grain samples ($n = 25$) submitted to PDS for routine analysis from July 2015 to January 2016 with a minimum of one visible ergot body were retained for further analysis. After visual inspection, samples were homogenized by shaking the sample within a sealed bag then hand mixed until the sample appeared uniform. In addition to the shaking and hand mixing the grinding process in the preceding steps aided in the homogenization of the sample. Samples larger than 1000 mL ($n = 13$) were homogenized and subsampled into 1000 mL portions. For samples smaller than 1000 mL ($n = 12$), the entire sample was analysed. The data were divided into two groups. The first group included all values from samples collected and the second included only samples with total ergot alkaloid concentrations of 350 $\mu\text{g}/\text{kg}$ or less. Diagnostic laboratories in both Canada and the United States recommend a no effect ergot alkaloid concentration of 100-200 $\mu\text{g}/\text{kg}$ (T. J. Evans, 2011). Therefore, the purpose of grouping samples under 350 $\mu\text{g}/\text{kg}$ was to determine the correlation in the vicinity of diagnostically relevant concentrations.

2.3.1.2 Ergot quantification

Count and weight measurements were performed following separation of the ergot sclerotia from each subsample. Sclerotia were identified by their purplish/black color and their cylindrical shape with round ends. The whole ergot sclerotia were counted and the broken ergot sclerotia (ergot body fractions) were matched with other ergot body fractions to represent a full sclerotia and added to the count. The ergot sclerotia were weighed. Once the count and weight were recorded, the ergot sclerotia were re-homogenized with the grain in the original corresponding subsample. The homogenized subsample was weighed in order to calculate the count and weight concentrations.

Prior to the analytical testing, each subsample was ground entirely using the UDY cyclone sample mill belt drive model 3010-060 (Fort Collins, USA). The ergot alkaloid extraction, clean-up and HPLC-MS/MS analysis were completed following the processes as stated below.

2.3.1.3 Ergot extraction and clean-up procedure

Ground grain (5.0 g), taken at random, was weighed using a Sartorius BP2100 scale (Elk Grove, USA) and a 25 mL volume of 85/15 extraction solvent (85% acetonitrile 15% 10mM ammonium acetate, v/v) was added and the mixture was stirred on a magnetic stir plate for 10 minutes. The supernatant was filtered through a filter paper (Whatman 41 110 μ m, ashless). A 1 mL volume of the filtrate was added to 50 mg Agilent Bondesil-PSA 40 μ m (Santa Clara, USA) and agitated using a IKA-Vibrax shaker (Oakville, Canada) for 5 minutes to clean the matrix. The supernatant was transferred to an Agilent auto-sampler vial with insert (Santa Clara, USA) for high performance liquid chromatography – tandem mass spectrometry analysis (HPLC-MS/MS).

2.3.1.3 High performance liquid chromatography – tandem mass spectrometry analysis

The HPLC-MS/MS quantified the 8- β -isomers ergosine, ergocornine, ergocristine, ergocryptine, ergotamine and ergometrine. The instrument used was an Agilent 1100 HPLC system with a Micromass Quattro Ultima Pt mass spectrometer operated in positive mode. The column used was an Agilent Zorbax Eclipse XDB-C18 narrow bore 2.1 x 150 mm, 5 μ m p/n 993700-902 (Santa Clara, USA). Multiple reaction monitoring was used where the “parent” mass was monitored in the first quadropole and the “daughter” mass in the second quadropole, after the collision cell. Accuracy and precision at three concentrations (10, 3 and 0.75 mg/mL) were verified by the laboratory falling below 11% CV for all six alkaloids. The recovery rates were 82, 75, 87, 81, 101 and 51% for ergosine, ergocristine, ergocornine, ergocryptine, ergotamine and ergometrine respectively. These recoveries obtained are comparable to previous studies (Krska et al., 2008). The limit of quantification was 1.25 μ g/kg for each alkaloid.

Prior to analysis of the samples, three 5 ng/mL standards and a blank consisting of the 85/15 extraction solvent were analyzed to standardize the system. A calibration curve was produced next (0.25, 0.75, 1.25, 2.5, 7.5 and 12.5 ng/mL) followed by all test samples with a run time of 21 minutes per sample. A sample volume of 20 μ L was injected into the system for each

analysis. The results were acquired after cross-checking the sample concentrations against the calibration curve. In the case of highly concentrated samples appearing beyond the calibration curve, a 1:100 and/or a 1:1000 dilution were made and the samples were retested. To obtain the total ergot alkaloid concentration, the concentrations of the six ergot alkaloids, ergosine, ergocornine, ergocristine, ergocryptine, ergotamine and ergometrine, were summed.

2.3.2 Grinder comparison study

2.3.2.1 Sample preparation

Ten ergot sclerotia of the same weight (0.0148 ± 0.0002 g) were randomly selected from the highly ergot-contaminated spring wheat. Each ergot sclerotia was combined with 80 g of clean grain. Samples were stored at room temperature in plastic bags prior to ergot alkaloid extraction and analysis.

To compare the variation presented by particle size, two grinding methods were used. Five samples were individually ground using a household “Black & Decker smart grind” coffee grinder (model# CBG100W). Five additional samples were individually ground using a commercial “UDY cyclone sample mill”.

Five subsamples of 5 g each, taken at random, from the ground samples ($n = 5$ coffee grinder, $n = 5$ UDY sample mill) were analyzed separately. Extraction and clean-up process was completed using HPLC-MS/MS as described above.

Particle size from both grinders was examined using the LA-950 Particle Size Analyzer (PSA; Mississauga, Canada) following the standard operating protocol. All five samples for each the coffee grinder and UDY cyclone sample mill were analyzed for particle size. The PSA is fully automated, and auto dilutes and evaluates the sample. The PSA only requires the user to add sample to the reservoir when indicated to do so. Each sample was shook in a sealed bag prior to being added to the PSA. Once the ground grain was added, the PSA auto diluted the sample. The PSA recorded the particle size and displayed the particle size distribution. When finished, the PSA completed a rinse cycle to prepare for the next sample.

To determine if the particle size produced by the grinding methods had an effect on the analytical assessment, the coefficient of variation (CV) for each set of subsamples ($n = 5$ per sample) was calculated and these values ($n = 5$ per grinding method) were used to compare the two grinders by T-test.

2.3.3 Sample volume comparison to assess analytical variability

In order to evaluate the effect of sample volume and sample concentration on the variability of the analytical concentration determined, four volume sizes were considered at five concentrations.

2.3.3.1 Sample preparation

The highly ergot-contaminated spring wheat (very high group) was mixed with clean grain to create four additional (20 kg) ergot-contaminated grain concentrations to give a total of five ergot alkaloid concentrations (very low, low, mid, high and very high). Each of the ergot-contaminated spring wheat samples were homogenized using a Hobart mixer model D-300-T from the Hobart MFG Company Ltd. (Don Mills, Canada) for 15 minutes. Three subsamples of each 1000, 500, 250 and 75 mL were collected from each of the samples using a glass 1 L (1000, 500, 250 mL volumes) or 100 mL (75 mL volume) graduated cylinder and stored in a labelled plastic bag. This process was repeated a total of three times to obtain a total of 9 subsamples of each volume at each of the five levels of contamination. There is no real theoretical limitation to using volume as a measure for grain. Volume rather than sample weight was employed to provide a more practical, industry comparable approach. Grain weight varies with grain type. Ergot sclerotia weigh less than the grain itself. Using volume as opposed to weight ensured the sample volume remained consistent between the very high and very low ergot contaminated grain concentrations. Each subsample was ground entirely using the UDY cyclone sample mill and stored at room temperature until (4 volumes x 9 subsamples x 5 concentrations = 180) analytical measurements were conducted. The ergot alkaloid extraction, clean-up and HPLC-MS/MS analysis were completed following the process stated previously.

2.3.4 Statistical analysis

All statistical analyses were completed using IBM SPSS statistics 23 (Armonk, USA). Pearson product-moment correlations were used to determine correlations. Independent t-tests were used to determine statistical differences between two groups. To determine differences between groups of more than two, one-way ANOVA followed by Tukey's test for significance was used. A P -value < 0.05 was considered a statistical difference. Levene's test of homogeneous variances was used to assess variability among groups. A one-sample Kolmogorov-Smirnov test was used to test for normality of the data. Coefficients of variation (standard deviation divided by the mean) were calculated to identify the impact of sample volume and concentration on the overall variability.

2.3 Results

2.3.1 Sclerotia count, weight and analytical correlation study

Both the number of ergot sclerotia and weight of ergot sclerotia were plotted against the total ergot alkaloid analytical concentration analyzed using HPLC-MS (Figure 2.1).

The number of ergot sclerotia ($R^2 = 0.95$, $P < 0.001$) and weight of ergot sclerotia ($R^2 = 0.96$, $P < 0.001$) compared to the total ergot alkaloid concentration were highly correlated (Pearson) when all grain samples were included (Figure 2.1a, b). In contrast, samples with ergot alkaloid concentrations, below $350 \mu\text{g}/\text{kg}$, demonstrated no correlations related to the number ($R^2 = 0.0002$, $P = 0.956$) and weight ($R^2 = 0.0064$, $P = 0.769$) of ergot sclerotia and the total ergot alkaloid concentration (Figure 2.1c, d).

2.3.2 Grinder comparison study

The samples ground with the coffee grinder ($n = 5$) had a mean particle size ($\pm\text{SD}$) of $516 (\pm 79) \mu\text{m}$ with 85% of the particles smaller than $1020 \mu\text{m}$. The samples ground with the UDY cyclone sample mill ($n = 5$) had a mean particle size ($\pm\text{SD}$) of $192 (\pm 37) \mu\text{m}$ with 85% of the particles smaller than $395 \mu\text{m}$. The standard deviations (SD) of the total ergot alkaloid concentrations analysed were larger using the coffee grinder than those ground by the sample

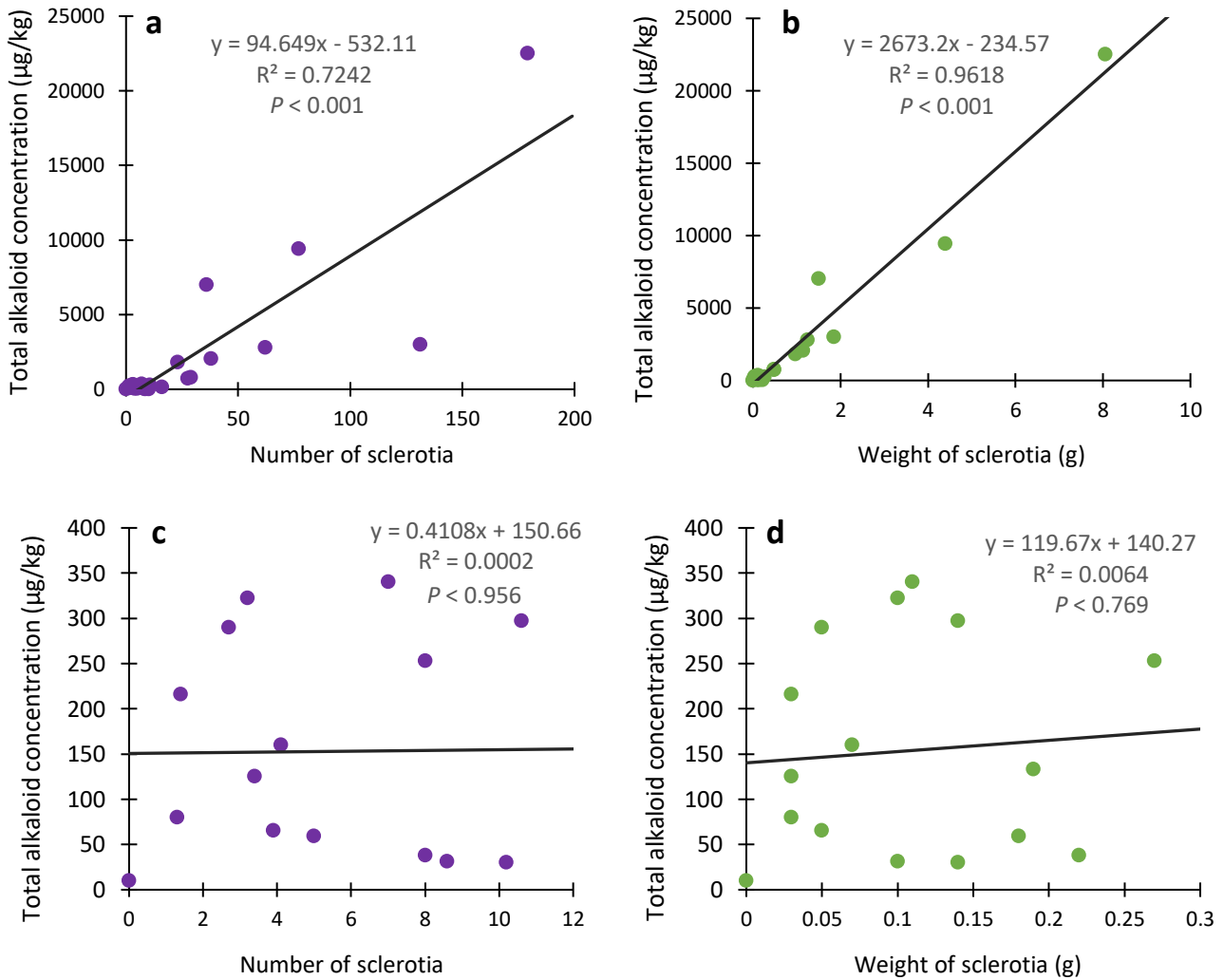


Figure 2. 1 Correlation of number of sclerotia (a, c) and weight of sclerotia (b, d) to the total alkaloid concentration. (a,b) $n = 25$ (12 wheat, 10 barley, 2 durum, 1 triticale) at all concentrations (10 to 22521 $\mu\text{g}/\text{kg}$). (c,d) $n = 16$ (10 wheat, 5 barley, 1 durum) at concentrations below 350 $\mu\text{g}/\text{kg}$. P -value represents Pearson product-moment correlation.

mill (Figure 2.2). Ergot sclerotia of the same weight were found to have different (ANOVA) total ergot alkaloid concentrations. The variation, evaluated using the CV, was higher using the coffee grinder as opposed to the UDY cyclone sample mill (T-test, $P = 0.041$) (Figure 2.3). Further chemical analysis demonstrated that the concentrations of individual ergot alkaloids were also variable among ergot sclerotia of the same weight from the same ergot contaminated grain sample (Figure 2.4). Individual ergot alkaloid concentrations differed (ANOVA) in at least one sample for all alkaloids ($P < 0.001$) except ergocornine ($P = 0.140$).

2.3.3 Sample volume comparison to assess analytical variability

The total ergot alkaloid and ergot sclerotia net weight concentrations of each of the five ergot contamination levels were recorded in Table 2.1.

In general, visual comparison of the coefficient of variations (CV) of different sample volumes at different levels of ergot contamination based on the total ergot alkaloid concentration revealed that variation increases with a decreased sample volume and a lower degree of ergot contamination (Figure 2.5). The CV declined as sample volume increased (97% CV for 75 mL to 64% CV for 1000 mL for the mean of all concentrations) but increased as sample concentration declined (17% CV for very high to 284% CV for very low for the mean of all sample volumes).

Equal variance was not achieved in the very low or low degrees of contamination (Table 2.2). The remaining degrees of contamination, mid, high and very high, were non-significant (ANOVA, $P > 0.05$).

2.4 Discussion

The objectives of this study were to determine the correlation between ergot sclerotia count and weight compared to the total ergot alkaloid concentration, to evaluate the effect of grinding process (i.e., particle size) on ergot alkaloid analysis using HPLC-MS, and to determine the impact of sample volume on analytical variability.

