Effects of Ergot Alkaloids from *Claviceps purpurea* on Sperm Characteristics and Prolactin Concentration in Yearling and Mature Angus Bulls

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ABSTRACT

Ergot alkaloid contamination of livestock feed is a growing concern in western Canada. There are reports on ergot alkaloid contamination from tall fescue in the United States, but few studies of effects of ergot alkaloid contamination from cereal crops, grains, and grasses. The overall objective of this dissertation was to examine effects of ergot alkaloid-contaminated feed on reproduction in Canadian bulls and to ascertain whether current limits for ergot alkaloids in Canadian national feed regulations are adequate.

In the first study, our objective was to determine whether feeding yearling Aberdeen Angus bulls with the highest permissible Canadian limit of ergot alkaloids (~3 mg/kg of daily dry matter intake, DMI; 60µg ergot alkaloid/kg body weight, daily) for 9 weeks would affect sperm characteristics and plasma prolactin concentrations. Plasma prolactin concentrations decreased markedly (mean ± SEM, 16.74 ± 3.70 in Exposure and 33.42 ± 3.08 ng/mL in Post-Exposure periods; $P<0.01$; n=7) compared to Control (67.54 ± 21.47 and 42.59 ± 15.06 ng/mL; n=7). Ergot treatment did not affect ($P\geq0.17$) body weight gain, sperm concentration, sperm count/ejaculate, motility or percent live sperm. However, averaged over the exposure and post-exposure durations, scrotal circumference was 2.7% smaller ($P=0.02$) in the Ergot group. Progressive motility remained unchanged from 59.92 ± 2.31% in Exposure to 59.61 ± 2.59% in Post-Exposure periods, compared to a marked increase in Control (61.42 ± 1.60% to 67.52 ± 1.47%; $P=0.02$). Straight-line sperm velocity decreased (-3.15 ± 1.53 µm/s) from exposure to post-exposure periods in Ergot group ($P=0.04$) versus an increase (2.96 ± 2.17 µm/s) in Control. Midpiece defects decreased from Exposure to Post-exposure periods in the Control group, but remained unchanged in the Ergot group (trt*age, $P<0.01$). Ergot feeding reduced proportion of sperm with medium mitochondrial potential (Ergot: 22.65 ± 0.98%, Control: 24.35 ± 1.05%, $P=0.04$). In conclusion, Ergot fed at the Canadian permissible limit for 9-wk resulted in a 4-fold decrease in plasma prolactin concentrations. Semen end points were not significantly affected, although there were subtle effects on progressive motility, midpiece defects and mitochondrial membrane potential. Results supported our hypothesis that prolonged low-level ergot will adversely affect plasma prolactin concentrations, and semen end points were partially affected, consistent with similar work on fescue toxicosis.

In the second study, our objectives were to determine if feeding mature and yearling Angus bulls ergot alkaloids (from *Claviceps purpurea*) within the Canadian permissible limit (~3 mg/kg) affect post-thaw sperm quality. In Experiment 1, mature Angus bulls consumed ergot alkaloids (~1 and ~2 mg/kg of daily dry matter intake (DMI); n=8 and n=6 bulls, respectively) for 61 d, with semen collected and cryopreserved bi-weekly. In Experiment 2, yearling Angus bulls (n=7/group) were fed placebo or ergot alkaloids (3.4 mg/kg of DMI) daily for 9 wk, with semen collected and cryopreserved weekly. All frozen semen was assessed 0 and 2 h post-thaw. In Experiment 1, post-thaw total and progressive sperm motilities decreased ($P \leq 0.05$) from pre-exposure to exposure periods, then rebounded. During exposure, average path velocity (VAP) and straight-line velocity (VSL) decreased ($P \leq 0.01$) at 0 h compared to pre-exposure and subsequently rebounded. Live sperm with intact acrosomes at 2 h post-thaw was affected by ergot ($P=0.01$). Medium mitochondrial membrane potential increased ($P \leq 0.01$) during exposure compared to pre-exposure and subsequently decreased. In Experiment 2 on yearling bulls, total and progressive sperm motilities at 0 and 2 h increased ($P \leq 0.01$) steadily throughout the study. During post-exposure, curvilinear velocity (VCL), VAP and VSL at 0 h increased ($P \leq 0.01$), whereas VSL at 2 h increased ($P \leq 0.01$) from pre-exposure to exposure to post-exposure. Live sperm with an intact acrosome increased ($P \leq 0.01$) at both 0 and 2 h during post-exposure. Medium mitochondrial membrane potential increased ($P \leq 0.01$) from pre-exposure to exposure, followed by a slight decrease in post-exposure. Our hypothesis is partially supported in mature Angus bulls , with only transient effects of ergot on sperm motilities and velocities after cryopreservation. Post-thaw sperm characteristics in yearling bulls underwent expected age-related improvements, with any effects of ergot alkaloids potentially masked by sexual maturation.

Overall, in both studies, results partially supported our hypotheses that ergot has no detectable long-term adverse effect on fresh or post-thaw sperm characteristics in yearling or mature bulls. This work will provide important evidence for producers working with ergot-contaminated grain or ergot-exposed cattle.

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LIST OF ABREVIATIONS

BW	Body weight
CASA	Computer-assisted sperm analysis
°C:	Degree Celsius
CFIA	Canadian food inspection agency
ELISA	Enzyme linked immunosorbent assay
EY	Egg yolk
FITC-PNA	Fluorescein isothiocyanate peanut agglutinin
h	Hour
IACR	Intact acrosome
IPM	Intact plasma membrane
IU	International unit
IVF	*In vitro* fertilization
kg	Kilogram
L	liter
LC	Liquid chromatography
µg	Microgram
µL	Microliter
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mo.	Month
MtDR	MitoTracker® deep red
n	Number
P	Probability
PDS	Prairie diagnostic services
ppb	parts per billion (µg/kg
ppm	parts per million (mg/kg)
PI	Propidium iodide
PRL	Prolactin
RIA:	Radioimmunoassay
ROS	Reactive oxygen species
SE	Standard error
SEM	Standard error of the mean
TCA	Tris-citric acid
TLC	Thin layer chromatography
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity
y	Year

CHAPTER 1:
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Claviceps purpurea is a fungus that produces ergot alkaloids poisonous to humans and livestock (Belser-Ehrlich et al., 2013; Lee, 2009). Ergot contamination can be easily identified by purplish/black structures in the grain or grass seeds; these structures are known as sclerotia or ergot bodies. Ergotism occurs when an animal consumes a toxic threshold of sclerotia that contains ergot alkaloid mycotoxins. These mycotoxins may produce several effects, depending on various factors i.e., the species of *Claviceps*, host species, growing conditions, as well as the size of the sclerotia. Consequently, the concentrations and exact chemical composition of this fungus can fluctuate (Grusie et al., 2018a; Lorenz and Hoseney, 1979). In humans, ergot poisoning is uncommon today; however, a risk to livestock species is still present. Livestock may become exposed to ergot whilst grazing contaminated pastures or consuming rations deemed unsafe for humans but used as animal feed. Ergotism has been observed in the majority of livestock species.

1.2 Ergot

1.2.1 History of Ergot

Ergot alkaloids are complex compounds produced by certain fungi. The Middle Ages had frequent epidemics of ergotism following consumption of breads made from contaminated grains (mostly rye). Those at the bottom of the social class were more susceptible to these outbreaks as they were more likely to consume easily available food sources, especially in an era with frequent famine. Ergot was originally considered to be sunbaked kernels of grain and many of the ergot-associated symptoms were thought to be the reason behind “witchcraft” and a likely factor in the Salem Witchcraft Trials in late 1600’s (Caporael, 1976). Ergot was also used by mid-wives as an aid to promote birth and abortion. It also has some current medicinal uses as a treatment for migraines and Parkinson’s disease (Haarmann et al., 2009; Schiff, 2006).

The first widely reported case of ergotism was in France (AD 944-945) when approximately half of the affected population died (Schiff, 2006). Russia suffered from a severe outbreak in 1926-1927. Over 10,000 cases of convulsive ergotism were reported during this period, despite government efforts to regulate acceptable concentrations in grains (Kent and Evers, 1994). In 1927, Manchester, England also reported cases of ergotism resulting from poor screening and improper cleaning procedures. The rye meal was tested, and ergot concentrations were toxic (0.90%), although upon re-testing, there was conflicting evidence that infected grains were at 0.13%, i.e., below the toxic level. However, the symptoms presented by people were consistent with gangrenous ergotism and the divergent concentrations may have been due to miscalculations. Regardless, following the appearance of symptoms, patients were given a rye-free diet for 14 days, which resulted in a decline in ergotism (Morgan, 1929). The most recent outbreak of ergotism was reported in Ethiopia in 2001. The actual number of cases was not reported; however, at least three patients died (Urga et al., 2002).

Human outbreaks are not the only reported cases of ergotism. Livestock, especially cattle, are known to be affected by ergot. In cattle, primarily gangrenous or convulsive ergotism is common. However, convulsive ergotism is more common in humans than cattle (Bourke, 2000). In modern outbreaks, hyperthermic ergotism has been reported in cattle. Ergotism is primarily associated with alkaloids from *C. purpurea* (Schneider et al., 1996a). Until the 1980s, cattle ergotism was not well reported. In July 1981, heifers suffering from suspected chronic ergotism, with signs of hind limb lameness, were reported in Australia. A positive identification of *C. purpurea* was not made, but rye grass seed heads had similar characteristics (Fraser and Dorling, 1983). During June 1984, ergotism was reported in the United States. There were 52 cattle affected, of which 3 heifers died, 20 had necrosis on their ears, tailheads, and pasterns, and 20 had lameness due to necrosis or cracked skin near their hooves, with many other symptoms of hyperthermic ergotism. In laboratory tests, 100% of the seed heads were infected with ergot (Coppock et al., 1989). Since these documentations, ergotism has been reported regularly around the world (Belser-Ehrlich et al., 2013).

1.2.2 Ergot in Canada

Annual variations in prevalence and severity of ergot toxicity are heavily dependent on the climate and weather conditions. This likely also accounts for increased interest in ergotism in years with a higher prevalence, but lower interest in years with little to no outbreaks. Cool damp weather supports ergot infection of plants by promoting germination of the sclerotia. Management of ergot today is mainly through limiting its presence in cereal grain. Currently, many jurisdictions, including Canada, have tolerances for ergot sclerotia (Tittlemier et al., 2015). Ergot, a fungal pathogen, infects most grasses and cereals across Canada. Ergot has become a problem for Canadian farmers and producers over the past 10-20 years. Ergot is more prevalent when rain is persistent during the flowering stages. With continued changes in weather patterns and more precipitation predicted due to climate change, this problem may worsen (Coufal-Majewski et al., 2016). Rye is the predominant substrate of C. purpurea, due to a longer flowering stage (Lee, 2009).

In Canada, there was no substantial reports on ergot infestations throughout the 1980s or 1990s. In Manitoba, ergot infestation was observed in 10% of Canadian Western Red Spring (CWRS) wheat samples in 2005. This was followed by ergot infestation in CWRS wheat samples by 12, 15, and 13% in Alberta, Saskatchewan, and Manitoba, respectively, in 2008. Following a decline in 2009, the incidence of ergot was elevated to 29, 19, and 15% in Alberta, Saskatchewan, and Manitoba, respectively, in 2011. There was again a decrease in 2012, but levels were still significantly higher than those reported in the early 2000s. The incidence of ergot in durum wheat samples also increased over the same period (Menzies and Turkington, 2015). Ergot also reduces small grain cereal crop yield by 5-10% (Wegulo and Carlson, 2011). With increased refusal of affected grains for human consumption, livestock are increasingly more likely to consume ergot-contaminated feed.

1.2.3 Biology and Life-cycle of Ergot

Ergot is easily identified by physical characteristics and follows a predictable life-cycle. Ergotism is the common name given to a disease of grass inflorescences caused by the fungi of genus *Claviceps*. It also refers to dark black/purple fungal structure produced within the floret and known as sclerotium. These sclerotia are characteristically cylindrical and round ended, straight to curved, and tapered at one end. The genus *Claviceps* encompasses a unique group of

species, only infecting ovaries of grasses. *Claviceps* species are not able to penetrate through closed glumes; therefore, cross-pollinated crops are the most threatened. Specifically, *Claviceps purpurea* has a broad host range of approximately 400 grass species, including cereals and almost all forage grasses in temperate regions (Miedaner and Geiger, 2015).

The lifecycle of the ergot fungi begins in the spring/early summer. Wind-borne ascospores land on susceptible flowering host plants' hyphae, invade and colonize the ovary. This produces masses of spores that secrete a "honey-dew" like syrupy fluid. The "honey-dew" ultimately hardens into the characteristic kernel-shaped sclerotia and supplants the entire seed head of the grass or grain. Sclerotia take approximately 5 weeks to form. Head-to-head contact, rain, and insects may transfer "honey-dew" to neighboring blooming plants, thereby promoting fungal spread across a field(s). The number and size of sclerotia produced on each plant by *C. purpurea* varies according to grain. Generally, rye has a considerable number of sclerotia. During the autumn months, sclerotia fall to the ground and remain stable until the following spring. The sclerotia are very stable and thus annual contamination issues may arise, leading to completion of the life-cycle (Schiff, 2006).

Ergot alkaloids are biologically active secondary metabolites synthesized by *C. purpurea*. As secondary metabolites, they are not directly necessary for life; however, they may have profound influence on organisms that come into contact with them. The main ergot alkaloids in *C. purpurea* are ergometrine, ergotamine, ergosine, ergocristine, ergocryptine, and ergocornine (Figure 1.1). All these alkaloids are similar, except differing at C-8 (Krska and Crews, 2008). The common structural component of all naturally occurring ergot alkaloids is the ergoline ring (Figure 1.2), methylated on nitrogen (N-6) and variously substituted on C-8. Most ergot alkaloids also feature a double bond at C-8, C-9 or C-9, C-10 (Flieger et al., 1997). Dopamine, noradrenaline and serotonin, very important neurotransmitters, can be mapped almost entirely into the ergoline ring structure (Figure 1.2). Due to this structural compatibility, many ergot alkaloids act as agonists or antagonists towards receptors for dopamine, serotonin and adrenaline. Hence, they have high biological activity and a wide spectrum of pharmacological effects (Liu and Jia, 2017).

1.2.4. Epimers and Ergot

Epimers are diastereomers contain multiple chiral centers but differ in configuration at only one chiral center. Over 90 ergot alkaloids have been isolated and identified which can be separated into two main classes based on chemical structure, amide derivatives of lysergic acid and small peptides (Liu and Jia, 2017). The small peptides have two conformational forms, (*R*)-epimers, termed ergopeptines and ergopeptames, and (*S*)-epimers, termed ergopeptinines. Both epimers are available in nature in even amounts; however, with prolonged storage of raw material or inadequate storage conditions, larger amounts of ergopeptinines might form (Krska and Crews, 2008). Very few studies consistently measure and report the stereochemical configuration of ergopeptines used under experimental conditions. The storage and experimental conditions affect the epimerization of the ergot alkaloids (Smith and Shappell, 2002).

The shared structural feature of ergot alkaloids is the ergoline ring, methylated on the N-6 nitrogen atom, is substituted at C-8, and possesses a C8═9 or C9═10 double bond (Flieger et al., 1997). Historically the (*R*)-epimers are reported to be the biologically active epimers while the (*S*)-epimers are biologically inactive (Pierri et al., 1982). Ergot alkaloids produce a contractile response and the (*R*)-epimers have been studied using an *in vitro* tissue bath system where the contractile reaction of arteries is monitored after incubation with a particular (*R*)-epimer (Foote et al., 2011; Klotz et al., 2010; Oliver et al., 1993). A recent study examined the (*S*)-epimers contractile response and found (*S*)-epimers to also be biologically active, requiring further careful study and monitoring in animal feed (Cherewyk et al., 2020).

1.2.5 Current Canadian Standards

The Canadian Food Inspection Agency (CFIA) has established tolerance levels for ergot concentrations in feed. The CFIA recommends maximum ergot alkaloid content in feed 2-3 mg/kg or 2000 to 3000 parts per billion (dry weight) (CFIA, 2017).

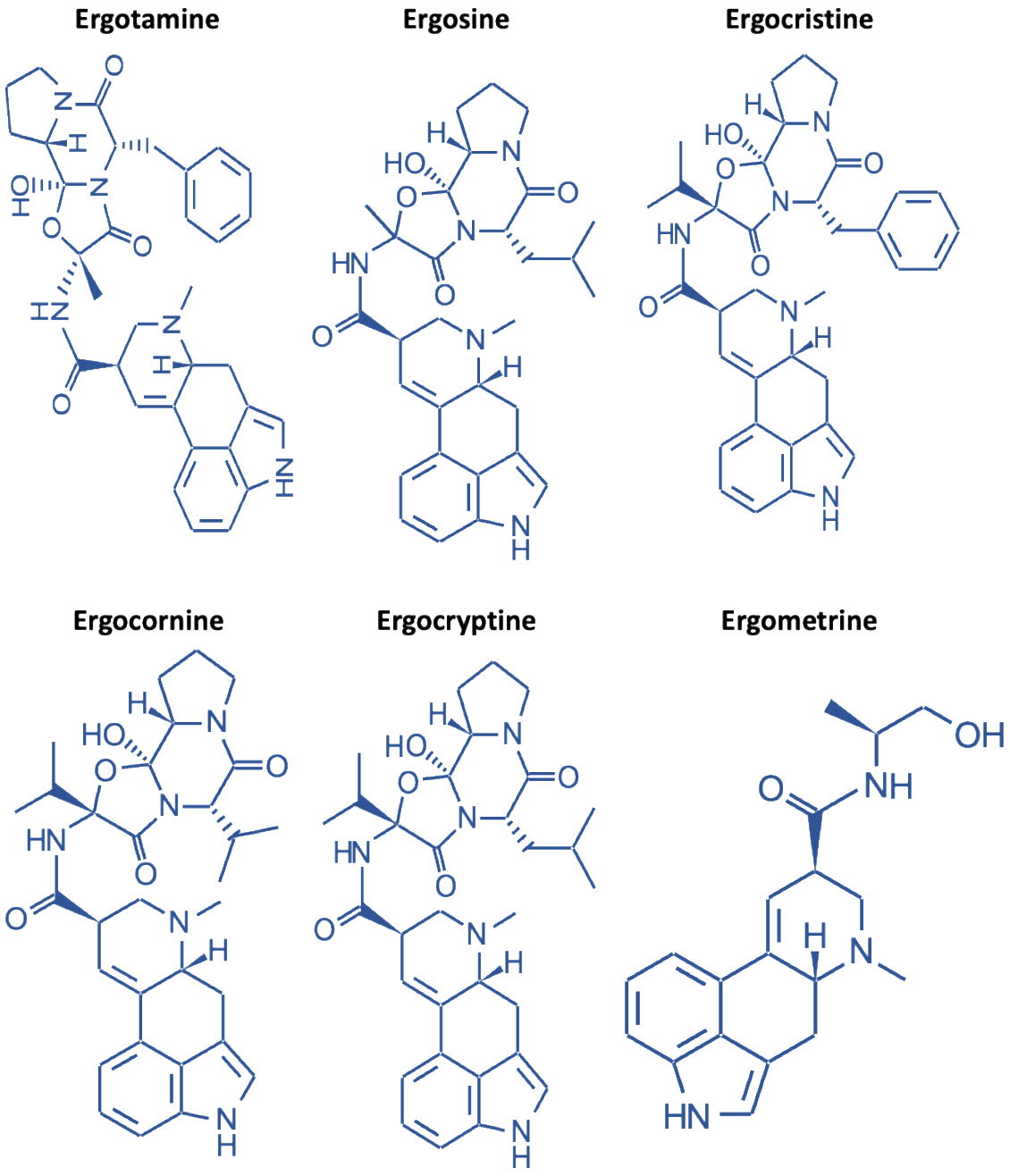

Figure 1.1 Structures of the toxicologically important ergot alkaloids produced by *Claviceps purpurea*. Structures from Wikimedia Commons under Creative Commons license (retrieved; 31 July, 2019).

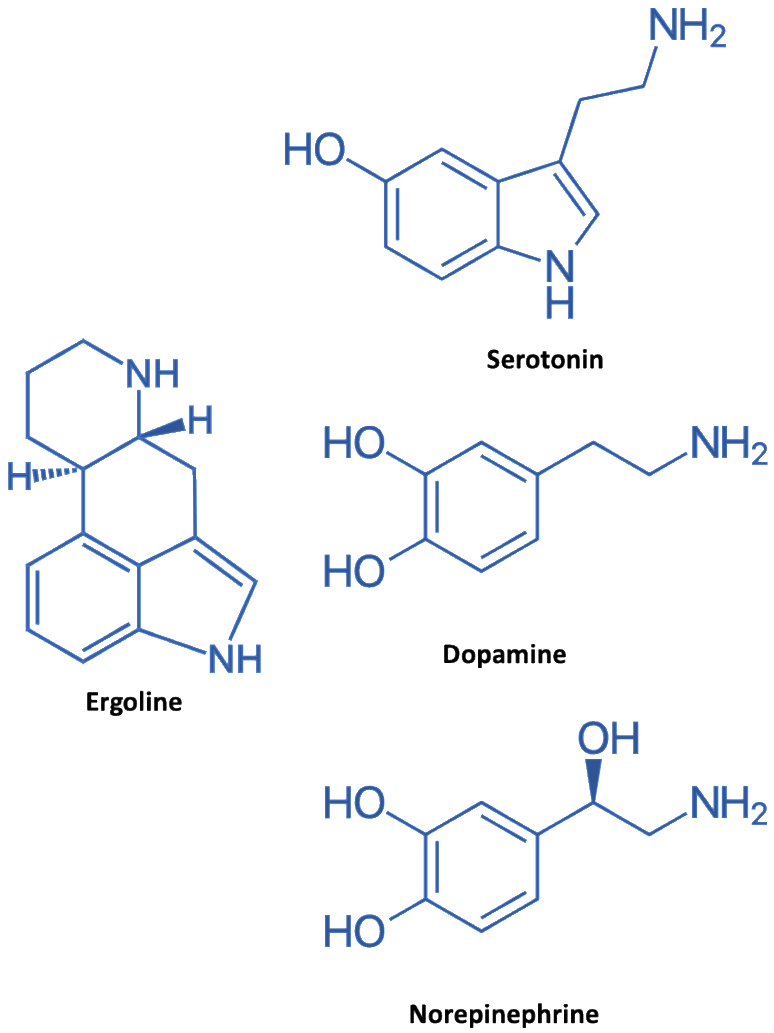

Figure 1.2 Structural similarities between the ergoline ring and serotonin, dopamine, and norepinephrine. Structures from Wikimedia Commons under Creative Commons license (retrieved; 31 July, 2019).

1.3 Ergotism in Livestock

1.3.1 Overview

Ergotism may refer to a variety symptoms and syndromes in livestock. The manifestation of ergot alkaloid-induced effects varies from species to species and animal to animal within a species. This variation may be in part due to the complex chain of interactions from plant-fungus-animal-environment and results in changing alkaloid concentrations, proportions, and distribution throughout the animal (Aiken and Strickland, 2013). In addition, ambient environment aspects such as temperature, humidity and other environmental factors, can also influence a herd's predisposition to ergot toxicosis (Klotz, 2015).

Ergotism from *C. purpurea* is broadly divided into four categories of symptoms/syndromes: convulsive, gangrenous, hyperthermic, and reproductive. After sclerotia are consumed by an animal, symptoms may appear within hours to months (Belser-Ehrlich et al., 2013). The pharmacological effects and form of ergotism ultimately depend on the type and ratio of ergot consumed.

1.3.2 Convulsive Ergotism

Convulsive ergotism is very rare in livestock; however, unique cases have been reported. Ergot alkaloids are serotonin agonists and have the ability to overstimulate the central nervous system (Eadie, 2003). The features of convulsive ergotism, also referred to as nervous ergotism, include distortion of the trunk and limbs, painful involuntary flexion of the fingers and wrists, and flexion or extension of the ankles. Drowsiness, deliriousness, lethargy, melancholy, hallucinations, and double vision are also part of the major symptoms in humans. Individuals may also present with sweating, fever, and twitching (Belser-Ehrlich et al., 2013; Eadie, 2003). Sheep have isolated incidences of convulsive ergotism (Greatorex and Mantle, 1973). However, convulsive nervous disorder, caused by ergot alkaloids, has not been reported in other livestock species (Bourke, 2000). Overall, little information is available on convulsive ergotism in cattle.

1.3.3 Gangrenous Ergotism

Gangrenous ergotism is the most acute and visible form, with obvious physical effects and is generally a result of blood vessel constriction (Klotz, 2015; Strickland and Aiken, 2014). This form of ergotism is the most common and thus described in detail. Sclerotia of *C. purpurea*, when ingested, cause vasoconstriction of extremities which leads to lameness and eventually gangrene of feet, tail, and ears. In pregnant females, it may also cause abortion (Mantle, 1969). First clinical signs of this form of ergotism are pain in the extremities, ataxia, and lameness; as the disease progresses, a line of demarcation will appear between healthy and non-viable tissue (diseased), followed by necrosis and eventual sloughing of the non-viable tissue (Williams et al., 1975). Other clinical signs may include constipation or diarrhea (Mostrom and Jacobsen, 2011). Gangrenous ergotism is characterized by extreme burning pain and cyanosis of extremities; affected cattle often stand in ponds or mud wallows and may have loss of affected hooves, ear tips, and tail switch (Klotz, 2015).

The clinical manifestations associated with persistent vasoconstriction are mainly due to ergot alkaloids, specifically ergotamine from *C. purpurea* (Klotz, 2015). This is due to a decreased blood flow to the extremities, damage to endothelial linings, edema, and thrombosis (Vuong and Berry, 2002). Horses tend not be affected by this form of ergotism (Cross et al., 1995); however, vasoconstriction in the distal palmer artery was noticed when horses were exposed to ergot in their diet (McDowell et al., 2013). Sheep and cattle have swelling of the fetlock joint and hoof when exposed to ergot alkaloids and cause lameness (Klotz, 2015). Exposure to ergot alkaloids reduces the luminal areas of the caudal artery in tails of cattle (Aiken et al., 2007; Cowan et al., 2018, 2019)and auricular artery in goats (Aiken and Flythe, 2014). Early signs of ergotism may be reversed if diseased animals are immediately removed from the ergot-alkaloid based diet; otherwise, they may progress and die (Klotz, 2015; Mostrom and Jacobsen, 2011).

Severe winters and generally wetter springs are more conducive to ergotism (Welty et al., 1994). Vasoconstriction in extremities due to ergot alkaloids is especially a problem in winter, as cold weather may exacerbate the gangrene. Canadian winter is often harsh, and livestock use peripheral vasoconstriction as a physiological mechanism to divert blood to critical organs to prevent heat loss. Ergot alkaloid toxicity in conjunction with this normal physiological response to cold conditions make livestock vulnerable to frostbite and freezing of limbs, thereby making gangrenous ergotism more severe. A number of ergot toxicity cases in cold weather were

reported and summarized (Craig et al., 2015); in Canada, tail loss was reported at -20°C in February, following consumption of 473 ppb total ergot alkaloid concentration, dry weight in feed, well below CFIA standards. In Idaho, 3500 ppb ergot alkaloids with temperature at -5°C led to term abortions, agalactia, and reduced milk production in cattle. In another case in Canada, approximately 40% of steers suffered sloughing of hooves, tails, and ears during April at ambient temperatures of -4°C, with ergot alkaloid concentrations measuring 11,538 ppb and ergotamine at 1,161 ppb. There is not a known concentration without some effect.

1.3.4 Hyperthermic Ergotism

This form of ergotism is more related to climate than other factors. The signs of ergot alkaloid exposure overlap with many signs of heat stress, including low feed intake, higher rectal temperatures, panting, and elevated respiration rates. Therefore, signs of heat stress and hyperthermic ergotism are difficult to separate. The same mechanism that governs gangrenous ergotism applies to the hyperthermic form. However, instead of cold being the factor, heat takes precedence (Klotz, 2015). Livestock that consume ergot alkaloids have a reduced thermo-regulatory ability that can be exacerbated by changes in climate.

Hyperthermic ergotism has been reported around the world. From 1998-1999, Australia had outbreaks of hyperthermic ergotism. During outbreaks of ergot in rye, poisoning occurred in sheep and cattle in central New South Wales. The ergot was consumed both directly from contaminated annual rye grass pastures and indirectly from barley grain contaminated with ergotized annual rye grass seeds. In some animals, hyperthermia was fatal during this outbreak. Bright sunlight and absence of shade were associated with mortality - consumption of 200 mg of ergot per kg of feed led to hyperthermia causing 10% mortality in two herds. In one herd, deaths occurred during two intervals of only 6 daylight hours in the summer with no shade available. In the other herd, deaths occurred sporadically over 25 days in winter with plenty of sunlight, but no shade available. Similar clinical signs were observed in sheep, but with less mortality than in cattle (Bourke, 2000). All animals had signs of reduced weight gain, weight loss, reduced milk production, feed refusal, seeking shade, standing in dams and water troughs and, excessive panting. They also had increased rectal temperatures (41-43°C). In another study from Australia, all animals exposed to ergot had clinical signs of hyperthermia within 3 days (Ross et al., 1989).

In yet another study, Hereford steers were exposed to 180 mg/kg body weight rye grass ergot. This group was exposed to 7 hours of sunlight for 5 days, with a control group (no ergot) exposed to the same conditions (Bourke, 2003). Ergot-treated cattle exposed to sunlight had above-normal body temperatures. Nine of 18 ergot-treated and sun-exposed cattle developed hyperthermia. Sunlight caused an increase in body temperature in control (no ergot consumption) cattle, which was attributed to solar radiant heat. However, ergot consumption caused an increase in body temperature in shaded cattle, attributed to ambient temperature and humidity effects. That response, however, was increased by sun exposure and attributed to solar and radiant heat. This study concluded that ambient temperature and humidity influenced hyperthermic ergotism, as does exposure to high solar radiation (Bourke, 2003). Hyperthermic ergotism is not an immediate threat in Canada, although it may become an issue due to unpredictable and unchecked climate change.