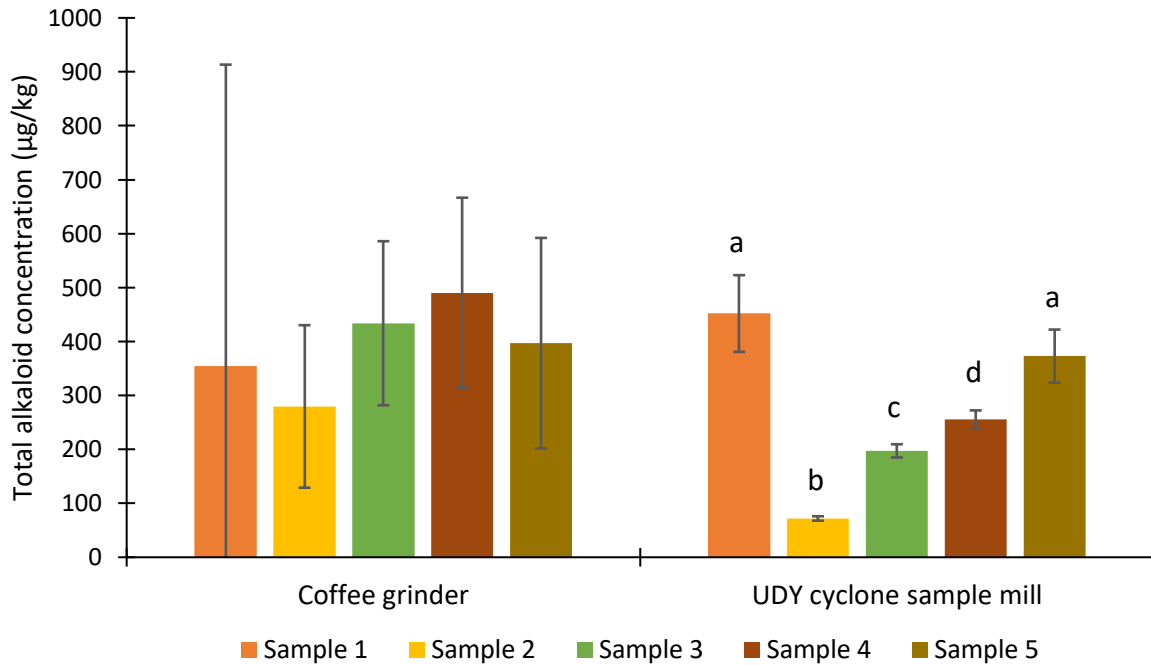


Figure 2. 2 Mean ($n = 5$ coffee grinder; 5 UDY cyclone sample mill) total alkaloid concentration (\pm SD) of a single ergot sclerotia of similar weight (0.0148 ± 0.002 g) ground with 80 g of clean wheat using either a coffee grinder or a UDY cyclone sample mill. Each sample was subsampled 5 times. Different letters denote a statistical difference (ANOVA coffee grinder $P = 0.876$; sample mill $P < 0.001$).

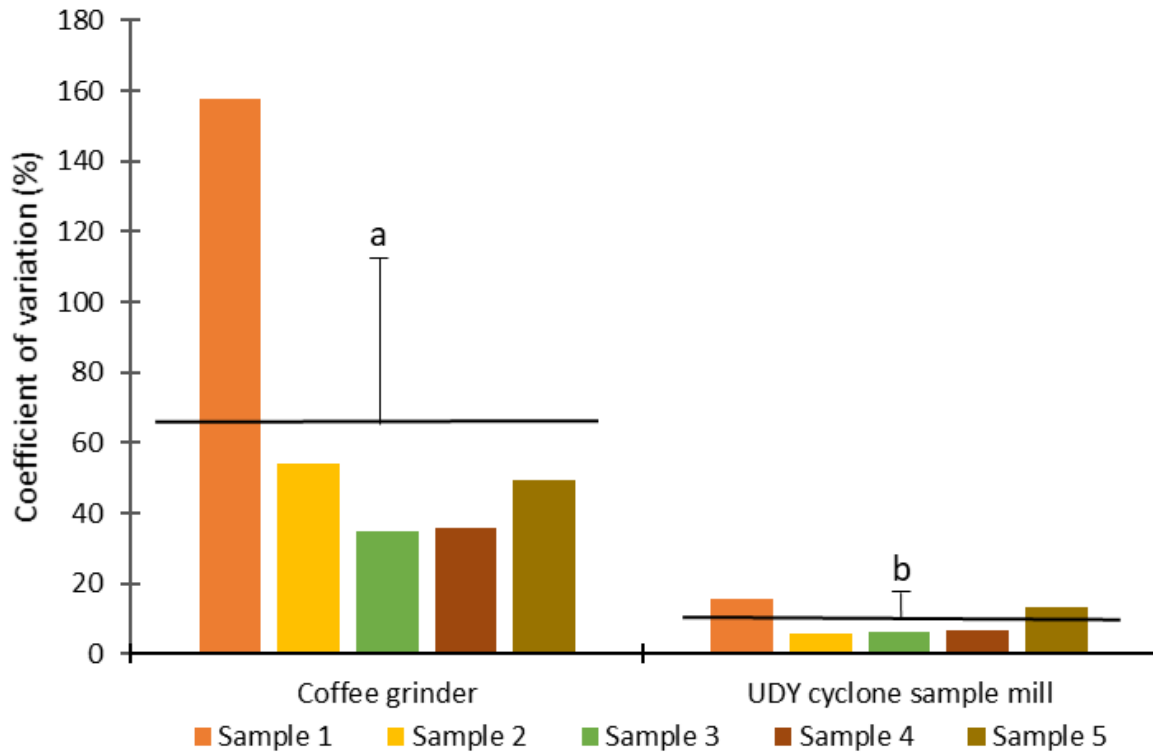


Figure 2. 3 Comparison of the percent coefficient of variation of 5 independent estimations of a single ergot sclerotia of similar weight (0.0148 ± 0.002 g) ground with 80 g of clean wheat using either a coffee grinder or a UDY cyclone sample mill ($n = 5$ subsamples per sample). Vertical bars represent the percent coefficient of variation (CV) of the individual samples. Horizontal lines represent the mean CV (\pm SD) of each grinder. Each replicate was subsampled 5 times. Different letters denote a statistical difference (Independent T-test $P = 0.041$).

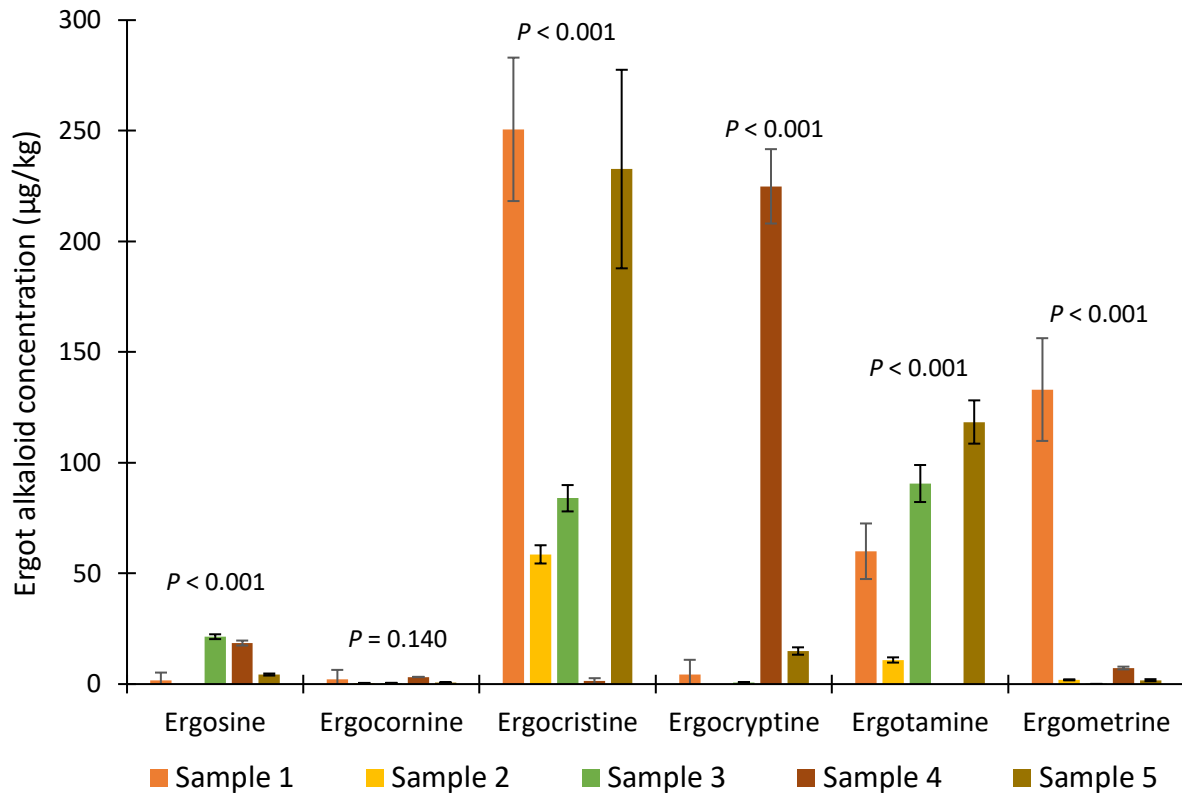


Figure 2. 4 Concentrations of six alkaloids (mean \pm SD) of a single ergot sclerotia ($n = 5$ samples) of similar weight (0.0148 ± 0.002 g), from the same ergot contaminated grain sample, ground with 80 g of clean wheat using a UDY cyclone sample mill. Each sample was subsampled 5 times.

Table 2. 1 Total ergot alkaloid concentration and percent ergot sclerotia by weight of all ergot contaminated wheat samples. (*n* = 36 per degree of contamination)

Degree of contamination	Mean total alkaloid concentration (\pm SD) (μ g/kg)	Percent ergot sclerotia by weight (\pm SD)
Very low	35 \pm 100	0.002 \pm 0.003
Low	419 \pm 1090	0.013 \pm 0.018
Mid	2050 \pm 903	0.095 \pm 0.046
High	8180 \pm 2160	0.432 \pm 0.079
Very high	81700 \pm 13700	4.780 \pm 0.387

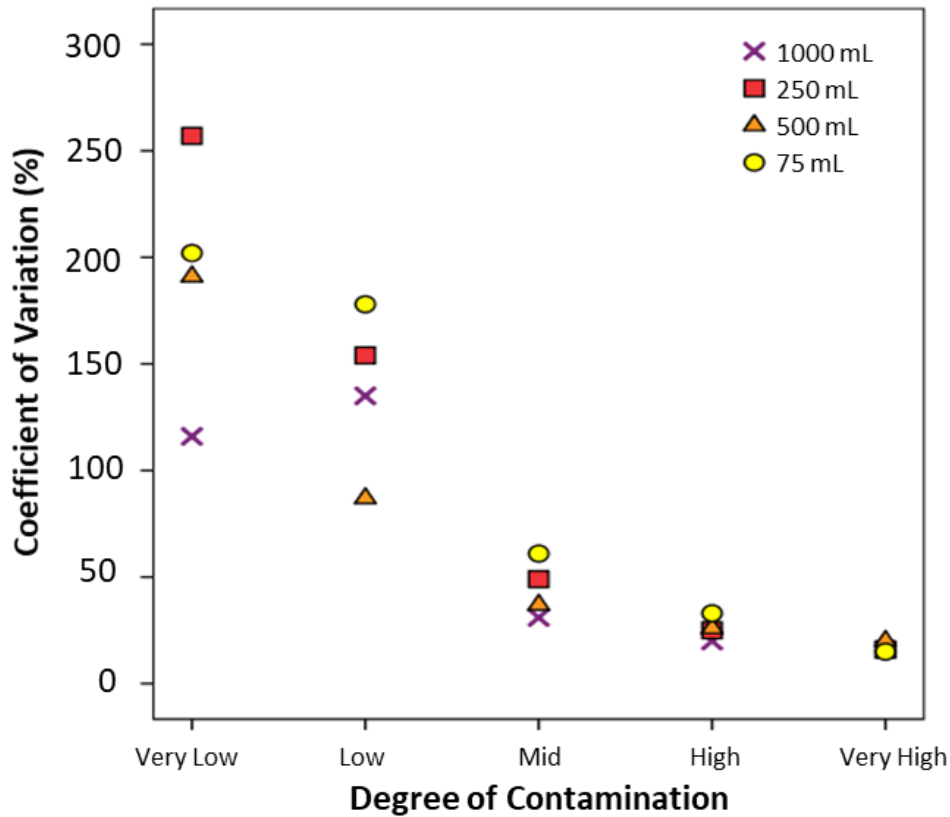


Figure 2. 5 Percent coefficient of variation of the total alkaloid concentration of varying degrees of ergot-contaminated wheat samples at four sampling volumes. *n* = 9 per volume at each degree of contamination. See Table 2.1 for mean contamination concentrations.

Table 2. 2 Statistical values comparing the total ergot alkaloid concentrations of five degrees of ergot-contaminated wheat at the four volume amounts. $n = 36$ for each degree of contamination.

Test statistic	Degree of contamination				
	Very low	Low	Mid	High	Very high
Equal variance ¹	0.025	< 0.001	0.197	0.653	0.950
Volumes with normal data ²	n/a	n/a	75, 250, 1000	75, 250, 500, 1000	75, 1000
<i>P</i> -value ³	n/a	n/a	0.952	0.588	0.418

¹ Levene's test for equal variance

² One-sample Kolmogorov-Smirnov test for normality

³ One-Way ANOVA

The first hypothesis was not supported. The data indicated that ergot count and weight were not predictive of diagnostically relevant ergot alkaloid concentrations. The second hypothesis was supported, confirming that a grinding method that produces finer particles will decrease the measurement variability as compared to a grinding method with a larger particle size. The third hypothesis was not supported, as the study determined that both 75 and 250 mL sample sizes are inadequate to reliably estimate ergot alkaloid concentrations in samples with low ergot contamination. The studies also confirmed that the total alkaloid content and relative amounts of the six alkaloids vary among individual sclerotia.

2.4.1 Sclerotia count, weight and analytical correlation study

Currently, count and weight ergot assessments have been used to determine ergot contamination (CGC, 2016; Government of Canada, 2015). This study was designed to determine the correlation between ergot sclerotia count and sclerotia weight and ergot alkaloid concentrations across the entire range of contaminations in field samples (10 to 22,521 $\mu\text{g}/\text{kg}$; 25 samples, Figure 1a,b) and low-level concentrations that are diagnostically relevant and frequently encountered (0 to 350 $\mu\text{g}/\text{kg}$; 16 samples, Figure 1c,d). In the context of Canadian grain intended for livestock consumption, the recommended tolerance concentrations of ergot alkaloids for cattle set by the Canadian Food Inspection Agency (CFIA, 2015) is 2,000 $\mu\text{g}/\text{kg}$. Over a wide range of ergot alkaloid concentrations, a correlation existed for both ergot count and ergot weight to the total ergot alkaloid concentration. These results were similar to the correlation reported by Tittlemier *et al.* (2015). However, in examination of samples with concentrations under 350 $\mu\text{g}/\text{kg}$, there was no correlation for ergot count or ergot weight as compared to the total ergot alkaloid concentration. This suggests that using ergot sclerotia count or weight assessment may not be an appropriate method to determine the ergot contamination of a sample at low diagnostically relevant field conditions frequently encountered by livestock. Piecing together the ergot sclerotia fragments to estimate sclerotia numbers was very difficult. This difficulty increases unreliability of the counting method. It is recommended that the count, weight and analytical evaluations to determine concentrations should not be used interchangeably.

The concentrations of concern that may produce subclinical symptoms of ergotism, including impaired growth, lactation and thermoregulation (J. L. Klotz, 2015; McMullen & Stoltenow, 2002) appear to be lower than currently available regulatory guidelines for many livestock species (Craig et al., 2015). Ideally it would have been beneficial to extend our lower range past 350 µg/kg to the current standard of 2,000 µg/kg for cattle feed, however, due to the lack of samples it was not possible for this study. The lack of samples also prevented the determination of a correlation for individual grain types rather than a combined overall grain group correlation. By combining all grain types the assumption is made that all grain types will follow the same correlation. This may not be true for all grain types as previously documented (Tittlemier et al., 2015).

The concentrations of alkaloids in a single ergot sclerotia in a grain sample were examined. A single ergot sclerotia of the same weight does not necessarily have the same total alkaloid concentration or relative amounts of individual alkaloid. This further supports the conclusion that count and weight concentration estimates are not as reliable. Our results are supported by the previous studies that have shown significant differences in the total alkaloid content of ergot sclerotia (Lorenz & Hosney, 1979; Schoch & Schlatter, 1985; Wolff & Richter, 1989) reviewed in Krska *et al.* (2008). Furthermore, ergot alkaloid concentrations did not appear to be related to the size of sclerotia (Blaney et al., 2009). These differences may be due to individual fungal strain in a geographical region and the host plant (Krska & Crews, 2008), but may also be due to the environmental conditions during the development of the ergot sclerotia.

There are two variables that must be considered when examining the relationship between concentration of ergot alkaloids and the count or mass of sclerotia. The first being the alkaloid concentration within the sclerotia. The second is the alkaloid concentration within a sample. This discrepancy helps to explain why a meaningful correlation was found over a wide range of ergot alkaloid concentrations but not at concentrations <350 µg/kg. At concentrations below 350 µg/kg, the varying ergot alkaloid concentrations within individual sclerotia compromise the identification of a relationship between count or weight and alkaloid concentration. These lower concentrations generally contain fewer ergot sclerotia in the sample. When considering a wide range of ergot alkaloid contaminations, the different individual ergot

alkaloid concentrations in the sclerotia tend to have less impact, as samples with higher ergot alkaloid concentrations contain more sclerotia. This reduces variability and allows for the development of a better correlation between count or weight and ergot alkaloid concentration at higher concentrations.