Fescue toxicosis has been studied for over 50 years; however, ergot alkaloids differ between fescue and grains (Canty et al., 2014). There have been many studies and reviews on a spectrum of clinical symptoms in livestock exposed to ergot, mostly effects of ergotism from endophyte-infected tall fescue. Although it is assumed that ergotism from *C. purpurea* has similar effects, this warrants further research. The large spectrum of described clinical symptoms does have some commonalities of ergotism from endophyte infected tall fescue and *C. purpurea*. Suppressed serum prolactin concentrations, along with decreased feed intake, heat stress, poor weight gain, and reduced milk production, are important clinical signs for accurate diagnosis of ergotism. Annual fluctuations in ergot alkaloid concentrations, combined with age and genetic background of animals, variable environmental conditions, and hypoxic conditions at the cellular level, all influence the impact of ergot alkaloids on reproductive performance.

1.3.5 Reproductive Ergotism

Reproductive ergotism has been studied more in the female compared to the male. Ergot alkaloids have been used in obstetrics for centuries. It was suggested that three sclerotia could hasten labor and delivery in humans (van Dongen and de Groot, 1995). Ergometrine was used as a labor inducer until the early 1800s and then largely discarded, as uterine ruptures, stillbirth, and maternal death occurred too frequently (de Groot et al., 1998).

Primarily reproductive ergotism includes decreases in prolactin and progesterone concentrations, reduced milk production, subfertility, and birth defects (Burke et al., 2005; Mahmood et al., 1994; Strickland et al., 2011). Most studies have been conducted using endophyte-infected tall fescue, as alkaloids produced by *C. purpurea* act in analogous manner. The manner and mode of action of the alkaloids have not been fully elucidated. The vasoconstrictive effect of ergot alkaloids resulting in reduced blood flow could affect hormones involved in reproduction, digestion, central nervous system, nutrient delivery, and metabolism. There is a synergistic relationship between various factors, including animal age, ambient temperature, genetics, amount and duration of exposure to ergot alkaloids, and nutritional management with ergot alkaloid toxicity and dysfunction in reproductive systems in ruminants. There are also direct effects of ergot alkaloids on reproductive tissues (Strickland et al., 2011). Cattle distress due to ergot alkaloid-induced hyperthermia or lameness may also have indirect adverse effects on herd reproductive efficiency (Evans, 2011).

Clinical signs associated with reproductive ergotism are more typically attributed to ergopeptine alkaloid and are more common in horses than in ruminants. These negative effects, agalactia, prolonged gestation, dystocia, and embryonic death (Riet-Correa et al., 2013), may be subtle and vary from slight depressions in reproductive efficiency to substantial reductions in pregnancy rates and increased abortions (Evans, 2011). The impact of ergot alkaloids is very much dependent on concentrations in feed. Ovarian follicular dynamics could be adversely affected by ergot alkaloids via interactions involving hyperthermic and prolactin-inhibiting actions of toxins with thermal stress (Burke et al., 2005). Recently, some studies indicated that genes were adversely affected by ergot toxicity, leading to altered embryo quality and development (Jones et al., 2004). Ergot alkaloids produced by *C. purpurea,* causing reproductive problems, are associated with lactational failure in swine (Diekman and Green, 1992). In the bovine reproductive system, these effects may be mediated through the dopamine-mimicking action of ergot compounds (Larson et al., 1995). Various reproductive hormone concentrations, such as progesterone, estrogen, prolactin, and prostaglandin $F_{2\alpha}$, may be altered by ingestion of toxic alkaloids, with any of these endocrine changes potentially causing pregnancy failure (Jones et al., 2004). More recently, a study conducted to assess the potential loss of productivity due to ergot alkaloids from *C. purpurea* exposure at 822 μg/kg of total DMI had no impact on cow

weight, calf weight, rectal temperature, prolactin concentration, progesterone concentration, or post-partum ovarian function (Grusie et al., 2018b).

Ergot alkaloid influences on the reproductive system include altering the hypothalamus, and pituitary and pineal gland axis (Porter et al., 1990). Ergot alkaloids have the ergoline ring structure, with structural similarities to dopamine; this allows many alkaloids to bind D2-dopamine receptors (Sibley and Creese, 1982) in the anterior pituitary, reducing prolactin concentrations. The full extent of this action to cause reduced reproductive efficiency is not well characterized. In sheep and mares, prolactin has its influence on gonadotropin release (Strickland et al., 2011). Although direct effects of prolactin in cattle have not been demonstrated, there are prolactin receptors in bovine corpora lutea (Poindexter et al., 1979) and granulosa cells (Strickland et al., 2011). Furthermore, diameter of the largest ovarian follicle after estrous synchronization was correlated with serum prolactin concentrations (Flores et al., 2008). Therefore, a decreased prolactin concentration may be involved in reduced reproduction (Strickland et al., 2011). As prolactin is necessary for the sustainability of pregnancy and milk production, reduced concentrations would have adverse effects on livestock, including decreased milk production due to underdeveloped mammary glands and agalactia. Plasma prolactin concentration was not affected by ergot treatment in cows (Cowan et al., 2018; Grusie et al., 2018b), however the plasma prolactin levels in cows decreased from pre-exposure period through to post-exposure period in the Cowan et al., 2018 study.

There were lower blood progesterone concentrations post-ergot exposure (Strickland et al., 2011) in heifers, cows, ewes, and mares (Burke and Rorie, 2002; Jones et al., 2004; Klotz, 2015). As progesterone is vital for establishing and maintaining pregnancy, effects of ergot alkaloids on the endocrinology of pregnancy have been evaluated in detail. The critical period for ergot alkaloids negatively affecting conception was the interval between ovulation and the first 6 days of embryonic development in beef cattle (Burke and Rorie, 2002). In one study, 11 of 36 cows aborted 7-10 days after introduction of a rye grass pasture infested with ergot (Appleyard, 1986). In a recent study on cows, progesterone concentration was not affected by ergot alkaloids nor a difference in pregnancy rates in cow control or treatment groups (Grusie et al., 2018b).

1.4 Prolactin and Ergot in Livestock

1.4.1 Prolactin effects on livestock

Prolactin is a polypeptide hormone, discovered almost 90 years ago, identified by its ability to stimulate mammary gland development and lactation. Since then, > 300 actions of prolactin have been reported in various vertebrates (Torner, 2016). These include effects on water and salt balance, growth and development, endocrinology and metabolism, brain and behavior, reproduction, and immune regulation. Prolactin is secreted by lactotrophs of the anterior pituitary. It is universally accepted that the pituitary prolactin secretion is both positively and negatively regulated. It is, however, principally controlled by inhibitory factors originating from the hypothalamus. The most important of these is dopamine, acting on the D_2 subclass of dopamine receptors in lactotrophs (Bouilly et al., 2012; Goffin et al., 2002). Similarities between ergot alkaloids and dopamine in their chemical structures (Figure 1.2) enable alkaloids to occupy D_2-dopamine receptors in the anterior pituitary in an antagonistic mode, thus reducing prolactin secretion by lactotrophs (Klotz, 2015).

Decreases in serum prolactin concentrations are a marker for ergot alkaloid toxicity in cattle, sheep, and horses (Gooneratne et al., 2011; Hurley et al., 1980; Monroe et al., 1988). Due to inconsistencies in other symptoms of ergot alkaloid exposure and relatively consistent nature of prolactin response, this has become the most commonly used indicator of ergot exposure (Klotz, 2015). Decreased serum prolactin concentrations in lactating animals is not always directly associated with decreased milk production; however, an association between low milk production and ergot alkaloid toxicity was reported in cattle (Lean, 2001) and sheep (Stidham et al., 1982), and agalactia was reported in horses (Monroe et al., 1988). Elevated prolactin concentrations associated with daylight have been associated with hair shedding in multiple species (Thompson Jr. et al., 1997). Cattle that graze ergot alkaloid-contaminated tall fescue have shaggy hair coats despite summer heat, intensifying heat stress associated with ergotism (Mcclanahan et al., 2008). In previous studies, prolactin concentrations were too low in cattle grazing tall fescue to initiate winter coat shedding and delaying the onset of the summer hair coat (Aiken et al., 2011). Strickland et al. (2011) suggested that decreased prolactin concentrations affected reproductive ability of seasonal breeding animals more than non-seasonal breeding

animals (e.g., cattle). Prolactin concentrations rebounded once cattle are removed from ergot alkaloid containing diet (Aiken et al., 2013). Overall, the role of lowered prolactin in ergot alkaloid exposure requires further research.

1.5 Ergot and Male Reproduction

1.5.1 Ergot Alkaloid Effects on Sperm Quality

The effects of ergot alkaloids are less well documented in male versus female livestock. There are some reports that male reproductive ability may be altered from exposure to ergot alkaloids. Vasoconstriction, one of ergotism's major effects, is likely to reduce reproductive performance, for example, by impairing thermoregulation of the testes and affecting heat dissipation. Furthermore, ergot alkaloid toxicity could cause hypoxia of testicular tissue. Heat stress is also a major cause of decreased reproductive efficiency (Hansen, 2009). Bovine testes must be kept cooler than core body temperatures for normal spermatogenesis (Kastelic et al., 1997). In one study (Ross et al., 1989), scrotal insulation decreased semen quality, including reduced sperm motility, higher percentage of morphologically abnormal sperm, and lower concentration of sperm in the ejaculate, although there was no change in the rate of spermatogenesis. In a recent study, scrotal insulation was applied for 48 hours to bulls to mimic heat stress and semen was collected at 7-day intervals. There was a reduction in progressive motility, and higher percentage of abnormal sperm with head abnormalities, vacuoles, and tail defects in Holstein-Friesian and Belgian Blue bulls (Rahman et al., 2011). There is ample evidence that oxidative stress reduces sperm function and there is a correlation between lipid peroxide content of human sperm and severe motility loss (Aitken and Baker, 2006). Testes are metabolically active, making them susceptible to oxidative stress and generation of reactive oxygen species. Exposure of human sperm to extracellularly generated reactive oxygen species induces a loss of motility directly correlated with the level of lipid peroxidation. The loss of motility is also observed with overnight incubation of sperm at 37 °C, which is highly correlated with the lipid peroxidation status of the sperm (Gomez et al., 1998). Reactive oxygen species are a natural by-product of sperm metabolism. In equine sperm, reactive oxygen species reduced sperm motility and it was concluded that hydrogen peroxide was the major reactive oxygen species responsible for damage to sperm (Baumber et al., 2000).

1.5.2 Ergot Alkaloid Studies on Male Reproduction

There is not much literature regarding the effects of ergot alkaloids on sperm. Ergot alkaloids in tall fescue cultivars were the primary source of ergot for most studies on male reproductive performance, with none from cereal grains.

In one study, 16 bulls were used to study the effects of ergot alkaloids on bull semen. Bulls were evaluated for prolactin and testosterone concentrations, scrotal circumference, sperm motility, and morphology. There was no difference between experimental and control groups for any end point (Schuenemann et al., 2005a). The same group conducted a second study (containing 270 to 340 µg ergovaline per kg feed) to investigate *in vitro* potential of sperm and reported reduced cleavage rates in the experimental group versus the control group, but no differences in percentages of morphologically normal or motile sperm (Schuenemann et al., 2005b).

In an *in vitro* study to investigate the signaling pathways involved in inhibitory effects of ergotamine and dihydroergotamine on bovine sperm, motility was decreased via alpha adrenergic receptors (Wang et al., 2009). In an *in vivo* study, bulls were fed Kentucky 31 tall fescue for 155 d., Prolactin, a classic marker for ergot toxicity, was significantly decreased in the treatment group and sperm concentration was significantly decreased in experimental versus treatment groups, whereas there was decreased percent morphologically normal sperm in ergot-treated animals. Furthermore, motility and progressive motility of frozen-thawed sperm were decreased in the treatment versus control group (Pratt et al., 2015b). An interesting study investigated sperm velocities after subjecting the animals to ergot alkaloid-infested tall fescue (Looper et al., 2009). Sperm motility and morphology factors, along with prolactin concentrations, were investigated. Again, as with earlier studies, prolactin was significantly lower in the treatment group. Furthermore, percentage motile and progressively motile sperm declined significantly in the treatment group, with reductions in smoothed sperm path and progressive velocity in a straight line Recently, Angus bulls were used to evaluate the effects of ergot alkaloids from tall fescue on prolactin and sperm. Bulls grazing ergot-infested cultivar had lower concentrations of prolactin. However, there was no significant difference between treatment and control groups for ejaculate volume, sperm concentration, percent motile sperm, percent progressive motile sperm, percent normal sperm morphology, and velocities (Burnett et al., 2018). There are almost no

reports on the freezing ability of sperm from bulls consuming ergot alkaloids. In one recent study on the effects of ergot alkaloid consumption on sperm physiology, prolactin was used as a marker for verifying treatment effectiveness and urinary alkaloid concentrations confirmed the presence of ergot alkaloids. Motility and progressive motility of frozen-thawed sperm were significantly lower in the treatment group, with lower progressive motility post-thaw up to 56 days after removal of ergot from the diet (Burnett et al., 2017). Most studies also had a range of results and there is no consistent altered sperm pattern due to ergot alkaloid exposure. Regardless, serum prolactin concentration, sperm motility, and sperm morphology were consistently negatively affected.

1.5.3 Prolactin and Male Reproduction

Since prolactin is present in human seminal plasma, it is thought to be important in male fertility (Sheth et al., 1975). It has a "prosurvival" effect on sperm, prolonging motility and suppressing capacitation, with prolactin concentrations 500 ng/mL or greater significantly preserving sperm motility up to 19 hours post-treatment (Pujianto et al., 2010). However, ergot alkaloids lower prolactin concentrations in livestock. Therefore, prolactin concentrations could serve as a model for assessing the impact of ergot or any other toxicant on male reproduction. Prolactin-dependent mechanisms have been proposed (Pratt and Andrae, 2015) to serve as a model for assessing the impact of ergot alkaloids on male reproduction, as prolactin has been detected in in bull seminal plasma (Pratt et al., 2015b) and the prolactin receptor is present in the testis and sperm, including the epididymis and differentiating spermatids in bulls (Pratt et al., 2015a).

1.5.4 Potential Mechanisms of Ergot Alkaloid Effects on Male Reproduction

Limited information is available on the mechanism of ergot alkaloid on male reproductive functions. Prolactin has long been recognized to be a factor in male reproduction (Bartke, 2004). As previously mentioned, prolactin is present in biologically relevant concentrations in bull seminal plasma, whereas the prolactin receptor is present in the testis, epididymis, and on differentiating sperm (Pratt et al., 2015a). When bulls grazed KY31 compared to a nontoxic endophyte tall fescue, seminal fluid prolactin concentrations differed.

However, they did not differ when consuming a concentrate diet in the presence or absence toxic fescue seeds (Stowe et al., 2013). These studies suggest that prolactin may affect male fertility. If there is an effect on male fertility through changes in prolactin concentrations from fescue toxicosis, then the likely cause is prolactin with stress interactions that may lead to reproductive failure. This example would likely be an interaction of fescue and heat stress interaction, speculated in cattle and known to exist in rodents (Pratt and Andrae, 2015). There have been documented cases of direct actions of prolactin on sperm physiology. For example, in human sperm, addition of physiological concentrations of prolactin stimulated metabolism *in vitro* (Shah et al., 1976) causing presumptive motility effects (Gonzales et al., 1989). Prolactin reportedly has a pro-survival effect on sperm (Pujianto et al., 2010). As discussed earlier, in this same study, prolactin concentrations >500 ng/mL significantly maintained sperm motility for up to 19 hours post treatment. Based on studies in other species and confirmation of the prolactin receptor in bovine sperm, further study is required regarding the effects and/or alteration in expression with exposure to ergot alkaloids.