2.4.2 Grinder comparison study

Prior to ergot alkaloid extraction and analysis, the sample must be ground, therefore, this study examined the variability associated with sample preparation prior to ergot extraction and analysis by HPLC-MS/MS. The grinder comparison emphasizes the importance of particle size prior to the extraction procedure. The objective prior to weighing the 5 g for ergot alkaloid extraction is to ensure sample homogeneity in particle size. A smaller particle size obtained with the UDY cyclone sample mill preparation, resulted in a much more homogenous sample, and therefore, a less variable and more consistent and repeatable measurements of ergot alkaloid concentration was observed. In less homogeneous samples, as produced with the coffee grinder, there was a greater variation of ergot alkaloid concentration determination. To minimize variability, it is recommended that the grinder used for ergot alkaloid analysis produce a particle size less than 200 μm and with greater than 85% of the particles smaller than 400 μm .

The grinding process is also involved in the analysis of other mycotoxins. It was found that dry-milling wheat samples with ochratoxin A lead to an inhomogeneous subsample resulting in misclassification of acceptable or rejectable wheat lots (Lippolis, Pascale, Valenzano, & Visconti, 2012). However, when the same wheat samples were slurry mixed, a process that produces a smaller particle size (Spanjer et al., 2006), the ochratoxin was homogenous within the subsample. Wheat contaminated with deoxynivalenol appears to be less influenced by particle size as both dry-milling and slurry mixing have been found suitable for sample preparation (Lippolis et al., 2012).

2.4.3 Sample volume comparison to assess analytical variability

The greatest source of variability associated with the determination of the ergot alkaloid concentration under field conditions remains the sampling technique (Crews, 2015), this study examined the potential variability after sampling. The Canadian Grain Commission (CGC) has

suggested that in order to determine ergot sclerotia as a percentage of the net weight of a grain sample, the minimum representative portion is 500 g and the optimal sample size is 1000 g (CGC, 2016). Researchers (Krska & Crews, 2008; Scott, 2007) have identified the lack of research evaluating the sampling procedures for grains or grain products to be analyzed for ergot alkaloids.

Samples received at Prairie Diagnostic Services for ergot alkaloid analysis come in a wide range of volumes, ranging anywhere from less than 50 mL to over 3 L. Since only 5 g is required for extraction, typically only about 75 mL of the sample submitted is ground. The present study demonstrated that the amount of ground sample utilized prior to extraction can influence the estimate of the ergot alkaloid concentration of a submitted sample. The results document that generally, variability increased as sample volume used for analysis and sample concentration declined. Similar results have been found for other mycotoxins including aflatoxin, fumonisin and ochratoxin A (Lippolis et al., 2012; Nowicki & Roscoe, 2010; T. Whitaker, Dickens, Monroe, & Wisner, 1972; T. B. Whitaker et al., 1998).

For ergot alkaloid analysis, the analysis of small sample volumes such as 75 and 250 mL at low ergot alkaloid concentrations of less than 400 µg/kg may be unreliable for accurate interpretation. In some instances, the largest sample volume did not always have the lowest CV. This further demonstrates the high variability and difficulty of obtaining an accurate subsample, particularly at lower ergot alkaloid concentrations. When a sample contains few ergot sclerotia, grinding a small volume may over- or underestimate the total ergot alkaloid concentration of the whole sample. The non-uniform distribution of highly concentrated ergot alkaloids in the sclerotia can lead to inaccurate ergot alkaloid concentration estimates. To reduce this variability, the entire sample submitted should be ground to assess the ergot alkaloid concentration. This recommendation also applies to other mycotoxins such as aflatoxin (A. Campbell, Whitaker, Pohland, Dickens, & Park, 1986; Dickens, Whitaker, Monroe, & Weaver, 1979).

If a 35% CV is considered acceptable any total ergot alkaloid concentration exceeding 8000 µg/kg can be considered reliable so long as at least 75 mL sample was ground prior to extraction. Samples with a total alkaloid concentration between 2000 – 8000 µg/kg must be tested using a 1000 mL volume to ensure the CV remains below 35%. If a total alkaloid

concentration between 2000 – 8000 µg/kg was determined but a smaller sample volume than 1000 mL was ground prior to extraction the sample should be reanalyzed using the full 1000 mL to ensure the determined concentration is reliable. For total alkaloid concentrations at or below 2000 µg/kg the study determined that the volume required to obtain a CV of 35% well exceeds 1000 mL however, an exact volume cannot be determined as the study only examined up to 1000 mL. It should also be noted that a CV of 35% may be considered too high in which case the volumes required for a reliable analysis would also increase.

It has been recommended that nonhomogeneous distribution of ergot sclerotia in grain, requires 1000 – 5000 g of sample for optical assessment (Lampen & Klaffke, 2006). The present study demonstrates that an analytical assessment of ergot alkaloid concentration may require a larger sample depending on the concentration of that sample. This has also been found true for the analysis of other mycotoxins (Lippolis et al., 2012). An optimal volume for analytical analysis exceeds the 1 L volume if the concentration is below 2000 µg/kg. This emphasizes the importance of proper field sampling.

At high ergot alkaloid concentrations, greater than 8000 µg/kg, there is limited variation regardless of the volume of sample analysed. This however, is often irrelevant with most diagnostic cases submitted for ergot alkaloid analysis, since the samples with this degree of contamination are distinctly toxic and greatly exceed concentrations of diagnostic concern. The need for accurate, reliable contamination estimates is less important in these cases.

Researchers have developed mathematical models to describe the sample preparation for different mycotoxins, commodities and grinding methods (Johansson et al., 2000; T. Whitaker, Dickens, & Monroe, 1974; T. B. Whitaker et al., 1998). While an equation has not been developed for ergot alkaloids, the generalization for other mycotoxins is that if the average particle size decreases then the subsampling variance for a given size subsample decreases (T. Whitaker, 2006).

2.4.4 General discussion and conclusions

This study demonstrated that correlations exist between both ergot sclerotia count and ergot sclerotia weight compared to the total alkaloid concentration of 6 ergot alkaloids. However,

at ergot alkaloid concentrations below 350 µg/kg, ergot sclerotia count and ergot sclerotia weight were not correlated to the total alkaloid concentration. A lower variability was observed using a commercial UDY cyclone sample mill as compared to a household coffee grinder. Total amount and concentration of individual ergot alkaloids varied among sclerotia of similar weight. For the analytical method, the CV was numerically reduced as sample volume increased but increased as sample concentration decreased. This implies that analysis of small sample volumes at low ergot alkaloid concentrations will result in highly variable, potentially misleading results.

This study produced three general conclusions pertaining to ergot measurement and analysis: 1 – An analytical measurement should be used as opposed to number of ergot sclerotia or weight of ergot sclerotia to determine the ergot alkaloid concentration. 2 – The grinder used should produce a subsequent particle size equal to or smaller than 200 µm with more than 85% of the particles smaller than 400 µm in order to reduce the risk of unreliable estimates. 3 – In the case of commercial labs, the entire submitted sample should be ground to assess the ergot alkaloid concentration to minimize variability. The findings in this study indicate that regulatory agencies should standardize the methods of evaluation for ergot contamination. Relative to the analytical method the count and weight methods are inferior.

CHAPTER 3:
PROPORTIONS OF ERGOT ALKALOIDS (*CLAVICEPS PURPUREA*) DETECTED IN
WESTERN CANADIAN GRAINS

3.1 Abstract

Ergot alkaloid mycotoxins produced by the fungus *Claviceps purpurea*, are contaminants of cereal crops and cause a wide range of vascular, thermoregulatory, endocrine and neurologic disorders. Depending on the geographical region, climactic conditions and host plant, the relative composition of the individual ergot alkaloids can vary among samples. The objective of this study was to determine if the percentage of individual ergot alkaloids were similar across different cereal grains (barley $n = 39$, rye $n = 7$, triticale $n = 9$, wheat $n = 94$) collected in Western Canada over two years. Ergocristine was the predominant alkaloid accounting for half of total alkaloids in all grain types in Western Canada. This study documented that barley, rye, triticale and wheat collected across Western Canada had similar percentages of ergocornine ($6\% \pm 1$, $P = 0.201$), ergocristine ($48\% \pm 2$, $P = 0.939$), ergocryptine ($17\% \pm 2$, $P = 0.302$) and ergosine ($5\% \pm 0.5$, $P = 0.239$). There were differences between grain types for ergometrine ($P = 0.027$) and ergotamine ($P = 0.011$), which ranged between 6 to 13% and 11 to 24% respectively, of the total alkaloid content in different cereals. Both barley and wheat alkaloid percentages were similar between 2015 and 2016; ergocornine ($7\% \pm 1$, $P = 0.969$), ergocristine ($47\% \pm 2$, $P = 0.680$), ergocryptine ($18\% \pm 2$, $P = 0.572$), ergometrine ($8\% \pm 1$, $P = 0.080$), ergosine ($15\% \pm 1$, $P = 0.119$) and ergotamine ($15\% \pm 1$, $P = 0.189$). The ergocornine percentage was higher in wheat ($P = 0.017$) as compared to barley for 2015/2016 samples. Ergometrine was higher in barley ($P = 0.002$) as compared to wheat for 2015/2016 samples. While two of the alkaloid proportions varied statistically, overall the proportions of the six ergot alkaloids were comparable among the four grain types collected across Western Canada. The proportions of the six ergot alkaloids were found to be similar across Western Canada, which suggests a total ergot alkaloid concentration guideline can be considered acceptable so long as the grain was produced in Western Canada under typical environmental conditions.

Keywords: ergot alkaloid proportions, alkaloid analysis, *Claviceps purpurea*

3.2 Introduction

Ergot is a recognised infection caused by the fungus *Claviceps purpurea* which contaminates cereal crops and grasses. Ergot contamination is easily identified by purplish/black structures on the grain or grass replacing the seeds known as sclerotia. References to ergot date back to 1100 BC but is often associated with the Middle Ages (Haarmann et al., 2009; Schiff Jr, 2006). Today, increasing ergot contamination in places such as Western Canada (Tittlemier et al., 2015), presents a problem not for human ingestion but rather for animal consumption.

Claviceps purpurea produces several toxic ergot alkaloids which can cause hallucinations, convulsions, gangrene, hyperthermia and agalactia when ingested (Burrows & Tyrl, 2012; Haarmann et al., 2009; J. L. Klotz, 2015). While over 16 ergot alkaloids have been identified (Eadie, 2003), the most common alkaloids found and tested for in Western Canada are ergocornine, ergocristine, ergocryptine, ergometrine, ergosine and ergotamine (Krska & Crews, 2008). These six alkaloids are the 8- β -isomers and are considered biologically active (Burrows & Tyrl, 2012). The 8- α -isomers of these six alkaloids, identified by names ending in -inine, are of limited toxicological concern (Berde & Stürmer, 1978; Pierri et al., 1982) and therefore, have less diagnostic relevance.

Ergot development occurs in the spring and early summer when moisture is available at the soil surface (Seaman, 1980). Therefore, the weather is a major factor in the prevalence and severity of ergot contamination and the production of ergot alkaloids (Bailey et al., 2003). Krska et al. (2008) stated the concentration pattern of the ergot alkaloids produced in the sclerotia will vary depending on the geographical region and host plant (Schoch & Schlatter, 1985; Wolff & Richter, 1989).

Most jurisdictions that regulate ergot do so by weight of ergot sclerotia (FAO, 2004) however, improved regulations should be based on the ergot alkaloid concentration (Grusie, Cowan, Singh, McKinnon, & Blakley, 2017). Both Canada and Uruguay provide a recommendation based on ergot alkaloid concentration (CFIA, 2015; FAO, 2004). These recommendations are however based on total ergot alkaloid concentration rather than individual ergot alkaloid concentrations. Regulations, recommended ideal by the European Food and Safety Authority

(EFSA, 2005), would consist of maximum individual ergot alkaloid concentrations rather than a total ergot alkaloid concentration. This approach may be challenged by some toxicologists since it fails to recognize alkaloid interactions related to absorption, excretion, metabolism or possible receptor competition.

With the potential of jurisdictions revising ergot tolerance regulations to include individual ergot alkaloid concentrations, the objective of this study was to determine if the proportion of individual ergot alkaloid concentrations are similar across different cereal grains over two years in Western Canada.

3.3 Materials & Methods

Western Canadian grain samples were obtained from submissions to Prairie Diagnostic Services (PDS) for analysis. The majority of the samples submitted were from Saskatchewan with some samples received from Alberta and Manitoba. Ergot analysis was conducted using the standard operating protocols at PDS following established methods (Krska et al., 2008). Ergot alkaloid reference standards for ergosine, ergocornine, ergocristine, ergocryptine, ergotamine and ergometrine were obtained from Biopure/RomerLabs (Union, USA). All analytical reagents used were of HPLC grade.

3.3.1 Sample collection and preparation

Barley ($n = 39$), rye ($n = 7$), triticale ($n = 9$) and wheat ($n = 94$) samples submitted between November 2014 and September 2016 with a minimum total ergot alkaloid (ergosine, ergocornine, ergocristine, ergocryptine, ergotamine and ergometrine) concentration of 100 µg/kg were included in this study. This concentration was selected because it is well above typical normal background concentrations that may not reflect true time and plant species differences.

All samples were ground using a commercial UDY cyclone sample mill grinder which generates a mean particle size equal to or smaller than 200 µm with more than 85% of the particles smaller than 400 µm.

3.3.2 Ergot extraction and HPLC-MS/MS detection

Ergot extraction and high performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) analysis was completed at Prairie Diagnostic Services (PDS) at the University of Saskatchewan, Canada. Methods followed those as previously specified in Chapter 2 section 2.3.1 (Grusie et al., 2017). The ergot extraction solvent used was 85% acetonitrile 15% 10mM ammonium acetate, (v/v). The equipment used consisted of an Agilent 1100 HPLC system with a Micromass Quattro Ultima Pt mass spectrometer operated in positive mode with an Agilent Zorbax Eclipse XDB-C18 narrow bore 2.1 x 150 mm, 5 µm p/n 993700-902 column (Santa Clara, USA). Only the six 8-β-isomers ergosine, ergocornine, ergocristine, ergocryptine, ergotamine and ergometrine were quantified. A 1.25 µg/kg limit of detection was achieved for each of the six alkaloids.

3.3.3 Statistical analysis

All statistical analyses were completed using IBM SPSS statistics 23 (Armonk, USA). Each of the six alkaloids were calculated as a proportion of the total ergot alkaloid concentration. The proportion data was transformed using arcsine of the square root. A one-way ANOVA was used to find any proportion differences between the grain types for each ergot alkaloid. A Tukey's adjusted least significant difference test was used for post-hoc multiple comparisons. Alkaloids failing homoscedasticity were analyzed using a Kruskal-Willis with a Mann-Whitney U test for significance using a Tukey's adjustment. A two-way ANOVA was used to compare grain type and year ergot alkaloid proportion differences in grains with an *n* larger than 20. Alkaloids failing homoscedasticity were ranked prior to running the 2-way ANOVA.