Dopamine, serotonin, and adrenergic receptors on sperm have been reported in various species. Wang et al., (2009) indirectly identified the presence of alpha-adrenergic receptors present on bovine sperm. They incubated sperm with ergotamine and dihydroergotamine which decreased motility. Further research is required to fully evaluate the presence of ergot alkaloids in bull seminal fluid after consumption, and their role in semen quality and fertility.

1.6 Research Rationale

The purpose of this investigation was to study the effects of *Claviceps purpurea* ergot alkaloid exposure on bull reproductive health. Effects of ergot, especially from fescue toxicosis, on female reproduction have been extensively studied and reviewed (Poole and Poole, 2019); however, ergot effect on male reproduction has not been well characterized. The research focused on two studies involving Angus bulls. In the first study, the objective was to determine the effects of feeding the upper permissible CFIA limit of ergot alkaloids for 9 weeks on fresh sperm quality, sperm functions and plasma prolactin concentrations of yearling bulls during and after the treatment period. In the second study, the objective was to determine if feeding mature and yearling Angus bulls ergot alkaloids (from *C. purpurea*) within the Canadian permissible limit (~3 mg/kg) affect post-thaw sperm quality.

1.7 Overall Research Objectives

For this thesis, objectives were to:

1. assess the impact of long-term ergot alkaloid exposure on sperm characteristics of yearling bulls;
2. assess the impact of long-term ergot alkaloid exposure on serum prolactin concentrations in yearling bulls;
3. predict the maximum feed ergot concentrations that will adversely affect bull fertility;
4. assess the nature of ergot toxicity, whether transient or permanent;
5. assess the impact of cryopreservation on sperm characteristics following prolonged ergot alkaloid exposure in yearling and adult bulls;
6. produce information relevant to Canadian conditions with regards to ergot exposure in livestock.

1.8 Experimental Hypotheses

We tested the following null hypotheses:

1.8.1 Study 1

1. Ergot alkaloids (~3 ppm of dry matter intake) do not affect semen quality (sperm concentration, total number of sperm, motility, progressive motility, velocity and morphology) or sperm structural parameters (plasma membrane integrity, acrosome integrity, proportion of sperm with high and medium mitochondrial membrane potential) during the exposure or post-exposure periods;

2. Prolactin concentrations will not decrease by eating ergot-contaminated feed compared to the Control group.

1.8.2 Study 2

1. Post-thaw sperm characteristics of yearling bulls are not negatively affected by prolonged ergot alkaloid exposure.
2. Post-thaw sperm characteristics of mature bulls are not negatively affected by prolonged ergot alkaloid exposure.

CHAPTER 2:

FEEDING YEARLING ANGUS BULLS LOW-LEVEL ERGOT DAILY FOR 9 WEEKS DECREASED SERUM PROLACTIN CONCENTRATIONS AND HAD SUBTLE EFFECTS ON SPERM END POINTS

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This was the first study conducted on bulls to determine the effects of ergot alkaloids on sperm and breeding potential, as reproductive effects of ergot alkaloids in females are much more widely studied than in males. We used the high limit of the CFIA to establish whether or not there are any effects at the recommended levels.

2.1 Abstract

Our objective was to determine whether feeding yearling bulls with the higher recommended Canadian limit of ergot alkaloids (~3 mg/kg dry matter intake, DMI) would affect sperm characteristics and plasma prolactin concentrations. Aberdeen Angus bulls (12-13 mo old, *n* = 7/group) allocated by blocking for sperm concentration and body weight, were fed placebo or ergot alkaloids in gelatin capsules (60 µg/kg body weight daily, 3.4 mg/kg of DMI) for 9 wk. Semen samples were collected weekly by electroejaculation and examined with a computer assisted semen analyzer (CASA) and flow cytometry, for the intervals 5 wk before (Pre-exposure period), 9 wk during (Exposure period) and 9 wk after (Post-exposure period) treatment. Weekly plasma samples were analyzed for prolactin by radioimmunoassay. Plasma prolactin concentrations decreased markedly (mean ± SEM, 16.74 ± 3.70 in Exposure and 33.42 ± 3.08 ng/mL in Post-Exposure periods; $P<0.01$) compared to Control (67.54 ± 21.47 and 42.59 ± 15.06 ng/mL). Treatment did not affect ($P\geq0.17$) body weight gain, sperm concentration, sperm count/ejaculate, motility or percent live sperm. Averaged over the exposure and post-exposure durations, the scrotal circumference was smaller ($P=0.02$) by 2.7% in the Ergot group. Progressive motility remained unchanged from 59.92 ± 2.31% in Exposure to 59.61 ± 2.59% in Post-Exposure periods, compared to marked increase in Control (61.42 ± 1.60% to 67.52 ± 1.47%; $P=0.02$). Straight-line sperm velocity decreased (-3.15 ± 1.53 µm/s) from exposure to post-exposure periods in Ergot group ($P=0.04$) versus an increase (2.96 ± 2.17 µm/s) in Control. Midpiece defects decreased from Exposure to Post-exposure periods in Control group but remained unchanged in Ergot group (trt*age, $P<0.01$). Ergot feeding resulted in a smaller proportion of sperm with medium mitochondrial potential (Ergot: 22.65 ± 0.98%, Control: 24.35 ± 1.05%, $P=0.04$). In conclusion, feeding ergot at Canadian permissible limit for 9-wk resulted in a 4-fold decrease in plasma prolactin concentrations. Semen end points were not significantly affected, although there were subtle effects on progressive motility, midpiece defects and mitochondrial membrane potential. Clinical relevance of observed changes requires further evaluation. Results supported our hypothesis that prolonged low-level ergot will adversely affect plasma prolactin. However, semen parameters were partially affected, supporting similar work on fescue toxicosis.

Keywords: *Claviceps purpurea*; Ergot alkaloids; Flow cytometry; Prolactin; Semen; Sperm

2.2 Introduction

The fungus *Claviceps purpurea* infects many cereal plants and produces ergot sclerotia containing alkaloids that are toxic to humans and livestock (Belser-Ehrlich et al., 2013). Although ergot poisoning is uncommon in humans, food animals are commonly exposed by grazing or consuming stored feeds. *Claviceps* infections are most prevalent in rye, triticale and wheat, although the fungus is able to infect all grasses and cultivated grains across Canada (Lorenz and Hoseney, 1979). Increasing ergot contamination has become a major problem in western Canada in the past decade (Tittlemier et al., 2015). After being subjected to moisture in the spring, ergot sclerotia germinate, giving rise to ascospores that target the flowering stage and infect the plant ovary. A few days later, the ovary is replaced by stroma that releases a sugary substance called "honeydew". Eventually, the entire seed-head is replaced with a dark, hard, kernel-like sclerotia (Wegulo and Carlson, 2011). Sclerotia are stable for 12 mo (Mitchell and Cooke, 1968) and may survive for >3 y (Rapilly, 1968) and therefore remain infective for multiple crop cycles. Sclerotia contain six primary alkaloid toxins: ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornine (EFSA, 2005) and a group of agroclavines that are less bioactive. In a single sclerotium, the amount of ergot alkaloids can range from 0.15 to 0.5% by weight (Schiff, 2006); therefore, total alkaloid content cannot be accurately estimated from the weight of ergot bodies. Ergot alkaloids are divided into three structural groups (clavines, lysergic acid amides and peptides) that are separated into two major groups: water-soluble amino alcohol derivatives and water-insoluble peptide derivatives (~20 and 80% of the total alkaloid mixture, respectively) (Schiff, 2006). Ergocristine and ergotamine are in the highest concentrations in most grains in Western Canada (Grusie et al., 2018a).

Ergot alkaloids act as receptor-binding agonists or partial agonists of dopaminergic, serotonergic and alpha-adrenergic receptors (Belser-Ehrlich et al., 2013; Pertz et al., 1999). The most well-described changes due to the ergot exposure are peripheral vasoconstriction with subsequent gangrene formation, hallucinations, and decreased peripheral prolactin concentrations. There are four forms of toxicosis associated with ergot exposure in mammals: gangrenous, convulsive, hyperthermic and reproductive forms (Evans et al., 2004). The convulsive form (nausea, convulsions, hallucinations and hyper-excitability) is common during human exposure, but rare in livestock. Gangrenous ergotism is associated with long-term feeding

of ergot alkaloids that cause vasoconstriction of small blood vessels, leading to necrosis of extremities and sloughing of tails, ears, hooves, or lameness. Vasoconstrictive effects have been reported in the testicular artery (Aiken et al., 2015) and extreme cold conditions in Canada may exacerbate these effects. Hyperthermic ergotism is associated with long-term exposure during hot weather. Due to the vasoconstrictive nature of ergot alkaloids, dissipation of body heat is reduced, and core body temperature rises, leading to hyperthermia. Reproductive toxicosis is caused by multiple mechanisms, with these effects of ergot better characterized in females than males (Craig et al., 2015; Grusie et al., 2018b; Hurley et al., 1980; Klotz, 2015; Porter and Thompson, 1992). Ergot alkaloids have primarily been linked with fertility effects in females, as reported in numerous studies. Some of these effects are delayed onset of lowered pregnancy rates (Paterson et al., 1995; Schmidt and Osborn, 1993), reproductive losses due to conception failure or early embryonic loss (Kallenbach, 2015), decreased milk production (Porter and Thompson, 1992), and abortion (Appleyard, 1986) in cattle. Few reports are available on the bull-specific reproductive toxicosis in literature (Burnett et al., 2017; Looper et al., 2009; Pratt et al., 2015b; Schuenemann et al., 2005a, 2005b; Stowe et al., 2013; Strickland et al., 2011).

Decreased blood prolactin concentrations are a major indicator of ergot alkaloid exposure in livestock. The ergoline ring of ergot alkaloids is structurally similar to dopamine and binds to D2 dopamine receptors in the anterior pituitary, mimicking the binding of dopamine (Klotz, 2015) and thereby reducing release of prolactin; this hormone is necessary for maintenance of pregnancy, corpus luteum function (Wegulo and Carlson, 2011), milk production (Grattan, 2015) and it also supports testicular function (Bartke et al., 1986; Pratt et al., 2015a). Suppressed serum prolactin concentrations have also been used as an indicator of ergot alkaloid consumption in tall fescue grass toxicosis (Looper et al., 2009; Pratt et al., 2015b; Schuenemann et al., 2005b; Stowe et al., 2013).

Tall fescue grass is grown on an estimated 16M ha of pasture in the mid-west and southern USA. Most of the grass is infected with a fungal endophyte *Neotyphodium coenophialum* that produces ergot alkaloids somewhat related to those from *Claviceps,* except ergovaline (main alkaloid produced by *N. coenophilium* but not produced by *C. purpurea*). The estimated economic loss due to tall fescue toxicosis during the summer months is ~$1B annually (Strickland et al., 2011). These losses are primarily attributed to decreased body weight gain and milk production. There is growing concern that semen quality may be sufficiently affected by

summer tall fescue toxicosis to result in suboptimal pregnancy rates (Aiken et al., 2013; Aiken and Flythe, 2014; Burnett et al., 2017; Hurley et al., 1980; Looper et al., 2009; Pratt et al., 2015b; Schuenemann et al., 2005b; Stowe et al., 2013). In one study in bulls, there were no significant differences between the experimental (40 µg/kg body weight of ergotamine tartrate) and control groups for prolactin, scrotal circumference, testosterone, sperm motility, or sperm morphology (Schuenemann et al., 2005a). The same investigators conducted a second study and reported no differences in percentage of normal sperm morphology or sperm motility (Schuenemann et al., 2005b). In contrast, an *in vitro* study designed to investigate the signaling pathways involved in inhibitory effects of ergotamine and dihydroergotamine recorded a decrease in percent motile sperm (Wang et al., 2009), likely mediated via alpha adrenergic receptors. In another *in vivo* study, bulls were fed endophyte-infected Kentucky 31 tall fescue and evaluated for serum prolactin concentration and semen quality (Pratt et al., 2015b); both sperm concentration and percent normal morphology were significantly decreased in treatment versus control groups. Further, percent motile and progressively motile sperm significantly decreased in bulls fed endophyte-infested tall fescue, whereas sperm velocities (smoothed sperm path and progressive velocity in a straight line) were lower in the group exposed to ergot alkaloids in pasture for 121 d (Looper et al., 2009). Clearly, effects of ergot alkaloids on semen end points were not consistent. In a more recent study involving yearling and adult Angus bulls conducted over 2 y, ejaculate volume, sperm concentration, percent motile sperm, percent progressive motile sperm, percent normal sperm morphology, and velocities were not significantly different between treatment and control groups (Burnett et al., 2018). It is noteworthy that a major limitation of these studies was the lack of a reliable estimate of ergot alkaloids consumed per animal due to pasture grazing. The Canadian prairies have an extreme winter climate and a milder spring and summer; therefore, mechanisms associated with thermoregulation and temperature stresses for cattle may differ from those in the USA and Europe. Furthermore, there are regional differences in crops, crop management and presumably kinds of ergot alkaloids produced. Despite some studies on the effects of ergot alkaloids from tall fescue on reproduction in bulls, to the best of the authors' knowledge, effects of cereal grain ergot on reproduction in bulls have not been reported.

The Canadian Food Inspection Agency (CFIA) permits up to 3 parts per million (PPMs) of ergot alkaloids in total mixed ration of cattle feed (CFIA, 2017). Our objective was to

determine the effects of feeding the upper limit of ergot alkaloids for a 9-wk period on semen quality, sperm functions and plasma prolactin concentration of yearling bulls during and after the feeding period. We tested the null hypotheses that: 1) ergot alkaloids (~3 ppm of dry matter intake) do not affect semen quality (sperm concentration, total number of sperm, motility, progressive motility, velocity and morphology) or sperm structural parameters (plasma membrane integrity, acrosome integrity, proportion of sperm with high and medium mitochondrial membrane potential) during the exposure or post-exposure periods; and 2) prolactin concentration will not decrease by eating ergot-contaminated feed compared to the Control group.

2.3 Materials and Methods

2.3.1 Statement of Animal Ethics

This study was reviewed and approved by the University of Saskatchewan University Committee on Animal Care and Supply (UCACS) and Animal Research Ethics Board (AREB) under Animal Use Protocol #20170032.

2.3.2 Animal Husbandry

Fourteen Aberdeen Angus yearling bulls (12 to 14 mo of age; 417 to 549 kg body weight) were group-housed in a single open-air pen with shelters at the Goodale Research Farm of the University of Saskatchewan Livestock and Forage Centre of Excellence. Bulls had *ad libitum* access to water and to trace mineral salt blocks (CO-OP® Agro Centre, Saskatoon, SK, Canada). Bulls were observed daily for changes in health and wellbeing throughout the study period.

2.3.3 Experimental Design

Yearling bulls were assigned randomly by blocking for body weight and sperm concentration and were allocated to one of the two experimental groups: Control or Ergot group (n =7 bulls per group). Bulls did not undergo a standard breeding soundness evaluation (BSE) before the experiment; however, their initial scrotal circumference (mean ± SEM) was 33.50 ±

0.49 cm in Control group and 33.21 ± 0.43 cm in Ergot group. Data on scrotal circumference, and semen production and quality were collected during pre-exposure, exposure, and post-exposure periods. Data were gathered from all bulls for an initial 5-wk pre-exposure period during which routine feed (without ergot) was offered. The exposure period was 9 wk, to encompass one full (61 d) spermatogenic cycle (Amann, 1962a, 1962b; Barth, 2013; Staub and Johnson, 2018). Ergot group bulls were given ergot screenings (467 mg ergot alkaloids/kg of screening, Canadian Feed Research Centre, North Battleford, SK, Canada) daily with an oral bolus gun (Balling-Gun Metal Ideal Multi Bolus 17.5 inch; Neogen, Lexington, KY, USA) in 2 to 4 gelatin-capsules pre-weighed for each yearling bull to deliver 60 µg ergot alkaloids/kg body weight, equivalent to 3.4 mg/kg (3.4 ppm) of dry matter intake (DMI). Video clip demonstrating the feeding procedure is included (Supplementary video; online version only). The estimation for ergot alkaloids in the grain screening samples were done by the University of Missouri Veterinary Medical Diagnostic Laboratory on February 13th, 2018, wherein reported concentrations were: ergocornine, 47.1 mg/kg; ergocristine, 254.2 mg/kg; ergocryptine, 62.2 mg/kg; ergosine, 24.1 mg/kg; and ergotamine, 79.9 mg/kg. Ergot group bulls were fed from 26.54 to 36.47 mg total ergot alkaloids each day. The dosage of ergot alkaloids was adjusted at the end of Weeks 3 and 6 (Week 0 = beginning of the feeding) to account for increased bull weight. During the exposure period, the Control group was giving a single placebo capsule daily containing either cornmeal or oat flour using the same bolus gun and were not exposed to ergot at any time. Capsules were packed with ergot screenings flour, by researchers wearing respirators and working in a biosafety cabinet, at the Western College of Veterinary Medicine, University of Saskatchewan. The daily ration for bulls was set at 1.75% of body weight, which included (on an as-fed basis) a combination of silage (12.7 kg/head/d), hay (3.2 kg/head/d) and barley (1.8 kg/head/d) group-fed in their pen.

2.3.4 Sample Collection and Animal Handling

Bull weight, scrotal circumference and rectal temperature were recorded at weekly intervals. Blood and semen samples were also collected from each bull once a week (on Mondays). If a bull was non-cooperative, a second collection was attempted 2 d later (Wednesdays) in the same week. During the pre-exposure period, bulls were only moved from

their pen on collection days, with health and well-being observations done in the pen, for issues related to gait, changes in body coat, behavioral changes, and any visual effects of ergot such as possible gangrene, heavy panting, excessive salivation, or nasal discharge. Throughout the study, care was taken to minimize stress. During the exposure period, bulls were walked from their pen into the barn daily. Bulls in the Control and Ergot groups were fed either placebo or ergot capsules on a daily basis (~24 h intervals) during the exposure period (Supplementary video). To administer capsules, collect blood, semen and scrotal circumference data, each bull was individually restrained in a cattle handling chute, mildly squeezed and its head secured by the front gate.

2.3.4.1 Semen Collection

Semen ejaculates were collected weekly from each bull by electroejaculation, using a Pulsator IV battery pack and rectal probe (Pulsator IV, Lane Manufacturing Denver, CO, USA). The operator wore a protective palpation sleeve, lubricated her/his hand with lubricating gel (Priority Care; General Lube, First Priority, Inc., Elgin, IL, USA), emptied the rectum of feces, transrectally massaged the ampulla (terminal vas deferens), seminal vesicles and prostate gland, then inserted a lubricated electroejaculator probe into the bull's rectum. There were alternating intervals of stimulation and rest, with voltage increased progressively, according to the bull's response. Ejaculates were collected in a cup lined with a disposable plastic cone and held close to the tip of penis. After semen collection, the ejaculate was transferred to a pre-warmed 15-mL Falcon tube and ejaculate volume recorded. Tubes were then placed in a Styrofoam box containing water at 37° C. After collection of semen from all bulls (1 to 2 h), semen was transported to the laboratory (~25 min drive). At the laboratory, semen tubes were placed in a water bath (37° C) pending analysis.

2.3.4.2 Blood Collection

Blood was collected once weekly via caudal venipuncture into heparinized collection tubes. The tubes were placed on ice and subsequently centrifuged (3000 rpm; 12 min) and

plasma removed and stored at -20° C, pending a radioimmunoassay to determine prolactin concentrations.

2.3.5 Computer Assisted Sperm Analysis (CASA)

For CASA analysis, each semen ejaculate was diluted 1:20 in Tris-Citric-Acid buffer (TCA; 3.03% Tris base, 1.74% citric acid monohydrate, 1.2% fructose, Milli-Q distilled H_2O, adjusted to pH 7.1) prior to CASA evaluation. Diluted samples were loaded onto 4-chamber slides (depth 20 µm; Microtool, Cytonix, Beltsville, MD, USA) at a volume of 2.5 µL per well (1 sample/bull/well) and placed on the pre-warmed CASA microscope stage (Sperm Vision1 3.0; Minitube, Ingersoll, ON, Canada). The following semen/sperm end points were recorded by the software:

- Sperm concentration (million/mL)
- Total sperm motility (percent moving sperm)
- Progressive sperm motility (percent sperm moving in a straight line)
- Total sperm count (volume * sperm concentration/mL)
- Sperm velocities [average path velocity (VAP, µm/s; the spatial averaged path that eliminates sperm head wobble); curvilinear velocity (VCL, µm/s; total distance travelled by a sperm during the acquisition divided by time taken); and straight-line velocity (VSL, µm/s; the straight-line distance from beginning to the end of track divided by time taken)]

2.3.6 Sperm Morphology

Semen smears were made by placing 5-µL drops of semen and 5 µL of eosin-nigrosin stain next to each other on one end of a pre-warmed glass slide, mixed and spread across the slide. Differential counts of sperm morphology were done under oil immersion at 1000x magnification, using an upright, phase-contrast microscope (Zeiss Axioskop 40 Microscope, Zeiss, Oberkochen, Germany). At least 100 sperm were counted on each slide and counts recorded for each category. Morphologically normal sperm and those with defects of the head, midpiece, or principal piece, as well as detached heads, proximal droplets, and acrosome defects,

were recorded, as described (Barth, 2013). Percentages were calculated using the counts in each category.

2.3.7 Flow Cytometry for Sperm Structural Characteristics

Plasma membrane integrity, acrosome intactness, and mitochondrial membrane potentials of sperm were assessed with a flow cytometer, using propidium iodide (PI), fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) and Mitotracker deep red (MtDR), respectively, following protocols established in our laboratory (Anzar et al., 2011).

Semen was diluted to $1x10^6$ sperm/mL with TCA buffer. The fluorescent probes, 1 μL fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA; Sigma Chemicals, St. Louis, MO, USA; 1 mg/mL in PBS), 2 μL Mitotracker deep red (MtDR; Invitrogen, Burlington, ON, Canada; 0.1 mM in DMSO), and 3 μL propidium iodide (PI; Invitrogen; 2.4 mM in water) were added to 1 mL aliquot of semen and incubated for 10 min at room temperature. After incubation, 20 μL of 10% formalin was added to fix sperm.

Samples were analyzed by CyFlow Space (Partec GmBH, Munster, Germany). Data were obtained by FloMax software (Version 2.4) provided by Partec GmBH, using an established protocol [41]. Forward and side light scatters were used to identify sperm-specific events and a region drawn around them. Data for all three probes were collected from 10,000 sperm and displayed on a log scale. Sperm stained with PI were considered to have compromised plasma membranes (dead sperm), whereas unstained sperm were considered to have an intact plasma membrane (live sperm) (RN1 region; Fig. 1, Panel A). Sperm stained with FITC-PNA were considered those in which an acrosome reaction had occurred or outer acrosomal membrane is compromised (i.e., disrupted acrosome). Two-dimensional FITC-PNA/PI dot plot (Fig. 1, Panel B) recorded four sperm subpopulations: event count of sperm with a compromised plasma membrane but an intact acrosome (QB1); event count of sperm with a compromised plasma membrane and a compromised acrosome (QB2); event count of sperm with an intact plasma membrane and an intact acrosome (QB3); and event count of sperm with an intact plasma membrane and a compromised acrosome (QB4). Intensity of MtDR staining was gated to categorize sperm into those with high, medium or low mitochondrial membrane potential. The two-dimensional MtDR/PI histograms revealed six subpopulations of sperm (Fig. 1, Panels C

and D): event count of sperm with a compromised plasma membrane and low mitochondrial membrane potential (QA1; Fig. 1, Panel C); event count of sperm with a compromised plasma membrane and medium mitochondrial membrane potential (QA2-Q2; Fig. 1, Panel C, D); event count of sperm with a compromised plasma membrane and high mitochondrial membrane potential (Q2; Fig. 1, Panel D); event count of sperm with an intact plasma membrane and low mitochondrial membrane potential (QA3; Fig. 1, Panel C); event count of sperm with an intact plasma membrane and medium mitochondrial membrane potential (QA4-Q4; Fig. 1, Panel C,D); and event count of sperm with an intact plasma membrane and high mitochondrial membrane potential (Q4; Fig. 1, Panel D). Percentages of sperm belonging to each category were calculated based on event counts for a given category, divided by the total event counts * 100.

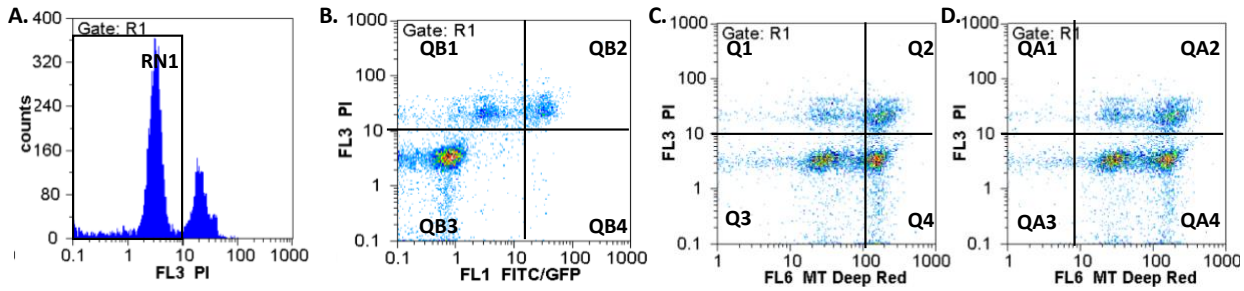

Figure 2.1 Flow cytometry of yearling bull sperm. Panel A is a one-dimensional histogram of PI fluorescence intensity (x-axis) and number of sperm-specific events (y-axis). Left peak (RN1) represents sperm with intact plasma membrane. Panels B, C and D represent two-dimensional plots wherein PI intensity (y-axis) is gated (horizontal line) to divide the sperm populations with intact plasma membrane (lower half; i.e., live sperm) versus those with compromised plasma membrane (upper half; i.e., dead sperm). Panel B is a two-dimensional FITC-PNA (x-axis) versus PI (y-axis) dot plot where FITC-PNA intensity (x-axis) gate (vertical line) separates the sperm counts with an intact acrosome (left half) versus those that are acrosome-compromised (right half). Panels C and D are two-dimensional MtDR (x-axis) versus PI (y-axis) dot plots. Sperm populations are divided by vertical lines between those having low + medium (left) versus high (right) mitochondrial potential (Panel C; Q3) and low (left) versus medium + high (right) mitochondrial potential (Panel D; QA4). Percent live sperm with intact plasma membranes and intact acrosomes = (QB3/(QB1+QB2+QB3+QB4))*100; Percent live sperm with high mitochondrial membrane potential (MtDr++) = Q4/(Q1+Q2+Q3+Q4)*100; Percent live cells with medium mitochondrial membrane potential (MtDr+) = ((QA4-Q4)/(Q1+Q2+Q3+Q4))*100.

2.3.8 Prolactin Concentration

Plasma prolactin concentrations were determined with an established radioimmunoassay in the Endocrinology Laboratory at the Western College of Veterinary Medicine. This is a double-antibody radioimmunoassay with reagents obtained from Dr A.F. Parlow, National Hormone & Pituitary Program (NHPP), Harbor-UCLA Med Ctr, Torrance CA, USA. Concentrations are expressed in terms of bovine prolactin (AFP4832B). For internal control, standards were prepared in 0.5 M phosphate buffered saline containing gelatin. The standard curve ranged from 2 to 128 ng/mL. The antibody used was AFP 753180 (0.2 mL per tube of 1:40,000 dilution in 0.1% Normal Rabbit Serum in 0.5 M phosphate buffered saline). Plasma sample (0.1 mL) and the antibody solution (0.2 mL) were mixed, and tubes were left overnight. Bovine prolactin AFP4832B was iodinated using the Chloramine T procedure (6 µg Chloramine T per µg prolactin to be iodinated). The working solution of tracer (12,000 counts/min in 0.1 mL 0.05 M phosphate buffered saline containing gelatin) was added to all tube and allowed to react overnight at room temperature. Bound and free fractions were separated using 0.5 mL of sheep-anti-rabbit double antibody in 5% polyethylene glycol (5 g Polyethylene Glycol (7000 - 9000 mol wt) dissolved in 100 mL phosphate buffered saline (PBS) with gel). Bovine growth hormone had a 0.06% cross-reaction, whereas bFSH, bLH and bTSH all had < 0.0001% cross-reactivity with the antiserum (data provided by NHPP). Intra-assay coefficients of variation were 7.5 and 6.2% for the reference standards with concentrations of 48.8 and 22.0 ng/mL, respectively. All samples were measured in a single assay.

2.3.9 Statistical Analyses

Statistical Analysis Software (SAS) version 9.4 with Enterprise Guide 6.1 (SAS Institute, Cary, NC, USA) was used for all statistical analyses. The repeated measures Mixed procedure was used to determine effects of treatment, experimental period, weekly animal age and interactions (syntax provided in the Supplementary Section, on-line version only). Variables analyzed included sperm concentration, sperm motility, sperm progressive motility, sperm velocities, sperm morphology, prolactin concentration, proportion of live sperm with intact acrosomes, and proportions of live sperm with high and medium mitochondrial membrane

potentials. Experimental week was used as a repeated variable. Data were analyzed using the animal weekly age for the exposure and post-exposure periods only, as no biological differences were expected in the pre-exposure weeks for all end points; however, data for the pre-exposure period weekly age are displayed for illustrative purpose in the left column graphs in Fig. 2 to 7. Data analysis for treatment periods (right columns in Fig. 2 to 7) included pre-exposure, exposure and post-exposure periods. The best fit model was selected based on the smallest Akaike information criteria (AICC) value from the nine tested covariance structures (simple, compound symmetry, heterogeneous compound symmetry, Toeplitz, banded Toeplitz, Huynh-Feldt, autoregressive, heterogeneous autoregressive and ante-dependence). Statistical significance was set at $P \leq 0.05$. If main effects or interaction term P-value was ≤ 0.05 (Type 3 Test of Fixed Effects), the final analysis of the data included post-hoc multiple comparisons using least square means. Data are presented as arithmetic mean ± SEM. Experimental means were obtained for week age and experimental periods by grouping bulls together in each experimental group.