3.4 Results

3.4.1 Alkaloid ratio comparison between grain types

Each of the six ergot alkaloids analyzed were plotted as their percentage of the total ergot alkaloid concentration for each grain type (Figure 3.1). Proportions of alkaloids ergocornine ($6 \pm 1\%$, $P = 0.201$), ergocristine ($48 \pm 2\%$, $P = 0.939$), ergocryptine ($17 \pm 2\%$, $P = 0.302$) and ergosine ($5 \pm 0.5\%$, $P = 0.239$) were not different between barley, rye, triticale or wheat. The percentage

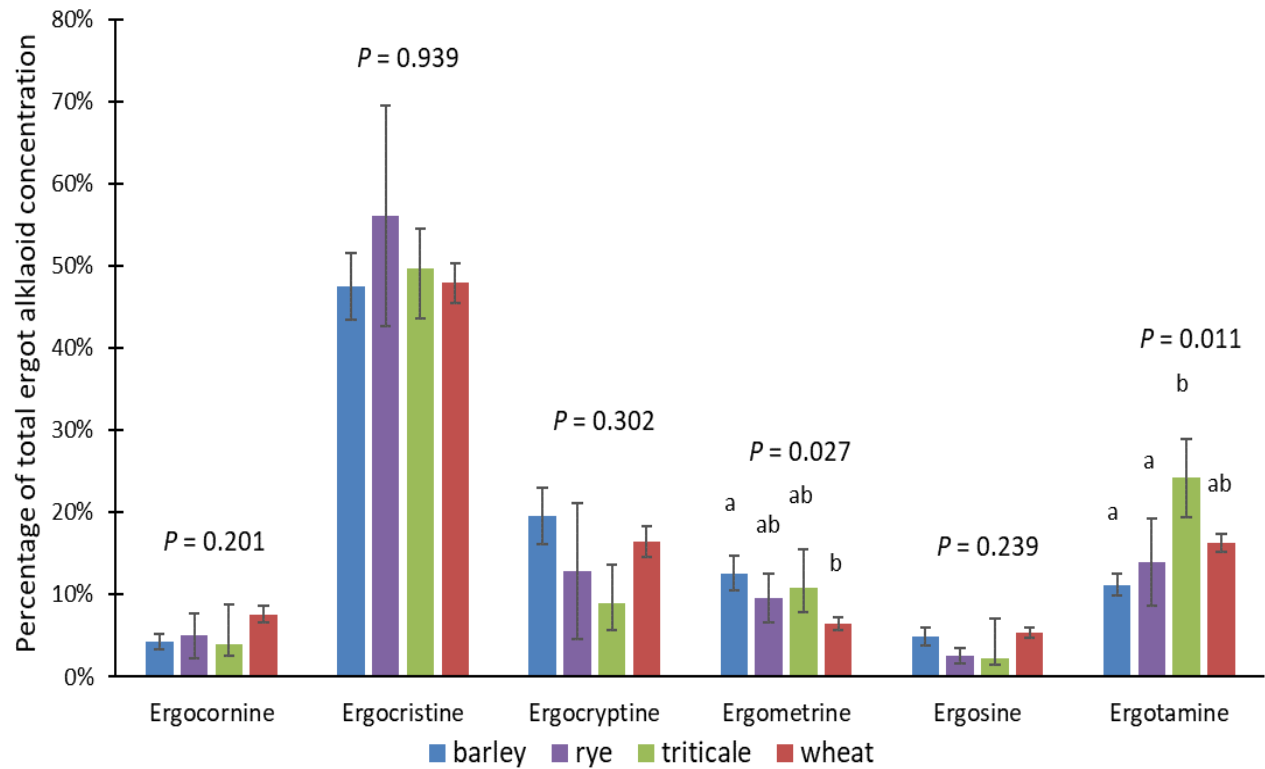


Figure 3. 1 Percentage (\pm SE) of each of the six ergot alkaloids that make up the total ergot alkaloid concentration in different grains sent for analysis to Prairie Diagnostic Services from across Western Canada between 2014 and 2016. $n = 39$ barley; 7 rye; 9 triticale; 94 wheat (ANOVA with Tukey post hoc). Ergometrine failed homoscedasticity therefore a Kruskal-Wallis and Mann-Whitney U post hoc tests using Tukey's adjustment was used.

P = probability of no differences between the grain types
 letters denote a statistical difference

of both ergometrine ($P = 0.027$) and ergotamine ($P = 0.011$) differed in at least one grain type. A higher percentage of ergometrine was found in barley ($13 \pm 2\%$) as compared to wheat ($6 \pm 1\%$); Rye and triticale values were intermediate. A higher percentage of ergotamine was found in triticale ($24 \pm 5\%$) compared to barley ($11 \pm 1\%$) and rye ($14 \pm 5\%$). The percentage of ergotamine in wheat ($16 \pm 1\%$) was similar to the other three grain types.

Ergocristine was the predominate alkaloid found consistently near the 50% value for all grain types analyzed.

3.4.2 Barley and wheat alkaloid ratio comparison

The six ergot alkaloids analyzed were plotted as their percentage of the total ergot alkaloid concentration for both barley and wheat which was separated into samples analyzed in 2015 and 2016 (Figure 3.2). Due to limited sample numbers, rye and triticale were not included in this comparison. There was no interaction between grain type and year for any of the ergot alkaloid comparisons. The ergot alkaloid percentages were not different between the years 2015 and 2016. Grain type differed for the alkaloids ergocornine ($P = 0.017$) and ergometrine ($P = 0.002$). Wheat had a greater percentage of ergocornine and barley had a larger percentage of ergometrine.

3.5 Discussion

This study examined the percentage distribution of the six ergot alkaloids ergocornine, ergocristine, ergocryptine, ergometrine, ergosine and ergotamine in the four grain types barley, rye, triticale and wheat sent for analysis to PDS between 2014 and 2016.

This study determined that for the most part the percentage of the six ergot alkaloids were similar between barley, rye, triticale and wheat. While differences were found between grain types for ergometrine, the percentage difference between the grain with the highest (barley) and lowest (wheat) percentage of ergometrine was only about 5%. The difference found in ergotamine was approximately 10%. These differences may be due to the low number of rye and triticale samples collected. With minimal variation between plant species, the necessity to develop plant-specific tolerance standards appears to be unnecessary.

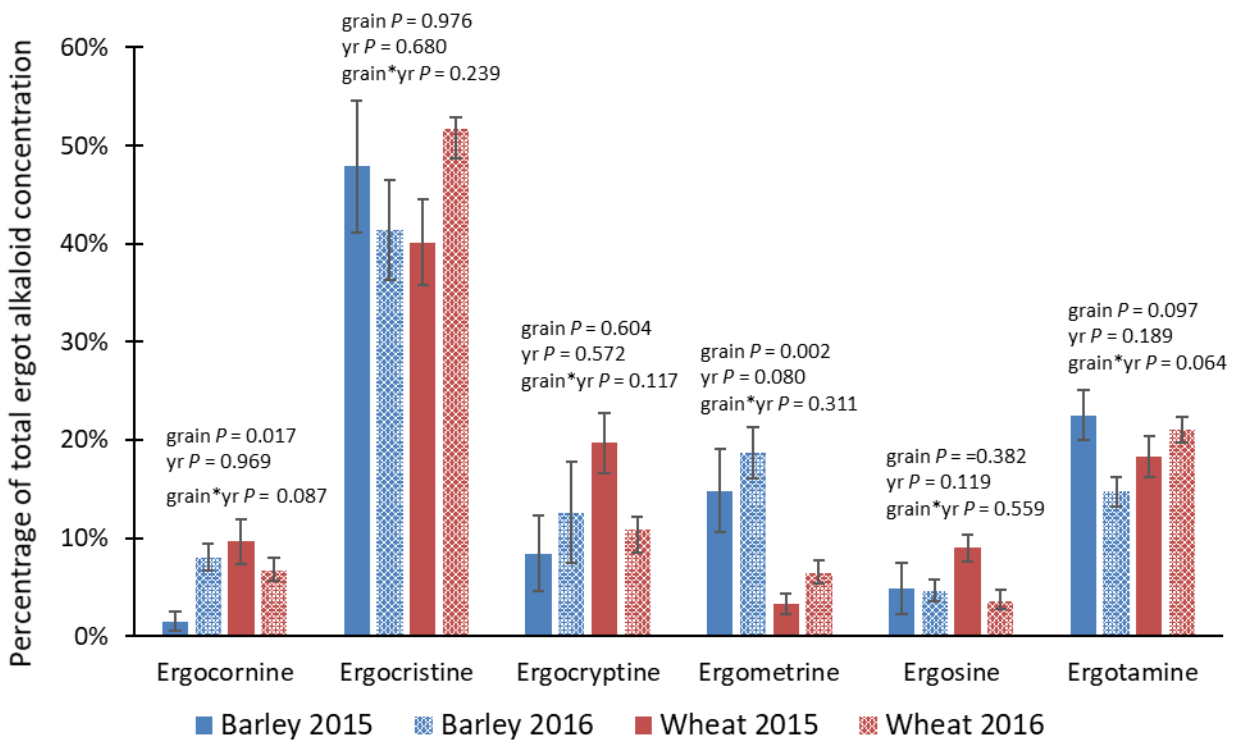


Figure 3. 2 Percentage (\pm SE) of each of the six ergot alkaloids that make up the total ergot alkaloid concentration in Barley and Wheat sent for analysis to Prairie Diagnostic Services from across Western Canada in 2015 ($n = 12$ barley;31 wheat) and 2016 ($n = 24$ barley; 59 wheat) (2-way ANOVA). Ergometrine failed homoscedasticity therefore, the data was ranked before running the 2-way ANOVA.

grain P = probability of no differences between grain types
 year (yr) P = probability of no differences between years
 grain*yr P = probability of no interaction between grain and year

In this study, the percentages of the ergot alkaloids in wheat and barley did not differ between 2015 and 2016. This may imply the weather conditions in the 2015 and 2016 growing seasons were similar. An extension of this study over at least a 10-year period with varied weather conditions would more clearly define the role of moisture and perhaps temperature related to alkaloid production. Other confounding factors such as crop rotation and no till husbandry practices (Schumann, 2000; Seaman, 1980) could also be evaluated.

While the current study found ergocristine made up the largest portion of the six ergot alkaloids, it is important to consider that the samples were collected in Western Canada. The alkaloid content and proportion distribution is heavily influenced on the geographical distribution, growing conditions, strain and host plant (Schoch & Schlatter, 1985; Wolff & Richter, 1989). The relative contribution of each factor and interactions among factors may also be highly variable. The inability to control environmental factors and perhaps husbandry practices makes it difficult to fully assess the impact of this multifactorial agricultural problem.

A previous Canadian study found fairly uniform individual alkaloid compositions in rye, wheat, triticale and barley samples with the exception of rye and barley from the maritime region (Young, 1981a; Young, 1981b; Young & Chen, 1982). It also determined that in Eastern Canada the major alkaloids were ergotamine followed by ergocristine whereas in the West these two alkaloids were reversed. The results of the present study agree with the exception that ergocryptine and ergometrine in some cases exceeded ergotamine. Both the previous Canadian study and a European study (Appelt & Ellner, 2009) determined that within a given field the individual alkaloid composition was highly variable but formed similar alkaloid patterns throughout a region.

This information may play a critical role to determine acceptable ergot alkaloid concentrations in grain for animal feed. Currently, guidelines recommend a maximum 'total' ergot alkaloid concentration (2-9 mg/kg depending on species) (CFIA, 2015). These guidelines fail to consider that all ergot alkaloids may not be similar in terms of potency and ability to cause adverse effects. While no studies to date have determined the effects of each individual ergot alkaloid, studies have determined that the affinities of the ergot alkaloids to bind to receptors

are different. A study done by Klotz et al. (2010) determined that the tall fescue alkaloid ergovaline binds to a receptor in vascular smooth muscle acting in a full agonistic manner, whereas alkaloids ergocristine and ergocornine functioned like partial agonists producing about 40 to 50% stimulation in the same vascular system.

Many toxicologists view individual alkaloid assessment as a low priority, since 'real world' exposure is always associated with multi-alkaloid exposure with varied alkaloid profiles. *In vivo* toxicological investigations in livestock species provide a more realistic measure of toxicology which reflects excretion differences. The extent of these multi-alkaloid interactions may vary considerably between animal species which should be reflected in species specific tolerances. Species differences including sheep, equine, rabbits and dogs have been identified to have biological and behavioural differences (T. Evans et al., 2004).

Once further studies have been conducted to demonstrate the potencies of each ergot alkaloid, ergot alkaloid guidelines can be adjusted to satisfy individual ergot alkaloid variability. This may result in a higher or lower total ergot alkaloid concentration considered safe to use as feed. For example, if two feed samples both contain 2000 µg/kg total ergot alkaloid concentration but the first sample contains over 50% ergotamine and the second sample contains only 10%, the first sample may cause adverse effects if fed to an animal where as the second sample may remain harmless clinically. In a sense, each alkaloid could be assessed using a Toxicology Equivalence Factor (Safe, 1998). The summation of the Toxicology Equivalence Factors would be used to define the tolerance guideline. This approach is frequently used to assess overall toxicity of complex mixtures encountered in water or soil samples (Van den Berg et al., 2006).

The proportions of the six ergot alkaloids were found to be similar across Western Canada, which suggests a total ergot alkaloid concentration guideline can be considered acceptable so long as the grain was produced in Western Canada under typical environmental conditions. The creation of guidelines for individual alkaloids would not be necessary for Western Canada. A total ergot alkaloid concentration guideline may not however, be appropriate for areas

that exhibit extreme or unique variation in the ergot alkaloid profile in which case individual ergot alkaloid guidelines would be more appropriate.

The contribution of each individual ergot alkaloid present in a sample will become more evident as the research and knowledge of the effects of the individual alkaloids expands.

CHAPTER 4:
**ASSESSMENT OF ERGOT (*CLAVICEPS PURPUREA*) EXPOSURE IN PREGNANT AND
POSTPARTUM BEEF COWS**

4.1 Abstract

Ergot alkaloids produced by the fungus *Claviceps purpurea* can cause a range of physiological disturbances which may lead to reduced productivity in cattle. The overall objective of this study was to evaluate the impact of ergot (*C. purpurea*) consumption in beef cow-calf operations. Cows were assigned randomly to treatment groups and were fed ergot containing mixed ration for 9 weeks at 5 µg/kg (control group, $n = 10$), 48 µg/kg (low group, $n = 10$), 201 µg/kg (high group, $n = 10$), and 822 µg/kg (very high group, $n = 6$) ergot alkaloid concentrations of dry matter intake. The study demonstrated that ergot alkaloid concentrations up to 820 µg/kg for 9 weeks did not alter the weight of peripartum and postpartum beef cows ($P = 0.931$) or nursing calves ($P = 0.077$). Concentrations up to 820 µg/kg also had no effect on rectal temperature ($P = 0.163$) or plasma prolactin concentrations ($P = 0.298$) during (overall 48 ± 1.2 ng/mL) or after the ergot treatment (36 ± 1.5 ng/mL) at moderate (5 to 29°C) ambient temperatures. Ergot exposure did not influence the time until first progesterone rise (>1 ng/mL; $P = 0.792$) postpartum or the progesterone concentration at the time of first rise ($P = 0.376$). The size of the first (14 ± 0.6 mm; $P = 0.403$) or second (13 ± 0.5 mm; $P = 0.414$) follicle to ovulate postpartum was unaffected by ergot exposure. The maximum size of the corpus luteum (CL) was 4 mm larger in the 820 µg/kg ergot treatment group compared to the control treatment ($P = 0.028$) for the first ovulation postpartum, but no differences in the CL size were observed between the control and 820 µg/kg groups by the second ovulation ($P = 0.113$). There was no effect of ergot exposure on the number of days until the appearance of the first (43 ± 4 days; $P = 0.949$) or second (52 ± 4 days; $P = 0.984$) CL postpartum. Ergot alkaloid concentrations up to 820 µg/kg did not affect pregnancy rates ($\chi^2 = 0.358$; overall pregnancy rate 91%). In conclusion, this study demonstrated that ergot alkaloid exposure for 9 weeks to concentrations as high as 820 µg/kg did not alter performance in pregnant and postpartum beef cattle at moderate ambient temperatures.

Key words: *Claviceps purpurea*, ergot alkaloids, productivity, prolactin, progesterone, ovarian function

4.2 Introduction

Animal productivity and performance are important for livestock producers to maximize economic return. Animal consumption of ergot alkaloids may cause a range of effects including but not limited to, convulsions, gangrene, hyperthermia, agalactia and reduced weight gain and feed intake (Burrows & Tyrl, 2012; Carson, 1977; J. L. Klotz, 2015; McMullen & Stoltenow, 2002). Animals grazing endophyte-infected tall fescue (*Lolium arundinaceum*) or consuming grain contaminated with *Claviceps* spp. will likely encounter ergot alkaloids, potentially causing adverse effects.

The alkaloids produced in tall fescue, which are commonly found in the United States, differ from those produced in grain infected by *C. purpurea* (Canty et al., 2014). While the clinical manifestations and effects of ergotism and fescue toxicosis are similar (Yates et al., 1985), most studies have focused on fescue rather than grain infected by *C. purpurea*.

The Canadian Food Inspection Agency (CFIA, 2015) has set 2-3 mg of ergot alkaloids/kg of cattle feed as the recommended tolerance concentration. The basis for this recommendation is unclear, however, we have speculated it to be based primarily on the clinical effects such as gangrene which can be viewed excessive, if subclinical disease such as decreased animal productivity and performance are considered. Clinical effects of ergot alkaloids have been documented at concentrations as low as 0.473 mg/kg, which is below the Canadian guidelines (Craig et al., 2015).

The main objective of this study was to evaluate the effects of low-concentration ergot consumption (*C. purpurea*) in cow-calf operations and the recovery from exposure. The endpoints examined included calf and cow weights, rectal temperature, prolactin and progesterone concentrations and ovarian function. Ergot exposure in pre- and postpartum beef cows was hypothesized to decrease both cow and calf weights, decrease cow prolactin concentrations, increase cow rectal temperatures and increase the time for the cows to return to normal cyclicity.

4.3 Materials & Methods

4.3.1 Grain collection and feed preparation

Contaminated ergot wheat screenings were collected from a seed cleaning plant in Weyburn, Saskatchewan using a sampling spear to ensure representative sample collection.

Treatment pellets were created from the ergot-contaminated wheat screenings by the University of Saskatchewan's Canadian Feed Resource Centre in North Battleford, Saskatchewan. Three ergot contaminated pellets at concentrations including 221, 731 and 2981 µg/kg were formulated for the study. Control pellets containing normal background ergot concentrations (18 µg/kg) were purchased from CO-OP Feeds in Saskatoon, Saskatchewan. All pellets were comprised of barley, oat hull, canola and wheat screenings which were formulated to meet the nutritional requirements of the beef cows when fed in combination with the remainder of the total mixed ration.

4.3.2 Ergot alkaloid extraction and measurement

Feed samples were evaluated for ergot alkaloid concentration using an extraction procedure followed by high performance liquid chromatography – tandem mass spectrometry analysis (HPLC-MS/MS) on an Agilent 1100 HPLC system with a Micromass Quattro Ultima Pt mass spectrometer operated in positive mode. An Agilent Zorbax Eclipse XDB-C18 narrow bore 2.1 x 150 mm, 5 µm p/n 993700-902 column was used. Ergot extraction and analysis was carried out as described previously in Chapter 2 section 2.3.1 (Grusie et al., 2017). Five gram samples of ground feed were extracted for 10 minutes using a 25 mL volume of 85/15 solvent (85% acetonitrile 15% 10 mM ammonium acetate, v/v). To clean the matrix, 50 mg Agilent Bondesil-PSA 40 µm was mixed with 1 mL of the filtered extraction. The solution (400 µL) was transferred to an Agilent auto-sampler vial with insert and placed into the HPLC auto-sampling tray. The total ergot alkaloid concentration was determined by summing the six ergot alkaloids, ergosine, ergocornine, ergocristine, ergocryptine, ergotamine and ergometrine.

4.3.3 Experimental design and animal husbandry

This study was approved by the University Committee on Animal Care and Supply before experimentation. Animals in this experiment were cared for in accordance to the guidelines of the Canadian Council on Animal care (Olfert, Cross, & McWilliam, 1993) under the University of Saskatchewan Animal Care Protocol 20140044. Animals were monitored using a humane intervention scoring system developed for the study. The scoring system monitored food and water intake, appearance and behaviour (pain and distress), vital signs and vasoactive and neurological signs.

Thirty-six pregnant Hereford cross beef cows ($576 \text{ kg} \pm 109$; mean \pm SD) were selected based on projected calving date (see Appendix Table A) at the University of Saskatchewan Research Farm. Cows were randomly assigned to treatment groups including, control ($n = 10$), low ($n = 10$), high ($n = 10$), and very high ($n = 6$) ergot alkaloid concentrations. Each of the groups were housed in an outdoor pen for a minimum of 2 weeks before the start of the study. During this period, the animals were acclimated to the new surroundings and introduced to the control pellet ration. Following birth, the calves remained in the same pen as their mothers.

Exposure to the contaminated feed began in April 2015 for a 9-week period and the study concluded at the end of August 2015. The experiment was designed to include 2 weeks of clean pellet consumption (wk -2 and wk -1) to collect baseline measurements on the cows. During the following 9 weeks (wk 0 to wk 8), the animals were fed their designated ergot-contaminated pellets. For weeks 9 and 10 (wk 9, wk 10) the animals were returned to the control pellets. During the final 7 weeks (wk 11 to wk 17) the animals were housed on a grass mix pasture. Pellets were not consumed during the final 7-week period.

Due to the large number of animals and practical considerations, the study was divided into 2 data collection days. Blood samples and other assessment endpoints from control and low groups were collected on Mondays, the high and very high groups were collected on Thursdays. On the collection day, the calves were separated from the cows.

4.3.4 Diets and feeding procedure

Animals were targeted to consume feed at 2% of their body weight (dry matter basis) during the study. The diets were based on the average weight of the animals in each of the groups. The diets consisted of 8.5 kg of dry chopped hay (grass/alfalfa mix), 2 kg of barley for energy and 3.5 kg of experimental pellets. The total average daily intake as fed was 14 kg per animal representing a total daily intake on a dry matter basis of 12.7 kg.

The targeted total daily intake of ergot alkaloids for each of the 4 groups based on the total mixed ration was 0 (control), 50 (low), 200 (high) and 800 µg/kg (very high) on a dry matter basis. To obtain these intake amounts in the animals, the control animals received 3.5 kg of the clean pellets, the low exposure animals received 2.7 kg of the 221 µg/kg total ergot alkaloid pellets and 0.8 kg of the clean pellets, the high exposure animals received 3.5 kg of the 731 µg/kg total ergot alkaloid pellets and the very high exposure animals received 3.5 kg of the 2981 µg/kg total ergot alkaloid pellets.

To minimize animal handling, the animals were group fed. The pellets for all the cows in each group were hand mixed and spread along a feed trough to reduce any feed competition between the cows in the morning. In addition, 70 g of 1:1 (calcium to phosphorous) mineral per animal was sprinkled on top of the distributed pellets. The chopped hay was spread along the trough using a tractor with a weigh scale and the barley was spread on top of the hay for each group in the afternoon to prevent selective consumption of feed type by the cattle.

Animals had ad libitum access to water and a CO-OP 2:1 Beef Cattle Range Mineral – Block (Saskatoon SK, Canada). The animals were administered 3 mL of Vétoquinol Vitamins AD-500, a mix of vitamin A (500,000 IU/mL), D (75,000 IU/mL) and E (5 IU/mL), during the acclimation period before the start of the study.

4.3.5 Animal weights

Animals were weighed approximately 1 hour after receiving their designated pellets. Any calves older than 4 days of age were run through the chute system and weighed. If a calf was

younger than 4 days their weight was obtained on the subsequent week on the appropriate collection day.

Cows were weighed after the calves were moved. Pre-partum cow weights were adjusted for fetal and conceptus weight according to the Nutrient Requirements for Beef Cattle (NRC, 2000).

A baseline weight was calculated to compare the weight change between the animals. This calculation was done by averaging the weights of the first 2 weeks before the study (wk -1 and wk -2). This weight was considered as the baseline value (100 %). A gain or loss of weight will result in a value greater than 100 % or less than 100 %, respectively.

4.3.6 Rectal temperatures

Rectal temperatures were recorded as the cows were weighed using a digital rectal thermometer. The temperature was taken twice to ensure a correct reading. In the case that the two readings were different, a third temperature was taken to determine the average reading. To compare rectal temperature between animals a baseline value was calculated for each animal in the same manner as the baseline weight values.

4.3.7 Blood collection

Blood was collected from the jugular vein of the cows at the same time they were weighed and 1 hour after receiving the experimental pellets. Approximately 20 mL of blood was collected. The side from which the blood was taken was alternated weekly to minimize vascular damage. Collection was done using 18 gauge needles and green-grey collection tubes with heparin separators (BD Vacutainer). The blood collection took approximately 2 hours in total. Following collection, blood samples were centrifuged for 15 minutes at 750 x g at room temperature. Plasma was collected in 5 mL storage vials creating 2 aliquots per animal. The plasma aliquots were stored at -20°C until further analysis.

4.3.8 Prolactin measurement

The prolactin concentration was determined using enzyme linked immunosorbent assay (ELISA) at the University of Saskatchewan Endocrine Lab in the Western College of Veterinary Medicine following the manufacturer's procedure. The ELISA kits used were prolactin bovine 96-well plates (Catalog # CEA846BO) purchased from Cedarlane Labs (Burlington, ON, Canada). The detection range for this kit was 2.47-200 ng/mL and the sensitivity was less than 0.98 ng/mL. The kits intra- and inter-assay coefficient of variation (CV) were 11% and 26%, respectively. To compare prolactin concentrations between animals a baseline value was calculated (in the same manner as baseline weights) for each animal.

4.3.9 Progesterone measurement

Progesterone concentrations were determined via radioimmunoassay (RIA) at the University of Saskatchewan Endocrine Lab at the Western Collage of Veterinary Medicine using ImmuChem Coated Tube Progesterone ¹²⁵I RIA Kits (Catalog # 07-270102; ICN Pharmaceuticals Inc.) following previously used techniques (Pfeifer et al., 2009). The detection range for the assay was 0.15-20 ng/mL with a sensitivity of 0.02 ng/mL. The intra- and inter-assay CV were 9% and 12%, respectively.

4.3.10 Ovarian parameters

All animals in the very high group ($n = 6$) along with the first six animals to calve in the control group were examined twice weekly (Monday and Thursday) starting approximately 2 weeks post-calving using Color Doppler and B-mode ultrasonography. Color-mode, which detects blood flow, was used to confirm the presence of a corpus luteum (CL). A linear 7.5 MHz trans rectal ultrasound probe was used with the MyLabFive[®] ultrasound system (Esoate North America Inc., Indianapolis, IN, USA). Ultrasound examinations took place after blood collection was completed for the day. Animals to be examined were gathered and ran through the locking chute system where video segments of both the left and right ovaries were recorded for further analysis. Immediately after all the examinations were conducted for that day, the recorded video segments were analyzed using the MyLabFive system. For each ovary, all follicles >4 mm and the

corpus luteum (CL; if present) were drawn onto a recording sheet. The sizes in mm of each of the follicles and the CL were measured using the MyLabFive system program and recorded with the drawing. Ultrasound examinations continued bi-weekly for all selected cows until two ovulations (i.e. a follicle was replaced by a CL) were detected. Once all ultrasound examinations were completed, the drawings were used to backtrack from the appearance of the CL to determine which specific follicle ovulated.

4.3.11 Pregnancy rates

Bulls were placed with the cows on week 9 of the experiment. All cows were checked for the presence of a fetus 17 weeks after the bulls were introduced to the cows (wk 26 of the experiment). Physical palpation and ultrasound were used to confirm pregnancy.

4.3.12 Statistical analysis

Animal variables were compared by calculating their change from baseline (wk -2 and wk -1) as described above. The change from baseline data during and following treatment was analyzed using IBM SPSS statistics 23 (Armonk, NY). A *P*-value of < 0.05 was considered a statistical difference. One-way ANOVAs were used to determine statistical differences between the treatment groups for cow weights, calf weights, rectal temperatures, prolactin and progesterone concentrations and time until first progesterone rise. T-tests were used to analyze ovarian follicle size, CL size and days to CL appearance. A chi-squared analysis was used to determine pregnancy rate differences. Weekly data for cow weights, calf weights, rectal temperatures, and prolactin concentrations were analyzed using the proc mixed model repeated measures procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC). The model included analysis of the ergot treatment effect (Tx; 4 levels), time effect (“During” versus “After” ergot exposure; D vs. A) and the interaction between the treatment and time (Tx * D vs. A). Calving month (fixed factor), location (pens versus pasture; random categorical variable) and ambient temperature (random continuous variable) were included in the analysis as confounding covariates. *P*-values of < 0.05 were considered to be significant. The following proc mixed model was initially tested for 11 time-series covariance structure (replacing ‘??’ with simple, cs, csh, ar(1), arh(1), toep,

toep(1), ante(1), hf, un (1), un)and the best model based on the AICC criterion was selected for final analysis. A final statement “lsmeans Tx / pdiff=all adjust=tukey;” was included if P-value for Tx reached significance level.

```
Proc mixed covtest cl plots=all;  
class ID Tx DvsA Week Calving_Month Pasture;  
model Percent_Cow_wt = Tx | DvsA | Calving_Month / DDFM=kr htype=3;  
random Pasture Ambient_Temp;  
  
repeated Week /subject=ID (Tx) type=??;
```

4.4 Results

For analysis, four animals were excluded from the study. One cow was removed from the control group as she was found to be nonpregnant during the study. One cow in the low ergot group died in week one. Post-mortem confirmed the death was caused by uterine perforation by the fetal feet which was unrelated to ergot treatment. The remaining two cows were removed from the high group. One cow from the high ergot group was removed due to nerve injury during parturition and the death of the calf. The second cow-calf pair was removed from the high group due to calving almost a full month later than the cohorts. With these changes, the number of animals in the control group was reduced to 9, the low group was reduced to 9, and the high group was reduced to 8. The number remained unchanged in the very high group at 9 cows.

4.4.1 Feed analysis, animal data and ambient temperatures

The feed components along with the actual ergot concentrations are shown in Table 4.1.

The mean raw data for cow weight, calf weight, rectal temperature and prolactin are shown in Table 4.2. Data compared to baseline values can be found in Tables 4.3, 4.4, 4.5, and 4.6 and Figures 4.1b, 4.2b, 4.3b, and 4.4b. Weekly data can be found in Figures 4.1a, 4.2a, 4.3a, and 4.4a.

The ambient temperature during the ergot feeding period (wk 0 to wk 8) was moderate ranging from 5 to 29°C with an average temperature of 21°C. The temperature after the ergot

Table 4. 1 Ration components and ergot concentration in each treatment diet. Animals were group fed daily.

Total mixed ration (As feed)	Ergot concentration in the pellet ($\mu\text{g}/\text{kg}$)	Amount fed per animal daily (kg)			
		Control	Low	High	Very High
Chopped grass hay	–	8.5	8.5	8.5	8.5
Barley	–	2.0	2.0	2.0	2.0
Control pellets	18	3.5	0.8	0	0
Low pellets	221	0	2.7	0	0
High pellets	731	0	0	3.5	0
Very High pellets	2981	0	0	0	3.5
Total daily intake as fed	–	14.0	14.0	14.0	14.0
Total daily intake dry matter	–	12.7	12.7	12.7	12.7
Ergot alkaloid concentration in ration ($\mu\text{g}/\text{kg}$ of dry matter intake)		5.0	48	201	822

Table 4. 2 Baseline (weeks -1 & -2 average; Mean \pm SD) weights, rectal temperatures and prolactin concentrations of the animals prior to ergot treatment.

Baseline measurements (weeks -1 & -2 average)					
Treatment		Cow weight (Kg \pm SD)	Calf weight* (Kg \pm SD)	Rectal temperature ($^{\circ}\text{C}$ \pm SD)	Prolactin concentration (ng/ml \pm SD)
Control	(n = 9)	554 \pm 123	48 \pm 10	39.0 \pm 0.34	50.5 \pm 15.0
Low	(n = 9)	595 \pm 99	48 \pm 10	39.2 \pm 0.46	55.9 \pm 24.6
High	(n = 8)	574 \pm 91	48 \pm 8	39.1 \pm 0.29	65.6 \pm 32.9
Very High	(n = 6)	578 \pm 131	49 \pm 8	38.9 \pm 0.17	54.9 \pm 18.4

* calculated using the first weight after birth

feeding period (wk 9 to wk 17) was also moderate ranging from 15 to 30°C with an average temperature of 23°C.

4.4.2 Cow weights

Cow weights were not affected by ergot treatment during ($P = 0.931$) or after ($P = 0.471$) the exposure period (Table 4.3). Furthermore, weekly cow weights showed no treatment ($P = 0.892$) or time (during vs. after ergot treatment; $P = 0.171$) effect (Figure 4.1a) after accounting for calving month ($P = 0.017$). Percent weekly cow weights (percent of baseline measurements) showed a treatment effect ($P = 0.002$) and an effect for time (during vs. after; $P = 0.049$) ergot treatment periods (Figure 4.1b). Based on the Tukey's adjusted post-hoc comparisons, the very high ergot group ($104.38 \pm 0.57\%$) had greater percent body weight change from baseline (averaged over during and after treatment period) compared to control ($104.01 \pm 0.34\%$) and low ergot ($102.94 \pm 0.33\%$) groups ($P < 0.05$). The high ($103.95 \pm 0.43\%$) ergot group had a greater percent body weight than the low ergot group, but had a lower value than the control group ($P < 0.05$). The high and very high groups did not differ; similarly, control and low ergot did not differ from each other.

4.4.3 Calf weights

Calf weights were not affected by ergot treatment during ($P = 0.077$) or after ($P = 0.613$) the exposure period (Table 4.4). Weekly and percent calf weights were not affected by ergot treatment effect ($P = 0.529$ and 0.623 , respectively), but did produce an effect for time (during vs. after ergot treatment periods; $P = 0.013$ and 0.037 ; Figure 4.2). Overall, calves were growing throughout the study (caving month $P = 0.078$ and 0.026) but there was no differential effect of treatment (treatment * during vs. after interaction $P = 0.892$ and 0.866).

4.4.4 Rectal temperatures

Cow rectal temperatures were found to be similar for all treatment groups both during ($P = 0.163$) and after ($P = 0.067$) the ergot treatment period (Table 4.5). Weekly rectal temperature data exhibited an interaction between treatment and time (during vs. after treatment periods; $P < 0.001$; Figure 4.3a). Weekly rectal temperatures compared to baseline measurements displayed

Table 4. 3 The mean cow weight expressed as a percent of baseline during and after ergot treatment.