2.4 Results

2.4.1 Body Weight, Rectal Temperature and Scrotal Circumference

Data on body weight, scrotal circumference and rectal temperature are shown (Fig. 2.2). Body weight changed over experimental periods ($P<0.01$), progressively increasing from pre-exposure period to exposure period to post-exposure period (Fig. 2.2A, right column). There was no effect of the treatment or treatment*time interaction ($P\geq0.66$), whether data were analyzed by weekly age or experimental period.

Rectal temperature (Fig. 2.2C) was not affected by treatment ($P\geq0.47$), irrespective of bull age or experimental period (Fig. 2.2C). There was no treatment*time interaction ($P\geq0.26$) when analyzed by weekly age (Fig. 2.2C, left column). Rectal temperature changed over time ($P\leq0.01$) when analyzed by experimental period (Fig. 2.2C, right column); there was a treatment*period ($P=0.05$) interaction when data were analyzed by experimental period.

The scrotal circumference was smaller (treatment, $P=0.02$) in the Ergot group (35.76 ± 0.18 cm, weekly data combined for exposure and post-exposure period) compared to the Control

(36.74 ± 0.20 cm) when data were analyzed for week age (Fig. 2.2B, left column). There was a tendency for increase in scrotal circumference with age ($P \leq 0.09$) over the exposure and post-exposure periods (Fig. 2.2B, left column). There was a treatment*period interaction effect on scrotal circumference ($P=0.04$) when data were analyzed by experimental period. Scrotal circumference increased in both Control and Treatment groups from Pre-exposure to exposure to post-exposure periods.

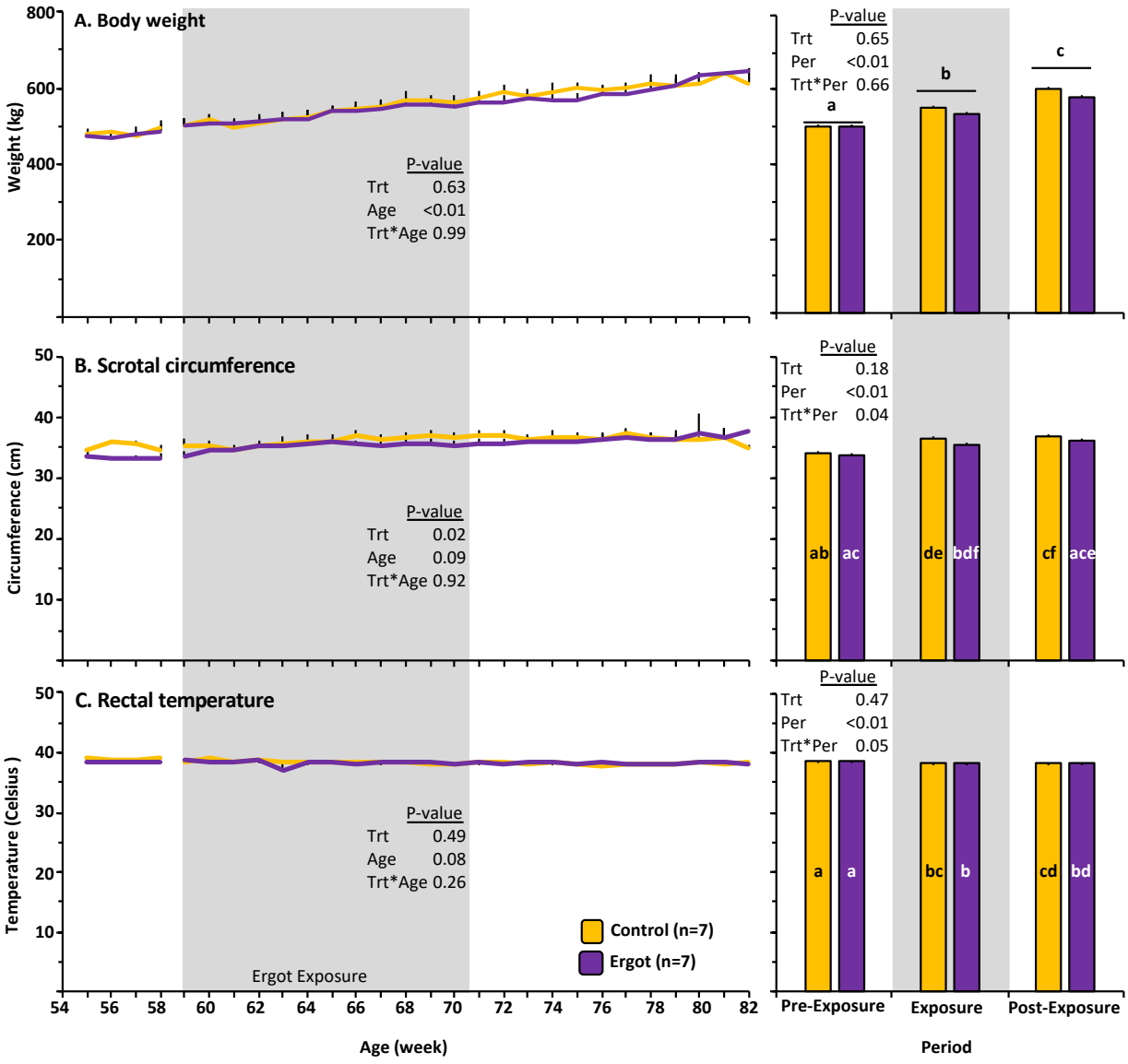

Figure 2.2 Effects of ergot alkaloid consumption (Mean ± SEM) between Control (*n*=7) and Ergot group (*n*=7) yearling bulls, based on weekly age (left panel; line graphs) and comparisons based on exposure periods (5-wk pre-exposure, 9-wk exposure and 9-wk post-exposure period; right panel - bar graphs). Effects of ergot feeding on (A) body weight (kg) and (B) scrotal circumference (cm) (C) rectal temperature (°C). Period of ergot exposure is shown by grey shading in left panel. Age data (left panels) were analyzed from Weeks 59 to 82; however, data for Weeks 54 to 58 are displayed for illustrative purpose. For Experimental period analysis (right panels), data were analyzed from Pre-exposure, Exposure and Post-exposure periods. [a-f] Within an end point, means without a common superscript differed ($P \leq 0.05$).

2.4.2 Computer Assisted Sperm Analysis (CASA)

Data on sperm concentration, total ejaculate sperm count, motilities, and velocities determined by CASA are shown (Fig. 2.3 and 2.4). Sperm concentration (number of sperm per mL) and total sperm count changed over experimental periods ($P<0.01$), increased from pre-exposure to exposure periods for Ergot and Control groups, but remained unchanged thereafter (Figure 3A and B, right panels). There was no effect of ergot exposure (treatment, treatment*age or treatment*period interaction $P\geq0.51$), whether data were analyzed by age or experimental period.

Sperm motility and progressive motility increased ($P<0.01$) from pre-exposure to exposure period for both groups. There was a treatment*period interaction ($P=0.02$) for progressive motility. Progressive motility increased from the pre-exposure to exposure periods for both the Control and Ergot group. However, a slight but significant decrease in progressive motility from the exposure to the post-exposure periods was recorded for the Ergot group (59.92 ± 2.31 to 59.61 ± 2.59 %), with an opposite increasing trend in the Control group (61.42 ± 1.60 to 67.52 ± 1.47 %, Fig. 2.3D, right panel).

Three sperm velocities, namely average path velocity (VAP), curvilinear velocity (VCL), and straight-line velocity (VSL) were analyzed. For experimental period analyses, there was a treatment*period interaction ($P=0.04$) for average path velocity and a tendency for a difference ($P=0.07$) for curvilinear velocity (Fig. 2.4, right column). VAP and VCL in the Control group decreased from pre-exposure to exposure period and returned to pre-exposure values during the post-exposure period, whereas no change in VAP or VCL were evident during these periods in the Ergot group.

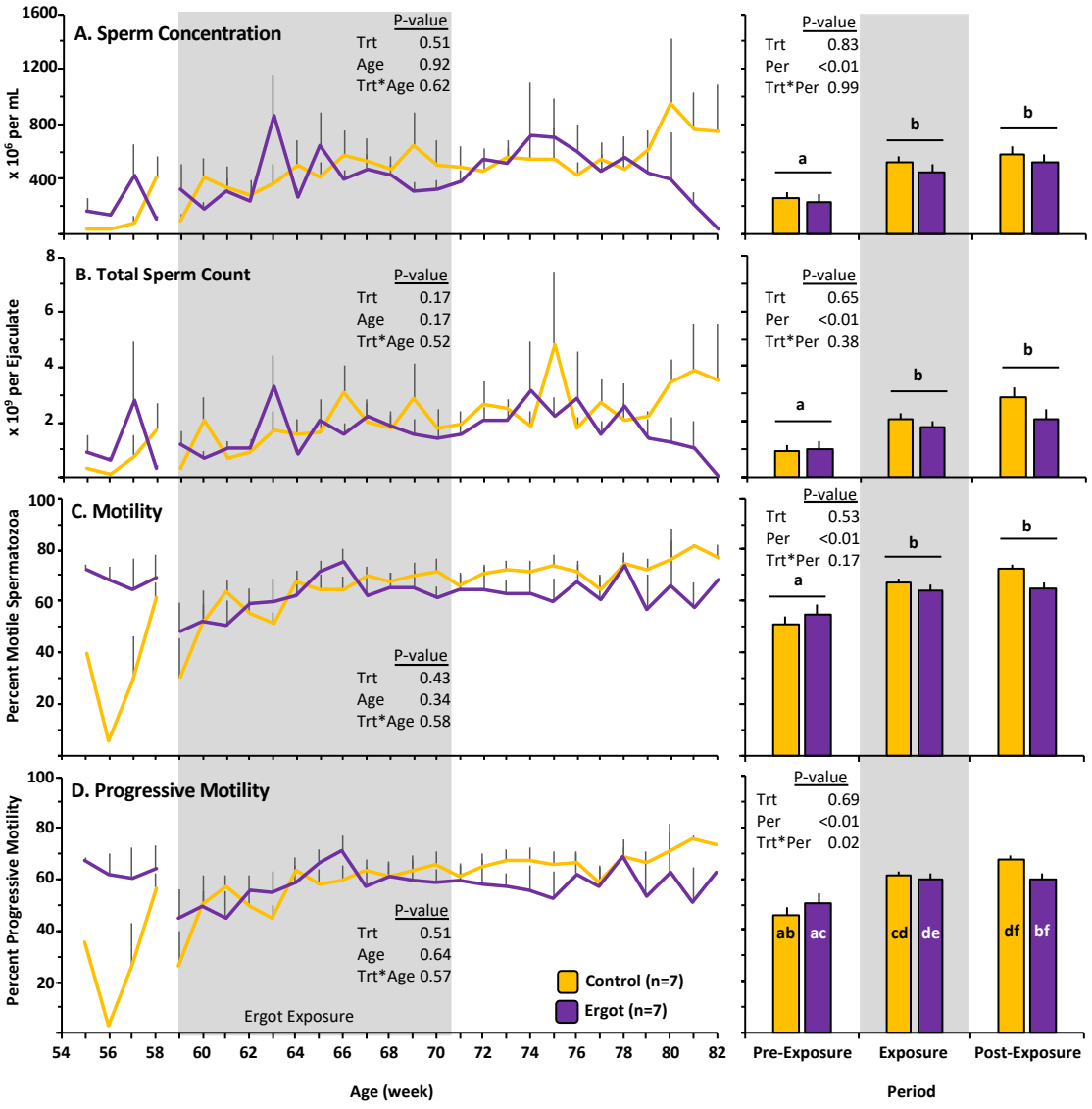

Figure 2.3 Effects of ergot alkaloid consumption (Mean ± SEM) between Control (*n*=7) and Ergot group (*n*=7) yearling bulls, based on weekly age (left panel; line graphs) and comparisons based on exposure periods (5-wk pre-exposure, 9-wk exposure and 9-wk post-exposure period; right panel - bar graphs). Effects of ergot feeding on (A) sperm concentration (10^6 per mL), (B) total sperm count per ejaculate (volume*concentration), (C) percent motile sperm and (D) percent sperm with progressive motility. Period of ergot exposure is shown by grey shading in left panel. Age data (left panels) were analyzed from Weeks 59 to 82; however, data for Weeks 54 to 58 are displayed for illustrative purpose. For Experimental period analysis (right panels), data were analyzed from Pre-exposure, Exposure and Post-exposure periods. [a-f]Within an end point, means without a common superscript differed ($P \leq 0.05$).

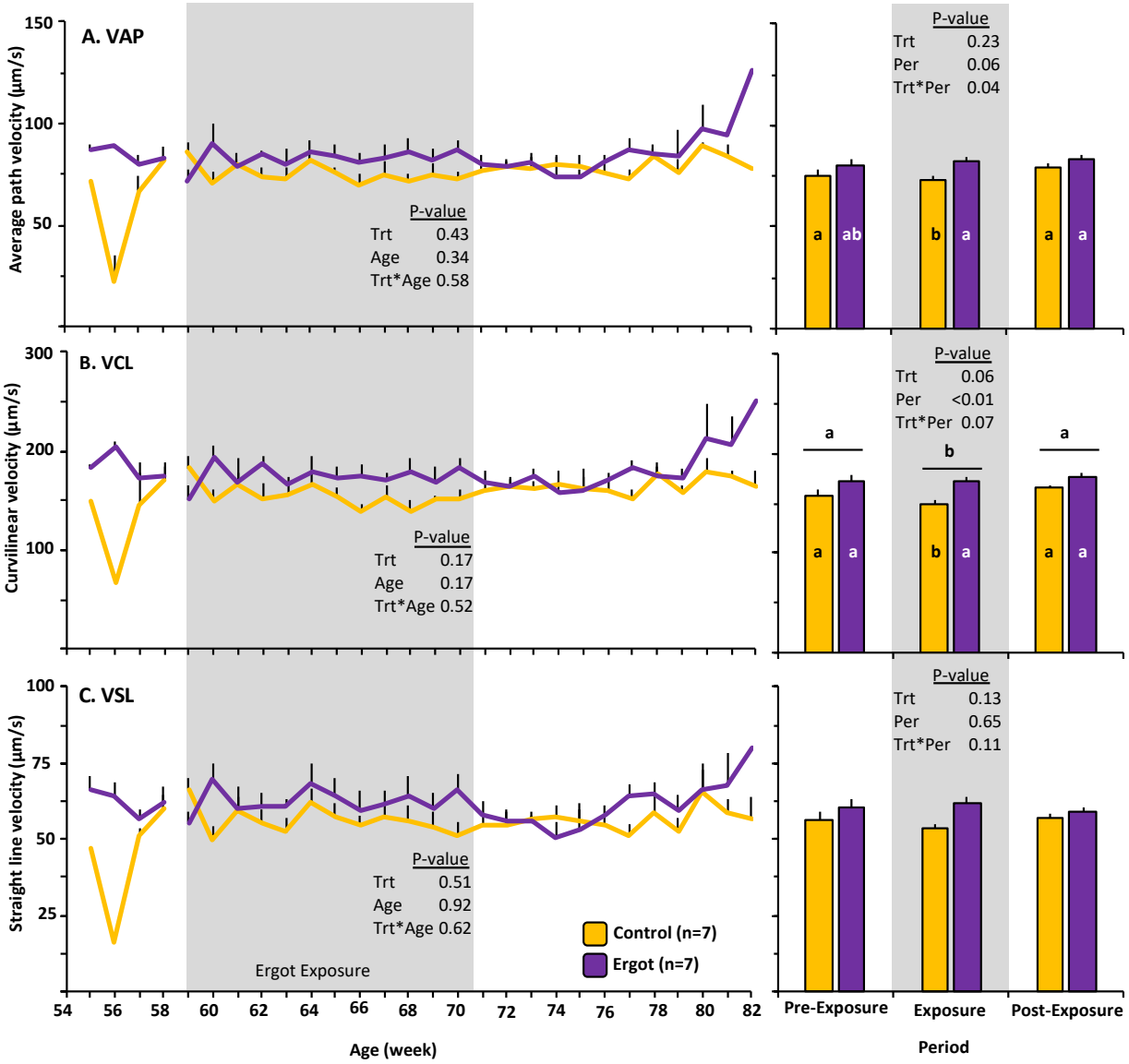

Figure 2.4 Effects of ergot alkaloid consumption (Mean ± SEM) between Control (*n*=7) and Ergot (*n*=7) yearling bulls, based on age (left panel; line graphs) and comparisons based on exposure periods (5-wk pre-exposure, 9-wk exposure and 9-wk post-exposure period; right panel - bar graphs). Effects of ergot feeding on (A) average path velocity (B) curvilinear velocity (C) straight line velocity. Period of ergot exposure is shown by grey shading in left panel. Age data (left panels) were analyzed from Weeks 59 to 82; however, data for Weeks 54 to 58 are displayed for illustrative purpose. For Experimental period analysis (right panels), data were analyzed from Pre-exposure, Exposure and Post-exposure periods. [a,b]Within an end point, means without a common superscript differed ($P \leq 0.05$).