Treatment ¹	During treatment (weeks 0 to 8)			After treatment (weeks 9 to 17)		
	Percent weight of baseline ²	SD	P-value ³	Percent weight of baseline ²	SD	P-value ³
Control (n = 9)	103.7	1.93	0.931	105.4	3.17	0.471
Low (n = 9)	103.0	2.67		102.9	3.99	
High (n = 8)	102.9	3.85		105.1	4.03	
Very High (n = 6)	102.9	4.17		105.9	5.72	

¹Control = 5; Low = 48; High = 201; Very High = 822 µg/kg total daily ergot alkaloid consumption

²Baseline = the average of w-1 and w-2, represented as 100%

³One way Analysis of Variance, P=Probability of no treatment effect

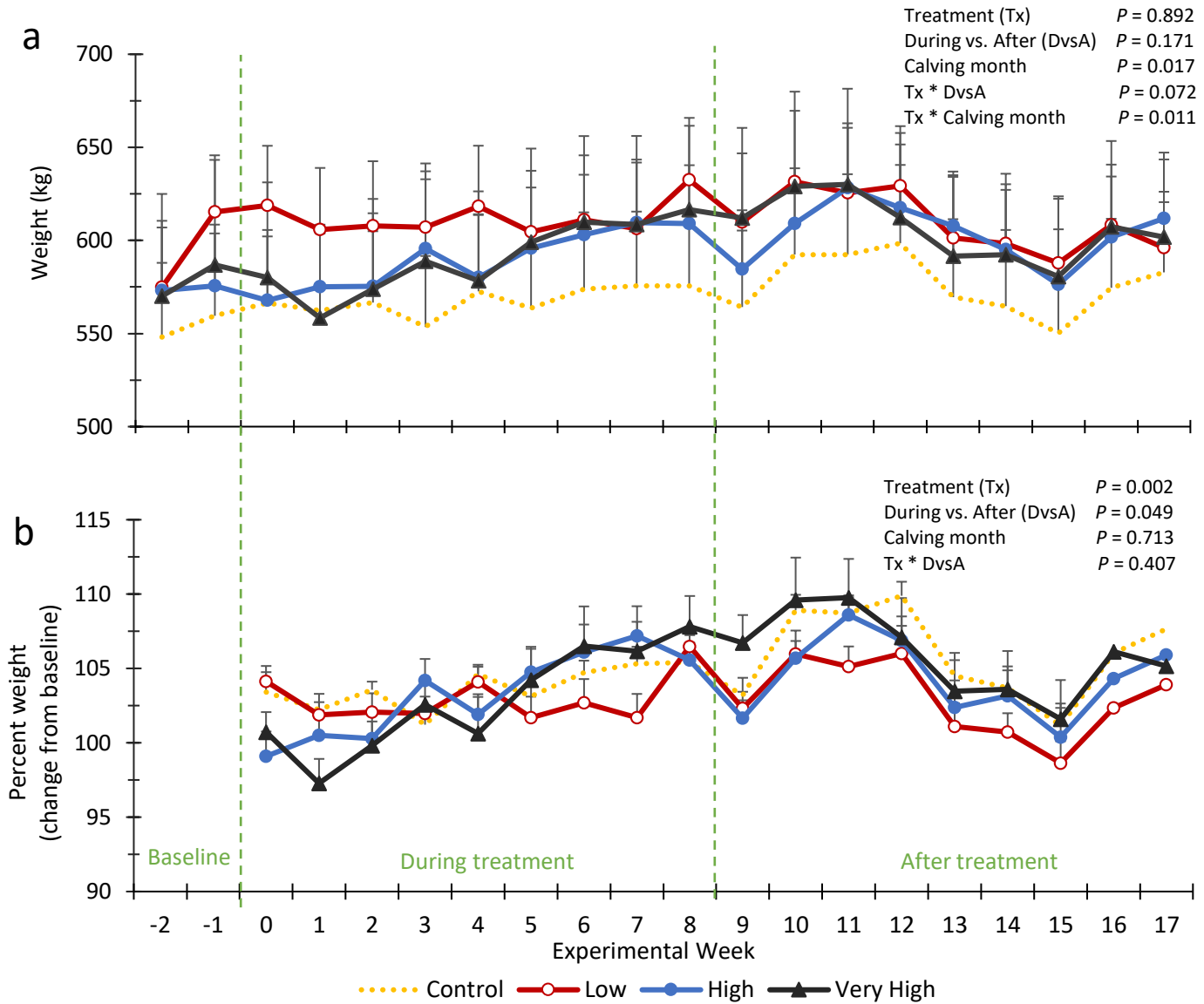


Figure 4. 1. Cow weights during (9 weeks) and after (9 weeks) ergot treatment feeding. Control ($n = 9$) received $5 \mu\text{g}/\text{kg}$, low ($n = 9$) received $48 \mu\text{g}/\text{kg}$ ergot, high ($n = 8$) received $201 \mu\text{g}/\text{kg}$ ergot and very high ($n = 6$) received $822 \mu\text{g}/\text{kg}$ ergot during ergot feeding. All cows received 2 weeks of control diet and 7 weeks of pasture for the duration of the after treatment feeding. Weekly mean ($\pm\text{SE}$) cow weight (a) and percent cow weight change from baseline (b). (Mixed model repeated measures, SAS)

Table 4. 4 The mean calf weight expressed as a percent of baseline during and after ergot treatment of the cows.

Treatment ¹	During treatment (weeks 0 to 8)			After treatment (weeks 9 to 17)		
	Percent weight of baseline ²	SD	P-value ³	Percent weight of baseline ²	SD	P-value ³
Control (n = 9)	136.5	23.9	0.077	219.6	46.9	0.152
Low (n = 9)	147.5	20.4		239.1	41.6	
High (n = 8)	162.6	19.1		261.2	26.1	
Very High (n = 6)	158.4	20.1		251.0	30.9	

¹Control = 5; Low = 48; High = 201; Very High = 822 µg/kg total daily ergot alkaloid consumption

²Baseline = the average of w-1 and w-2, represented as 100%

³One way Analysis of Variance, P=Probability of no treatment effect

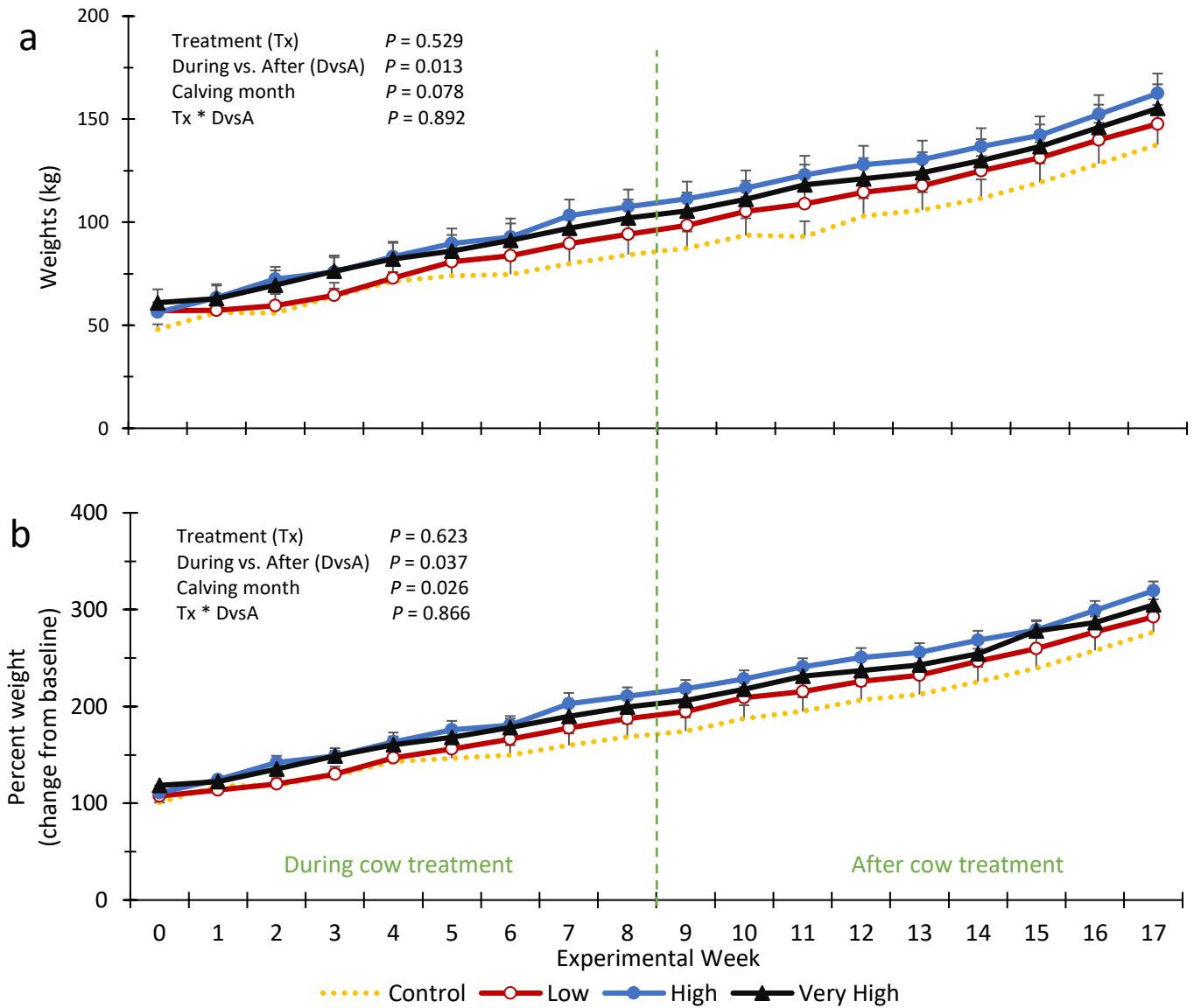


Figure 4. 2 Calf weights during (9 weeks) and after (9 weeks) ergot treatment feeding to the cows. Control cows received 5 $\mu\text{g}/\text{kg}$, low cows received 48 $\mu\text{g}/\text{kg}$ ergot, high cows received 201 $\mu\text{g}/\text{kg}$ ergot, and very high cows received 822 $\mu\text{g}/\text{kg}$ ergot during ergot feeding. All cows received 2 weeks of control diet and 7 weeks of pasture for the duration of the after treatment feeding. Control $n = 9$, low $n = 9$, high $n = 8$ and very high $n = 6$ once all calves were born. Baseline was calculated using the calves weight the first week after calving. Weekly mean ($\pm\text{SE}$) calf weights (a) and percent calf weight change from baseline (b). (Mixed model repeated measures, SAS)

Table 4. 5 Mean cow rectal temperature expressed as a percent of baseline during and after ergot treatment.

Treatment ¹	During treatment (weeks 0 to 8)			After treatment (weeks 9 to 17)		
	Percent rectal temperature of baseline ²	SD	P-value ³	Percent rectal temperature of baseline ²	SD	P-value ³
Control (n = 9)	100.4	0.21	0.163	98.9	0.20	0.067
Low (n = 9)	99.6	0.19		98.3	0.23	
High (n = 8)	99.9	0.33		99.2	0.21	
Very High (n = 6)	99.6	0.20		99.6	0.28	

¹Control = 5; Low = 48; High = 201; Very High = 822 µg/kg total daily ergot alkaloid consumption

²Baseline = the average of w-1 and w-2, represented as 100%

³One way Analysis of Variance, P=Probability of no treatment effect

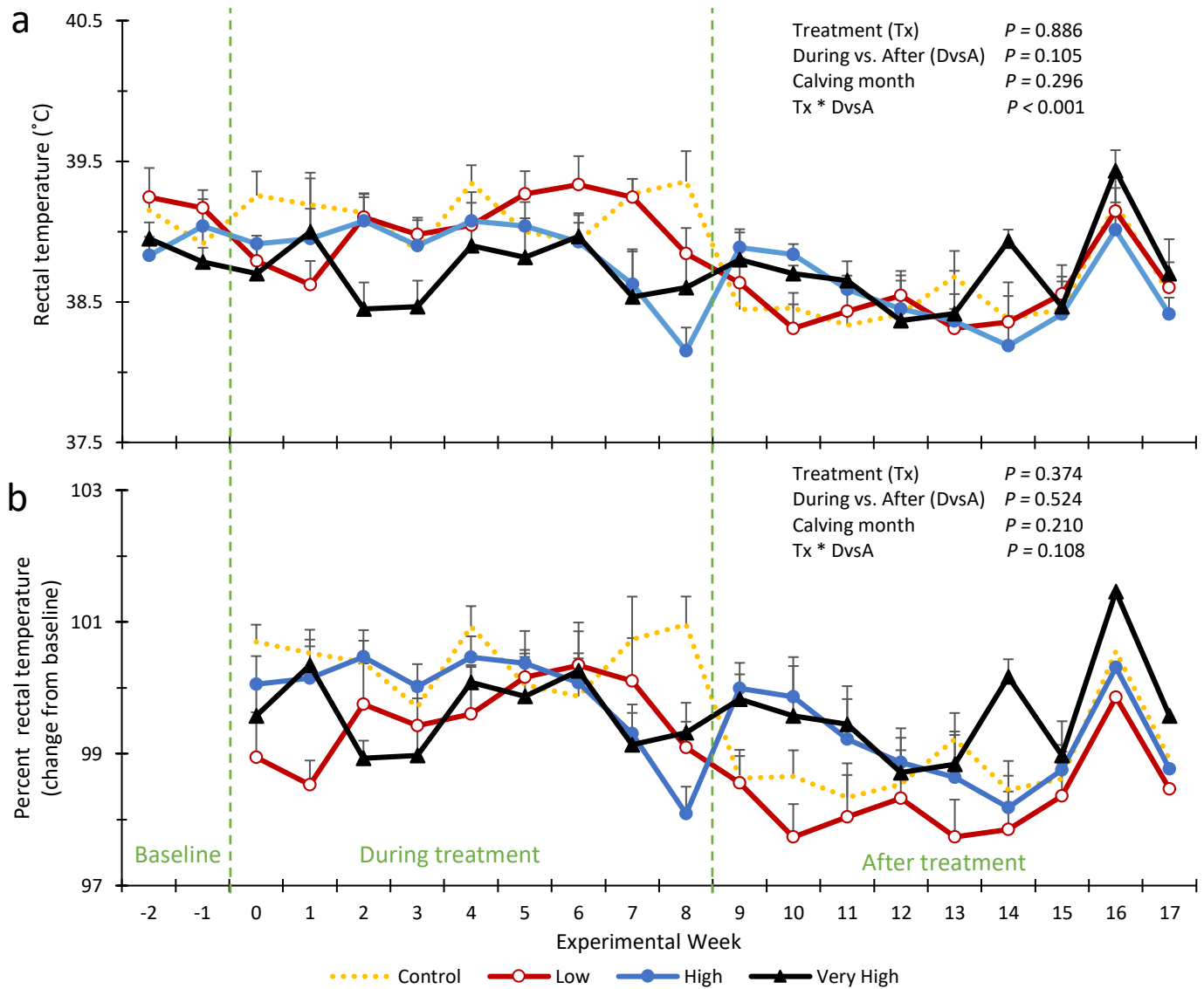


Figure 4. 3 Cow rectal temperatures during (9 weeks) and after (9 weeks) ergot treatment feeding. Control ($n = 9$) received 5 $\mu\text{g}/\text{kg}$, low ($n = 9$) received 48 $\mu\text{g}/\text{kg}$ ergot, high ($n = 8$) received 201 $\mu\text{g}/\text{kg}$ ergot, and very high ($n = 6$) received 822 $\mu\text{g}/\text{kg}$ ergot during ergot feeding. Weekly mean ($\pm\text{SE}$) cow rectal temperatures (a) and percent cow rectal temperature change from baseline (b). (Mixed model repeated measures, SAS)

no interaction between treatment and during vs. after ($P = 0.108$) nor a treatment ($P = 0.374$) or during vs. after effect ($P = 0.524$) (Figure 4.3b).

4.4.5 Prolactin concentrations

Cow plasma prolactin concentrations were not affected by ergot treatment during ($P = 0.298$) or after ($P = 0.870$) the exposure period (Table 4.6). Weekly prolactin concentrations showed no treatment ($P = 0.384$) nor time effect (during vs. after $P = 0.711$; Figure 4.4a). Weekly prolactin concentrations compared to baseline measurements also presented no treatment ($P = 0.431$) nor time effect (during vs. after $P = 0.631$; Figure 4.4b).

4.4.6 Progesterone measurements

The number of weeks until first progesterone rise postpartum (Figure 4.5a) and the progesterone concentration at that first rise (Figure 4.5b) were monitored. A rise in progesterone was considered a concentration greater than 1 ng/ml. Both the number of weeks until first progesterone rise postpartum ($P = 0.792$) and the concentration at that first rise ($P = 0.376$) were not effected by the ergot treatment.

4.4.7 Ovarian measurements

The largest follicle (Figure 4.6a), largest CL (Figure 4.6b) and days until CL was appearance (Figure 4.6c), were recorded by ultrasonography for the first and second ovulations postpartum. No differences were found for the largest follicle observed for the first ($P = 0.403$) or second ($P = 0.414$) ovulation postpartum between the control and very high ergot treatment groups. The size of the CL was found to be larger in the very high treatment group compared to the control group for the first ovulation ($P = 0.028$), however, this difference was not apparent for the second ovulation ($P = 0.113$). No differences were observed in the number of days until the appearance of the CL for the first ($P = 0.949$) or second ($P = 0.984$) ovulation comparing the control and very high treatment groups.

Table 4. 6 The mean cow plasma prolactin concentration expressed as a percent of baseline during and after ergot treatment.

Treatment ¹	During treatment (weeks 0 to 8)			After treatment (weeks 9 to 12)		
	Percent prolactin concentration of baseline ²	SD	<i>P</i> -value ³	Percent prolactin concentration of baseline ²	SD	<i>P</i> -value ³
Control (n = 9)	92.0	14.2	0.298	73.8	26.6	0.870
Low (n = 9)	98.0	25.9		73.0	32.1	
High (n = 8)	83.6	14.8		66.5	21.4	
Very High (n = 6)	81.6	16.5		63.7	30.0	

¹Control = 5; Low = 48; High = 201; Very High = 822 µg/kg total daily ergot alkaloid consumption

²Baseline = the average of w-1 and w-2, represented as 100%

³One way Analysis of Variance, P=Probability of no treatment effect

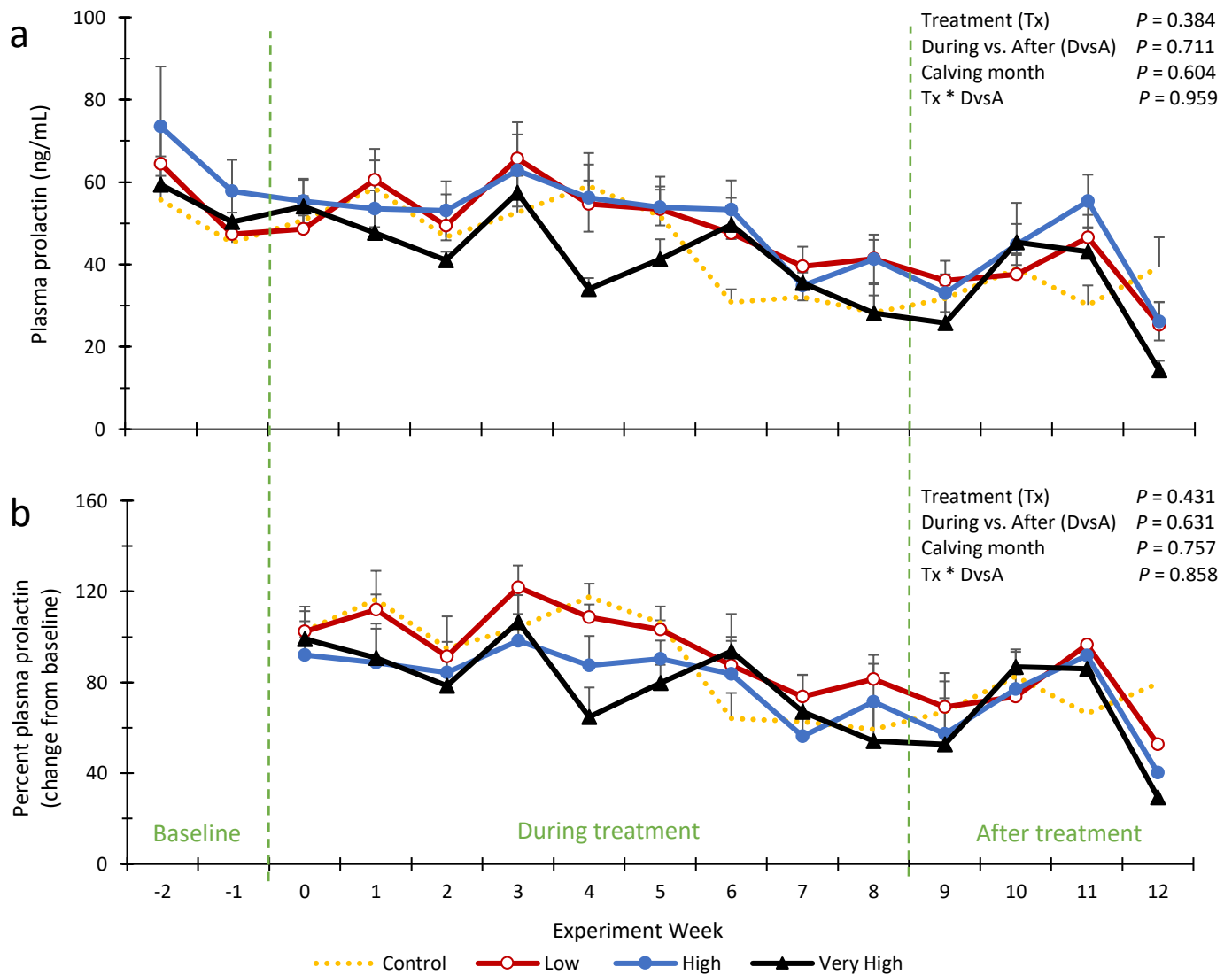


Figure 4. 4 Cow plasma prolactin concentrations during (9 weeks) and after (4 weeks) ergot treatment feeding. Control ($n = 9$) received $5 \mu\text{g}/\text{kg}$, low ($n = 9$) received $48 \mu\text{g}/\text{kg}$ ergot, high ($n = 8$) received $201 \mu\text{g}/\text{kg}$ ergot, and very high ($n = 6$) received $822 \mu\text{g}/\text{kg}$ ergot during ergot feeding. Weekly mean ($\pm\text{SE}$) plasma prolactin concentrations (a) and percent plasma prolactin change from baseline (b). (Mixed model repeated measures, SAS)

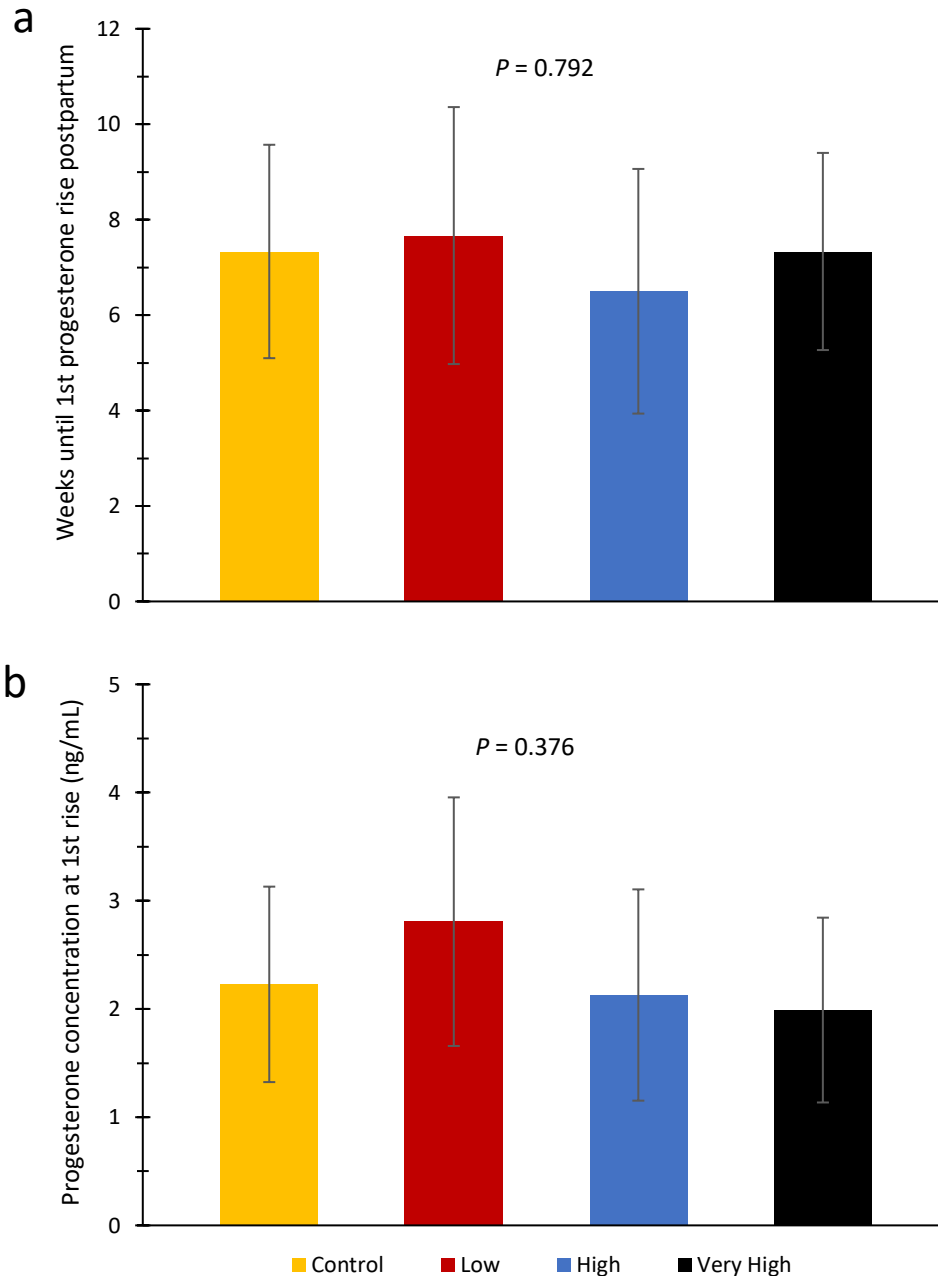


Figure 4. 5 Weeks (\pm SD) until 1st rise (>1 ng/ml) of progesterone postpartum (a) and progesterone concentration (\pm SD) at 1st rise postpartum (b) of cows receiving 9 weeks of ergot treatment feeding (One-way ANOVA, SPSS). Control ($n = 9$) received 5 μ g/kg, low ($n = 9$) received 48 μ g/kg ergot, high ($n = 8$) received 201 μ g/kg ergot and very high ($n = 6$) received 822 μ g/kg ergot during the exposure period.

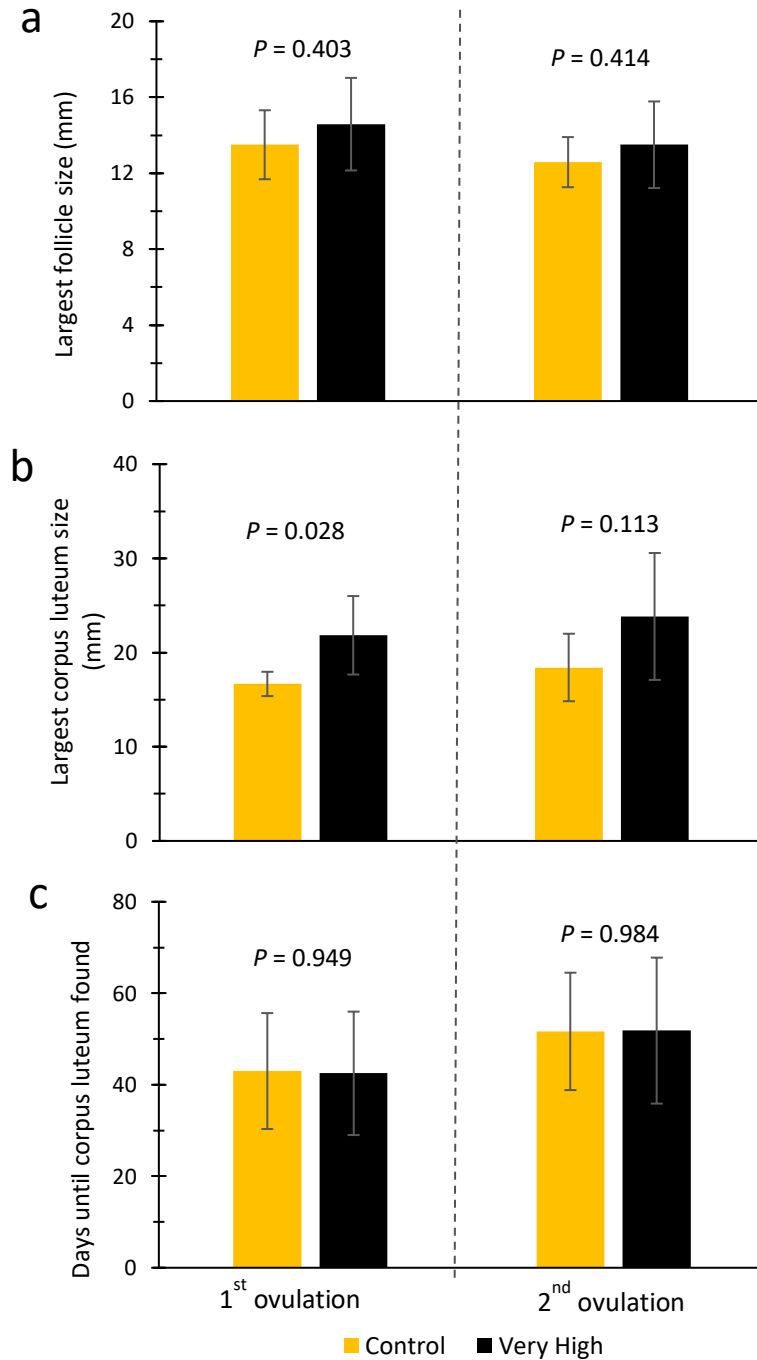


Figure 4. 6 Three ovarian parameters were compared between cows postpartum in the control ($n = 6$; $5 \mu\text{g}/\text{kg}$) and the very high ($n = 6$; $822 \mu\text{g}/\text{kg}$) ergot treatment groups. The parameters were observed for both the first and second ovulation. Largest diameter (\pm SD) measured of the ovulating follicle (A). Largest diameter (\pm SD) measured of the corpus luteum (B) and Number of days (\pm SD) until the corpus luteum was observed (C). (T-test, SPSS).

4.4.8 Pregnancy rates

Cows were checked for pregnancy 17 weeks (wk 26 of experiment) after bull exposure. There were no differences in pregnancy rates ($\chi^2 = 0.358$) between the ergot treatment groups (Table 4.7).

4.5 Discussion

This study examined the effects of ergot alkaloid consumption at concentrations up to 820 $\mu\text{g}/\text{kg}$ (Total Mixed Ration) in pregnant and postpartum beef cattle to assess performance and reproductive endpoints during the exposure and recovery period.

The study determined that low-concentration ergot exposure to pregnant and postpartum beef cows did not alter weight gain of the cows or the calves. Ergot concentrations up to 820 $\mu\text{g}/\text{kg}$ of total dry matter intake did not alter cow prolactin concentrations, rectal temperature or the return to postpartum cyclicity.

Calving month was incorporated into the weekly statistical analysis as a covariate in treatment effect; therefore, any statistical differences associated with calving month were not considered to be relevant in the discussion.

The findings in this study indicate that feeding up to 820 $\mu\text{g}/\text{kg}$ of total dry matter intake had no effect on cow weight gain during the early postpartum period. The interaction between treatment groups and calving month observed in the weekly weight data (Figure 4.1a) was most likely associated to the weight variation between the cows as this interaction disappeared when comparing the cows' weights to their baseline values (Figure 4.1b). It should be noted the control group remained amongst the middle of the treatment groups indicating there was no dose-response relationship or trend related to ergot alkaloid consumption up to 820 $\mu\text{g}/\text{kg}$ on weight gain. If ergot exposure had reduced weight gains, one would expect at minimum the very high ergot exposed treatment group to exhibit a reduced weight gain as the ergot exposure increased.

This finding is in contrast with Burfening et al. (1994) who found that average daily gain decreased linearly with ergot consumption from 0 to 1.6 percent of ergot in the diet. While it is

Table 4. 7 Pregnancy rates 17 weeks¹ after bull exposure of cows exposed to 9 weeks of ergot exposure.

	Cows pregnant	Total number of cows in group	Pregnancy Rate	Chi-square ²
Control	7	9	78%	0.358
Low	8	9	89%	
High	8	8	100%	
Very High	6	6	100%	

¹Bulls entered the pen on week 9 and cows were check for pregnancy on week 26

²Probability of a different pregnancy rate between treatment groups

difficult to determine the actual ergot alkaloid concentration in the cited study, it was likely much higher than the concentration used in the current study. Depending upon the feed type and growth conditions, the 1.6 percent ergot content represents approximately 10,000 $\mu\text{g}/\text{kg}$ alkaloid content. In the present study, if exposure concentrations had been increased by 10-fold, a linear decline may have been observed.

Most studies establishing reduced weight gain and intake as a consequence of ergot alkaloid consumption have been done using endophyte infected tall fescue (Foote et al., 2013; Koontz et al., 2015; Mahmood et al., 1994; Paterson et al., 1995). Estimated ergot concentrations in these studies range from approximately 5500 $\mu\text{g}/\text{kg}$ to unknown concentrations of up to 75% infectivity of endophyte in pasture. Alkaloids produced by *C. purpurea* are expected to act in a similar fashion by interacting with the serotonergic receptors involved in the regulation of gut motility, thereby, negatively affecting the motility and passage rate through the gut (J. L. Klotz, 2015). The lack of effect found in the present study may be due to the different alkaloid composition in the endophyte infected fescue compared to those found in *C. purpurea* or more likely related to the substantially lower ergot alkaloid concentration in the present study.