2.4.3 Sperm Morphology

Four end points were analyzed for morphology: percentages of normal sperm, and those with the head, midpiece or principal piece defects (Fig. 2.5). There was a progressive decrease in head defects for the Control and Ergot groups (period $P<0.01$) over the study period, with no difference between treatment groups ($P\geq0.55$). For midpiece defects, there was a treatment*period and treatment*age interaction ($P<0.01$) with a progressive decrease in midpiece defects in the Control group from pre-exposure to exposure and post-exposure periods. In contrast, although the Ergot group had a decrease between pre-exposure and exposure periods, the proportion of sperm with midpieces defects was maintained for the initial half of the post-exposure period (Fig. 4B, left column), resulting in similar percentage of defects between pre-exposure and post-exposure periods (Fig. 2.5B, right column). The percentage of principal defects did not change during the study period ($P\geq0.23$).

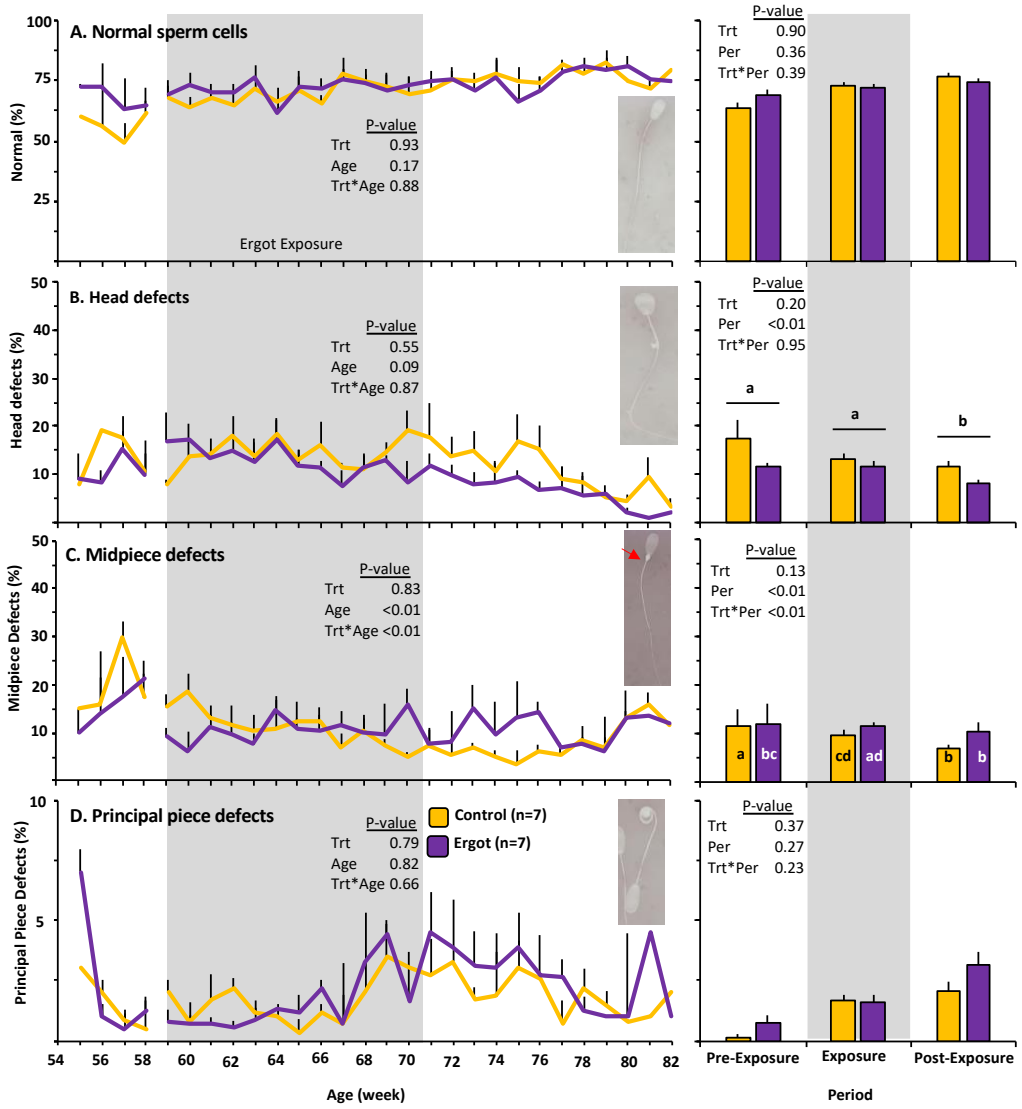

Figure 2.5 Effects of ergot alkaloid consumption (Mean ± SEM) between Control (*n*=7) and Ergot (*n*=7) yearling bulls, based on age (left panel, line graphs) and comparisons based on the exposure periods (5-wk pre-exposure, 9-wk exposure and 9-wk post-exposure period; right panel - bar graphs). Effect of ergot feeding on (A) percent normal sperm; (B) percent head defects [enlarged head defect shown] (C) percent midpiece defects [defect shown with red arrow] and (D) percent principal piece defects [coiled tail defect shown]. Period of ergot exposure is shown by grey shading in left panel. Age data (left panels) were analyzed from Weeks 59 to 82; however, data for Weeks 54 to 58 are displayed for illustrative purpose. For Experimental period analysis (right panels), data were analyzed from Pre-exposure, Exposure and Post-exposure periods. [a-d]Within an end point, means without a common superscript differed ($P \leq 0.05$).

2.4.4 Flow Cytometry for Sperm Structural Characteristics

Sperm populations categorized based on the PI, FITC-PNA and MtDR fluorescence are shown (Fig. 2.6). Percentage of live sperm with intact acrosomes increased ($P<0.01$) between the pre-exposure and exposure periods, with no further change between the exposure and post-exposure periods. There was no effect of ergot treatment ($P\geq0.28$) on the proportion of live sperm with intact acrosomes.

Percentage of live sperm with high and medium mitochondrial potential increased progressively from 5.28 ± 0.52 and 19.93 ± 1.67 during the pre-exposure period to 11.87 ± 0.95 and 21.59 ± 1.24 during the exposure period to 19.98 ±1.02 and 25.45 ± 0.69 during the post-exposure period (respectively, Period $P<0.01$; data for Control and Ergot groups were combined). There was no effect of ergot treatment on proportion of live sperm with high mitochondrial membrane potential; however, when exposure and post-exposure period data were analyzed by bull age (weeks), ergot treatment resulted in a decrease in the proportion of sperm with medium mitochondrial membrane potential when compared to the Control group (22.65 ± 0.98 in the Ergot group and 24.35 ± 1.05 in the Control group, $P=0.04$).

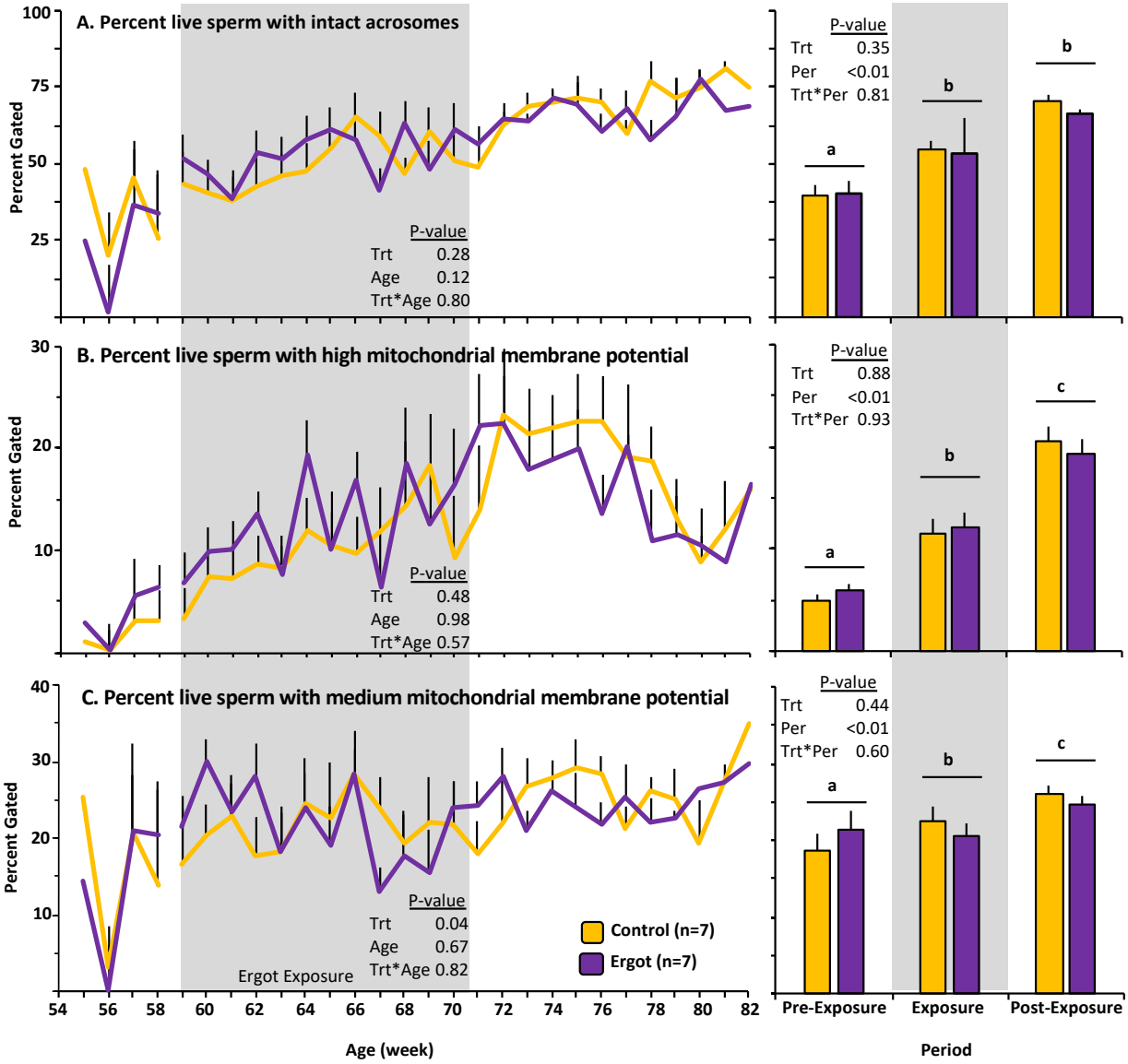

Figure 2.6 Effects of ergot alkaloid consumption (Mean ± SEM) between Control (*n*=7) and Ergot (*n*=7) yearling bulls, based on age (left panel, line graphs) and comparisons based on exposure periods (5-wk pre-exposure, 9-wk exposure and 9-wk post-exposure period; right panel - bar graphs). Effect of ergot feeding on (A) live cell-acrosome integrity (B) high mitochondrial potential sperm and (C) medium mitochondrial potential. Period of ergot exposure is shown by grey shading in left panel. Effect of age (left panel) was analyzed only for exposure and post-exposure periods (pre-exposure data presented for illustrative purposes).

[a-c]Within an end point, means without a common superscript differed ($P<0.05$).

2.4.5 Prolactin Concentration

Plasma prolactin concentrations are shown (Fig. 2.7). Based on age analysis during the exposure and post-exposure period (Fig. 2.7, left column), there was a treatment*age interaction (P<0.01) in plasma prolactin concentrations, with lower prolactin in the Ergot group compared to the Control group. Several prolactin values were exceptionally high in the dataset; therefore, we removed the outliers from the data by totaling the Control group mean + 3 times the standard deviation. Data including the outliers were analyzed for age (presented in Fig. 2.7, left panel). When data after excluding outliers were analyzed based on experimental period (Fig. 5, right column), there was a decrease (P<0.01) in the Ergot group versus the Control group. Overall, there was a tendency for a progressive decrease (P=0.06) in plasma prolactin from pre- to post-exposure period. Prolactin concentrations were 16.74 ± 3.70 and 67.54 ± 21.47 ng/mL during the exposure period and 33.42 ± 3.08 and 42.59 ± 15.06 ng/mL during the post-exposure period in the Ergot and Control groups, respectively.

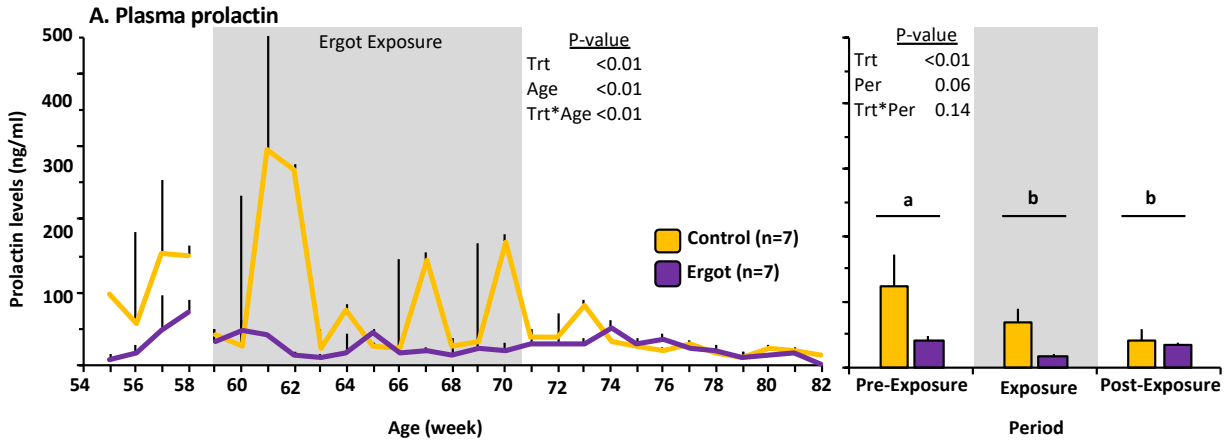

Figure 2.7 Mean ± SEM effects of ergot alkaloid consumption between Control (*n*=7) and Ergot (*n*=7) yearling bulls, based on age (left panel – line graphs) and based on exposure periods (5-wk pre-exposure, 9-wk exposure and 9-wk post-exposure period; right panel - bar graphs). Effect of ergot feeding on (A) prolactin concentrations. Period of ergot exposure is shown by grey shading. Weekly data (left panels) were analyzed from Weeks 59 to 82 (including outlier values); however, data for Weeks 54 to 58 are displayed for illustrative purpose. For Experimental period analysis (right panels), data were analyzed from Pre-exposure, Exposure and Post-exposure periods after excluding outlier values (outliers defined as Control group mean + 3*standard deviation). [a,b]Means without a common superscript differed ($P \leq 0.05$).