An effect of time (i.e., during vs. after treatment) and calving month was found in both the weekly and weekly change from baseline calf weight data (Figure 4.2). All of the treatment groups exposed to ergot demonstrated increased weight gains in the calves and numerically the control calves had the least body weight at the end of the study. It was anticipated that ergot exposure in the cows would have resulted in reduced milk production (prolactin inhibition) and consequently reduced nutrition (milk) would be available for the calves; however, this effect was not observed in the current study.

Prolactin has been functionally linked, together with other mechanisms, to the initiation and maintenance of milk secretion and mammogenesis (Fell et al., 1974; Houdebine et al., 1985). Decreased prolactin production in the lactating cow has the potential to negatively effect calf weight gain postpartum. Multiple studies have observed a decline in prolactin production in dairy cattle as a result of the consumption of ergot alkaloids (Carson, 1977; Ilha et al., 2003; Munkvold et al., 1997; Paterson et al., 1995; Strahan et al., 1987). However, this effect was not observed in

the current study. The difference related to the current study and past research may be attributed to the type of cow (i.e. dairy vs. beef), the source of ergot alkaloids (i.e. endophyte vs. *C. purpurea*) and/or the ergot alkaloid concentration. If the exposure to ergot by the cows had included treatment groups approaching 10,000 µg/kg, a negative impact on calf weight gain may have been observed. Milk production related to prolactin synthesis may be a more sensitive bioindicator of ergot exposure in high producing dairy breeds. Milk production was not evaluated in the present study.

Ergot alkaloids have the ability to cause arterial vasoconstriction thereby diminishing blood circulation (Seaman, 1980; Shelby, 1999; J. R. Strickland et al., 2009). Animals exposed to ergot alkaloids have been found to have a reduced ability to remove body heat particularly in hot climates or retain body heat in cold climates (Carson, 1977; Rhodes et al., 1991; Spiers et al., 2012; J. R. Strickland et al., 2009). Although an interaction between treatment and time (during vs. after) was found in the weekly cow rectal temperature data (Figure 4.3a), the values were within the normal body temperature range of 36.7 to 39.1°C for cows (Erickson, Goff, & Uemura, 2004). Furthermore, this interaction was not evident in the weekly change from baseline rectal temperature data (Figure 4.3b). Therefore, with interpretation based on both recorded rectal temperature and percent of baseline values, the anticipated dose-response hyperthermia with increasing ergot concentrations was not evident under the current ambient temperature conditions at the ergot alkaloid concentrations consumed in this study.

It is note worthy that, the ambient temperature was approximately 21°C and no extreme environmental temperature conditions were encountered. Thermoregulation was unlikely to be altered under the moderate climatic conditions encountered in this study. This conclusion may not be valid under extreme cold conditions encountered in Canadian prairies during the winter or during the extreme hot weather in the summer in Southern United States.

The return to normal ovarian cyclicity in postpartum cows in a timely manner is important for livestock farmers to maximize economic returns. This study evaluated the time of first postpartum progesterone rise and the concentration, timing of first postpartum ovulation and

size of the ovulatory follicle at that time to assess the impact of ergot exposure on the return to normal cyclicity in cows.

A progesterone concentration above 1 ng/mL is an accepted indication of the progression of the estrus cycle and the onset of ovarian activity (Díaz, Manzo, Trocóniz, Benacchio, & Verde, 1986; Patterson, Perry, Call, Beal, & Corah, 1989).

Some researchers have demonstrated decreased progesterone concentrations in cattle with ergot alkaloid consumption (Jones et al., 2003; Mahmood et al., 1994; Poole et al., 2016), while other studies found no effect of ergot alkaloids on progesterone (Burke et al., 2001; Schuenemann et al., 2005). The present study supported the latter conclusion, there were no observed effect on either the time of first progesterone rise above 1 ng/mL (all treatment groups) or the time of first ovulation, ovulatory follicle size, and the first or second corpus luteum (control versus 820 µg/kg ergot alkaloid group). The present results relating to no effect on the follicle size or diameter of the CL are consistent with other studies (Ahmed et al., 1990; Jones et al., 2003; Seals et al., 2005).

Mahmood *et al.* (1994) suggested that animal age can alter the effect of ergot alkaloids on progesterone. Grazing endophyte infected tall fescue reduced progesterone in weaned heifers (6-8 mo.) however, yearling heifers (11-13 mo.) were not as sensitive to the ergot alkaloids. It is plausible that the source of the ergot alkaloids, animal age, dose, duration, and time of ergot exposure may all contribute to the varied observations reported in literature related to plasma progesterone concentrations. It is interesting to note that pregnancy rates observed were not altered in the current study subsequent to ergot exposure. It should be noted that the limited number of animals used in this study makes it difficult to detect minor differences in pregnancy rates. However, considering all of the measurements together (timing of first progesterone rise, ovulatory follicle and corpus luteum size, timing of first ovulation, pregnancy rates) it appears that the consumption of ergot alkaloids at concentrations up to 820 µg/kg for 8 weeks in peri-parturient and early postpartum period in beef cows does not impact reproduction and return to cyclicity. This information is important for cattle producers as normal reproductive performance is necessary to keep cow-calf operations profitable. Delays in conception related to

ergot alkaloids can be a major production loss. Ergot alkaloid concentrations up to 820 µg/kg appear to be acceptable in beef cattle feed without adverse reproductive effects.

Since no clinically relevant alterations were observed during the treatment period, the assessment of recovery from ergot exposure in cattle from a reproductive perspective could not be evaluated. The lack of alterations in the 'after' treatment period for 9 weeks suggests there are also no delayed effects associated with the consumption of ergot alkaloid concentrations up to 820 µg/kg.

At the present time, there is considerable controversy related to current tolerance or feed guidelines related to the consumption of ergot-contaminated feed by cattle. Since no effects were observed at concentrations approaching 820 µg/kg, tolerance guidelines based on reproductive performance, prolactin concentration or weight gain could be established near the 820 µg/kg value. This recommendation may vary under extreme climactic conditions or perhaps with dairy cattle, with more sensitive metabolic requirements.

In conclusion, this study was conducted to assess the potential loss of productivity and cow-calf production due to consumption of ergot alkaloids produced by *C. purpurea*. Three concentrations of ergot alkaloids which were evaluated at or below 820 µg/kg of total dry matter intake. Endpoints measured which were unaffected by ergot exposure included: cow weight, calf weight, rectal temperature, prolactin concentration, progesterone concentration and postpartum ovarian function. There was no impact on the overall performance of cow-calf production at moderate ambient temperatures.

Further studies should explore the effects of ergot alkaloids produced by *C. purpurea* above 820 µg/kg but less than the current Canadian guidelines under varying climatic conditions and duration of exposure. Modifications of the guidelines may be influenced based on this updated species-specific dose-response information.

CHAPTER 5: GENERAL DISCUSSION

The overall objective of this thesis was to examine ergot contamination from an analytical, regulatory and biological approach by assessing methods used to evaluate ergot contamination and evaluating current Canadian ergot feed recommendations for cattle.

The first study (Ch.2) examined the correlation between number of ergot sclerotia and weight compared to the total ergot alkaloid concentration. This was important to identify as three different regulatory agencies in Canada: CFIA (2015), CGC (2016) and the Seeds Act (Government of Canada, 2015) all recommend maximum ergot concentrations based on different measurement criteria. The study found that a correlation did not exist at low diagnostically relevant concentrations suggesting that the different ergot measurement criteria (sclerotia count, sclerotia weight and total alkaloids concentration) cannot be interchanged. To put this into perspective a single ergot sclerotia can range from 18 to 450 µg/kg total ergot alkaloid concentration and a 0.1% net ergot weight sample can range from 1000 to 3000 µg/kg. Therefore, it was decided that using an analytical method to determine the total ergot alkaloid concentration was the most accurate considering the variability found within a single ergot sclerotia which has also been suggested by other researchers (Appelt & Ellner, 2009; Blaney et al., 2009; Young, 1981a; Young, 1981b; Young & Chen, 1982). This discovery led to the second objective of the first study: to evaluate the grinding process used prior to analytical analysis and to determine the impact of sample volume on analytical variability. It is important that analytical techniques are accurate at the recommended concentrations. Both particle size prior to the extraction process and sample size used was found to heavily influence the accuracy of obtaining a representative sample particularly at lower more diagnostically relevant concentrations.

The effect of particle size and the concentration of a sample on obtaining accurate results has also been shown with other mycotoxins (Lippolis et al., 2012; Schatzki & Toyofuku, 2003). In the overall process of assessing the contamination of ergot and other mycotoxins the greatest source of error remains field sampling. Johansson *et al.* (2000) found that in corn contaminated with aflatoxin, field sampling accounted for 77.8% of the variation whereas, sample preparation

and analytical variation only accounted for 20.5% and 1.7% respectively. Whitaker *et al.* (2015) who looked at wheat contaminated with ochratoxin found field sampling to be the highest source of variation at an astounding 95.3%. Ergot contamination would be expected to follow similar patterns where field sampling is the greatest source of error, sample preparation is a much lower source of error and the actual analysis is the least source of error. Therefore, after sampling the particle size is the most important factor in reducing the variability given the entire sample has been used for assessment.

The second study (Ch.3) examined the proportions of the individual ergot alkaloids across different cereal crops from within Western Canada. It has been demonstrated that not all the ergot alkaloids have the same potency (J. Klotz *et al.*, 2010). This may suggest that ergot recommendations should be based on individual ergot alkaloid concentrations rather than a total concentration. The proportions of the six ergot alkaloids in this study were comparable among cereal grains in Western Canada however, the proportions may not be comparable across all of Canada (Young, 1981a). Therefore, recommended total ergot alkaloid concentrations may need to be set based on region rather than country and regions that exhibit variation among the ergot alkaloid proportions should use individual ergot alkaloid concentrations rather than a total concentration.

The third study (Ch.4) evaluated the impact of ergot consumption in cow-calf operations. The majority of studies evaluating the effects of ergot consumption have used endophyte infected tall fescue as the ergot source (Foote *et al.*, 2013; Koontz *et al.*, 2015; Mahmood *et al.*, 1994; Paterson *et al.*, 1995) rather than *Claviceps purpurea* which is more commonly found in Canadian grain. The study focused on using ergot produced by *C. purpurea* and determined feeding concentrations up to 820 µg/kg will not alter cow-calf performance in moderate ambient temperatures. It is important to keep in mind that all factors including but not limited to temperature, plant type, alkaloid proportions, species and breed can influence what concentration is most acceptable.

At the present time, we feel the current Canadian ergot maximum tolerance recommendation of 2000 µg/kg for cattle feed has been considered outdated. This

recommendation has little to no scientific support and is assumed to be based primarily on clinical effects such as gangrene. Once an animal experiences clinical symptoms such as gangrene the effects are often irreversible (T. Evans et al., 2004). Due to finding no effects of ergot up to 820 µg/kg on pregnancy and postpartum beef cows in study 3 (Ch.4), a new maximum total ergot alkaloid tolerance recommendation of 820 µg/kg should be considered for beef cows. Further experiments should be conducted to reinforce the endpoints examined. While this recommendation does not account for extreme temperatures and may not be appropriate for all 'cattle' in general (i.e. bulls and dairy cows) it is likely that if an effect were encountered at this concentration it would present itself as a less severe and reversible subclinical effect rather than an irreversible clinical effect. Ideally with advancing research, the ergot tolerance recommendation would be raised or lowered to the highest concentration possible without the appearance of subclinical effects for each beef or dairy cows and bulls. It is also likely that these recommendations be based on different subclinical effects depending on what group of animals is being referred to. Based off production requirements it would seem fitting to base the ergot recommendations for female beef cows on body weight and reproduction, beef bulls on body temperature (spermatogenesis/reproduction) and dairy cows on prolactin concentration (milk production).

If the current ergot recommendation of 2000 µg/kg is lowered, accurate analytical techniques at lower concentrations will be required. It is also apparent that the inconsistency of ergot measurement methods needs to be addressed. Current methods lead to public confusion as the methods are not comparable which can also raise issues with importing and exporting grain internationally.

The studies completed for this thesis are only a starting point for future work regarding ergot analysis and regulations.

CHAPTER 6: MAJOR CONCLUSIONS & FUTURE DIRECTIONS

6.1 Major Conclusions:

The objectives of this thesis have lead to the following conclusions:

- An analytical measurement should be used as opposed to number of ergot sclerotia or weight of ergot sclerotia to determine the ergot alkaloid concentration

- The grinding method used prior to ergot extraction and analysis should produce a subsequent particle size equal to or smaller than 200 μm with more than 85% of the particles smaller than 400 μm in order to reduce the risk of unreliable estimates

- In the case of commercial labs, the entire submitted sample should be ground to assess the ergot alkaloid concentration to minimize variability

- Proportions of the six ergot alkaloids ergocornine, ergocristine, ergocryptine, ergometrine, ergosine and ergotamine were comparable among cereal grains collected from across Western Canada

- Ergot alkaloid exposure up to 820 $\mu\text{g}/\text{kg}$ will not alter performance (cow and calf weights, rectal temperature, prolactin concentrations and reproduction) in pregnant and postpartum beef cattle at modern ambient temperatures

6.2 Future Directions:

Ergot contamination of cereal crops and grasses has been of increasing concern, particularly in Western Canada. This concern puts pressure on the industries for improved knowledge, assessment and regulation of the ergot alkaloids.

While regulations are in place by different agencies within Canada (CFIA, CGC, Seeds Act), the ergot recommendations are somewhat unclear due to the inconsistent methods of evaluation. A standardization across all governing agencies should consider a maximum ergot alkaloid concentration recommendation.

Lowering current animal maximum tolerance ergot recommendations may put pressure on diagnostic laboratories and regulatory agencies to ensure accurate measurements can be obtained at the recommended levels. Future studies should aim to improve sample collection for analysis and assess what sample size is required for accurate ergot alkaloid measurement at concentrations below the current 2000 µg/kg recommendation.

The assessment of the effects of ergot alkaloids on cow-calf production as described in this thesis was only the first step in determining appropriate maximum tolerated ergot concentrations in feed. Future studies are needed to assess the same concentration of ergot alkaloids in 'extreme' climactic conditions (hot and cold) as this may lead to a lower ergot concentration tolerated by the animals. Furthermore, studies should explore bovine species and sex differences. Bulls may be more susceptible to the thermoregulatory effects of the ergot alkaloids in the process of sperm maturation. The main productive purpose of Dairy cows is significantly different than Beef cows (milk vs. meat) which might suggest that each should have their own ergot tolerance recommendation.

Current regulations consider a total ergot alkaloid concentration however it may be important to consider the effect of the individual ergot alkaloids. A study completed by Klotz et al. (2010) demonstrated receptor affinity differences between some of the ergot alkaloids. This suggests that not all ergot alkaloids have the same potency. Future studies should explore the effects and kinetics of the individual alkaloids. Once more information has been generated with regards to individual ergot alkaloids, regulations can be revised to satisfy individual ergot alkaloid variability.

In summary, the following directions are proposed:

- Standardization of the method used to evaluate ergot contamination across all Canadian governing agencies to reduce confusion
- Assessment of what sample volume/sample size is required for diagnostic laboratories to accurately measure ergot alkaloid concentrations below 2000 µg/kg
- Identify the kinetics and potencies of each individual ergot alkaloid to assess residues and withdrawal times
- Assessment of ergot alkaloid effects on cow-calf production at 820 µg/kg in 'extreme' climates
- Assessment of ergot alkaloids in bulls and dairy cows in order to establish specific recommendations
- Multi-mycotoxin exposure

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APPENDIX

Table A: Calving dates for all cows in Study 3 (Chapter 4)

Cow ID	Treatment group	Calving date (2015)	Experimental week
5	Control	16-Apr	0
10		24-May	5
38		18-May	4
51		18-Apr	0
77		30-Apr	2
84		14-Apr	-1
115		27-Apr	1
119		05-Apr	-2
149		15-May	2
4		Low	24-May
12	02-May		2
14	09-May		3
28	19-Apr		0
32	08-Apr		-1
40	21-Apr		1
56	25-Apr		1
75	14-Apr		-1
154	08-Apr		-1
47	High		20-Apr
79		30-Apr	1
92		09-Apr	-2
121		09-Apr	-2
126		06-Apr	-2
155		08-Apr	-2
157		11-May	3
197		08-Apr	-2
57	Very High	25-Apr	1
116		16-Apr	-1
118		09-Apr	-2
147		22-Apr	0
150		05-Apr	-2
179		04-Apr	-2