2.4.6 Differences between Treatment Periods

To further examine those factors where ergot effects were detected in the above analyses, we calculated differences in mean values of individual bulls between exposure periods (exposure – pre-exposure period and post-exposure - exposure period; Fig. 2.8) and analyzed data with a Student's *t*-test. Progressive motility decreased 0.75 ± 2.75 % between the exposure and post-exposure periods in the Ergot group, in contrast to an increase of 6.09 ± 2.06% in the Control group (tendency for a difference, P=0.07). Likewise, a decrease of 3.15±1.53 µm/s in VSL from exposure to post-exposure period was detected for the Ergot group (P=0.04) compared to an increase of 2.96 ± 2.17 µm/s in the Control group. Further, there was a tendency for a difference (P=0.08) in the magnitude of increase in VAP from the exposure to the post-exposure period, i.e., the Ergot group had a smaller increase (0.35±1.47 µm/s) versus the Control group (6.38±2.71 µm/s). Finally, the increase in percentage of sperm with principal piece defects between exposure and post-exposure period was greater (P=0.05) for the ergot group (1.57 ± 0.42%) compared to the Control group (0.42 ± 0.31%).

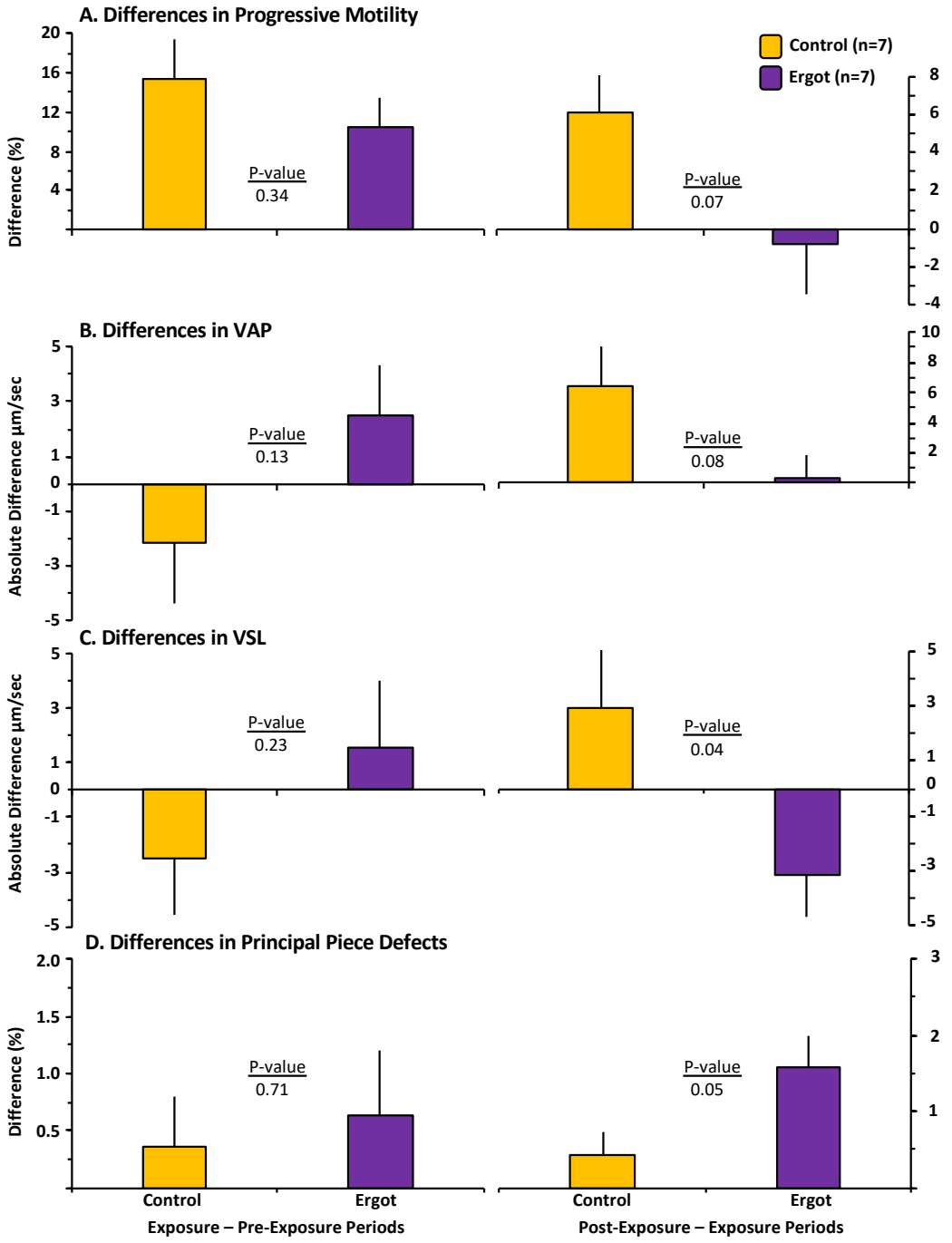

Figure 2.8 The differences between the exposure and pre-exposure period (left column), and the post-exposure and exposure periods (right column) for semen parameters were compared (Mean ± SEM of the difference) between the yearling bulls of Control (*n*=7) and Ergot (*n*=7) groups by T-test. (A) progressive motility (B) average path velocity (VAP) (C) straight line velocity (VSL) (D) principal piece defects. Data are shown for those end points where statistical differences were detected in previous analyses or those showing a tendency for a difference.

2.5 Discussion

Canadian Food Inspection Agency standards permit 2 to 3 mg/kg ergot alkaloids in animal feed. This study investigated effects of feeding the upper permissible limit of ergot alkaloids (3.4 mg/kg of dry matter intake) for a 9 wk period during the spring season on semen quality, sperm structural parameters and prolactin concentrations in yearling bulls. Plasma prolactin concentrations decreased by 4-fold in the ergot-fed bulls during the exposure period. Prolactin concentrations were 1.2-fold lower in the subsequent 9 wk after the exposure period. Since the present study was designed to encompass reproductive maturation, age-related expected improvements in body weight, scrotal circumference, sperm counts, progressively motility, and sperm velocities, along with decreasing trends in sperm defects, were recorded. The scrotal circumference was smaller by 2.7% in ergot-treated yearling bulls (averaged over exposure and post-exposure duration). The majority of CASA and flow-cytometric sperm end points were not affected by this concentration of ergot in feed. However, there were the following ergot-specific changes: 1) small decrease in progressive sperm motility from the exposure to the post-exposure period, in contrast to an expected increasing trend observed in the reference group; 2) a minor decrease in straight-line sperm velocity from the exposure to post-exposure periods in the ergot group, compared to an increase in the control group; 3) continuation of midpiece defects in the first half of the post-exposure period as compared to a progressive decrease in the placebo group; 4) increase in percentage of sperm with principal piece defects from exposure to the post-exposure periods was greater for the ergot group; and 5) a relative decrease in the proportion of sperm with medium mitochondrial membrane potential during exposure and post-exposure periods. The progressive increase in body weight and scrotal circumference was similar in both treated and un-treated yearling bulls and body temperature was maintained during and after ergot exposure. Overall, the present results supported our hypothesis that ergot alkaloids at low-levels markedly decrease plasma prolactin concentrations in yearling bulls. Prolactin concentrations appeared to recover somewhat in the 2-mo period after ergot feeding. Our second hypothesis related to ergot effects on semen quality and sperm characteristics was partially supported. Recorded semen effects were subtle, and it remains to be determined whether these changes affect bull fertility.

In the current study, sperm concentration and total ejaculate sperm count were not affected by the Canadian Food Inspection Agency (CFIA) recommended upper limit of ergot concentration (CFIA, 2017); however, these end points increased from 12-13 mo to 17-18 mo of age during the study period, as would be expected in maturing yearling bulls, indicating that spermatogenesis was not affected by low-level ergot alkaloid exposure. Although total sperm motility was unaffected by ergot exposure, progressive sperm motility in the ergot-exposed subtly decreased compared to the untreated Control group. These results were consistent with a reported decrease in progressive motility in yearling bulls exposed to endophyte-infected tall fescue grass containing related ergot alkaloids (Looper et al., 2009). Progressive sperm motility is an important factor for male fertility; therefore, a decrease in progressive sperm motility due to ergot toxicity cannot be ignored, especially if bulls are exposed to ergot alkaloids for an extended interval.

In the present study, we evaluated three sperm velocity parameters. In relative terms, straight-line velocity decreased ~3% between the exposure and post-exposure periods in the ergot group, compared to the untreated bulls recording a ~3% increase (Fig. 8); however, our study failed to detect a difference between ergot and untreated bulls when absolute velocity values were compared (Fig. 4). The positive changes in average-path velocity recorded due to ergot exposure (Fig. 4) were likely due to an unexplainable decrease in control group during the exposure period. Bulls fed toxic endophyte-infected fescue for 121 d had decreases in both straight-line and average-path velocities (Looper et al., 2009). However in another fescue grass toxicosis study in yearling bulls (Burnett et al., 2018), overall sperm velocity did not change. The straight-line velocity represents linear forward progression of sperm and is important in sperm motility; therefore, a reduction as observed in the present study in this velocity may have detrimental effects. It is, however, important to consider that although the difference was statistically significant, the magnitude of observed change in straight-line sperm velocity and the percentage of progressively motile sperm were subtle. Therefore, clinical implications of these observations on fertility needs further experimentation, e.g., in vitro fertilization procedures.

We critically evaluated sperm morphology defects for head, midpiece and principle-pieces separately, whereas prior studies generally reported only percent normal sperm as a single parameter or as combined major and minor defect parameters (Looper et al., 2009; Pratt et al., 2015b; Schuenemann et al., 2005b). No significant effect of ergot alkaloids was observed on

percent normal sperm in our study. Progressive decreases in head defects from the start to the end of the study were expected for maturing bulls and provided confidence in our experimental design to detect changes. It is interesting to note that the observed deleterious effect of ergot on midpiece defects may have resulted from the continuation of the defects for first half of the post-exposure period (Fig. 4C, left panel). Percentage increase in sperm with principal piece defects between exposure and post-exposure periods was greater in the Ergot group than the Control group (Fig. 8). Ergot may have had a long-term effect on semen quality, if observed midpiece or principal piece defects were exaggerated after longer duration exposure. However, based on our results, it is more likely that there was no biological relevance, and the bulls may have been maturing at different rates. Further, it would be difficult to speculate, based on sperm morphology analysis alone, whether bulls were exposed to ergot.

To our knowledge, this was the first study to simultaneously assess effects of ergot alkaloids on sperm structural parameters using flow cytometry, along with CASA assessment. Although the concept of using flow cytometry to assess plasma membrane integrity, acrosome intactness and mitochondrial membrane potentials is not new (Anzar et al., 2011), it has apparently not been reported for bulls consuming ergot alkaloids. Live sperm with uncompromised plasma membranes and intact acrosomes increased progressively throughout the experimental periods in both groups, consistent with post-pubertal improvements in sperm quality in yearling bulls, but there was no effect of ergot. When exposure and post-exposure period data were analyzed by bull age, ergot treatment resulted in a decrease in the proportion of sperm with medium mitochondrial membrane potential. Increase in overall proportions of sperm with uncompromised plasma membranes and increased high mitochondrial membrane potentials implied that these yearling bulls were maturing normally, and that ergot did not have a negative effect at the organelle level. Although a decrease in the proportion of sperm with medium mitochondrial membrane potentials may be a cause for concern, it was more likely that as the bulls matured, mitochondrial membrane potentials increased. In that regard, the observed change appeared to be within the limits of normal biological variation.

The prominent characteristic of this study was the distinctive ergot feeding process, i.e., using an oral bolus gun. The precise amount of ergot (60 µg ergot alkaloids/kg body weight/d; 3.4 mg/kg DMI) was ingested in a capsule(s) form at consistent intervals. In previous ergot toxicity studies, although the concentration of ergot alkaloids were measured, the total amounts

of ergot alkaloids being consumed by each individual bull or cow were not precisely known and day-to-day variations in consumption may have occurred due to variations in feeding or grazing conditions (Aiken et al., 2013; Burnett et al., 2017; Cowan et al., 2019, 2018; Grusie et al., 2018b; Schuenemann et al., 2005b). The current study represented the real impact of ergot alkaloids with certainty, as individual bull was fed with a fixed dosage of ergot alkaloids compared to previous studies in which grazing method of feeding was used. Interestingly, the prolactin concentrations between the present and previous methods of feeding were similar. Furthermore, this was apparently the first study in cattle to relate ergot alkaloid concentrations with body weight.

Feeding ergot reduced plasma prolactin concentrations by 4-fold during the exposure period. However, that prolactin concentrations rebounded after exposure indicated that delayed or persistent effects of ergot were unlikely. The rapid metabolism and excretion of ergot may be an important factor. This significant reduction in plasma prolactin concentrations in the ergot-fed bulls validated our ergot treatment efficacy and research findings. In recent studies, prolactin concentrations were significantly decreased during fescue grass toxicosis in bulls (Burnett et al., 2018; Looper et al., 2009; Pratt et al., 2015b; Stowe et al., 2013). Prolactin concentrations are sensitive to ergot alkaloids and are considered a clinical marker for ergot toxicity (Gooneratne et al., 2011; Hurley et al., 1980; Monroe et al., 1988). Prolactin may have a protective effect on sperm, as it prolongs sperm motility and suppresses capacitation (Pujianto et al., 2010). In a study conducted to evaluate prolactin in bovine testis and epididymis, bulls were fed ergot-infested feed for 126 d; prolactin concentrations in bovine seminal fluid were consistently higher in the control group compared to the treatment group (Pratt et al., 2015a). Although we did not measure prolactin in seminal plasma, ergot exposure could serve as a future model for assessing impacts of prolactin on male reproduction. In the present study, relatively subtle and variable changes in sperm characteristics compared to the marked reduction in Ergot group prolactin concentration makes prolactin a better indicator than sperm characteristics for ergot toxicity.

2.6 Conclusions

Yearling Angus bulls fed 3.4 mg ergot alkaloids/kg of daily dry matter intake (60 µg ergot alkaloids/kg body weight/d) for 9 wk had subtle changes in sperm parameters, namely

progressive motility, sperm velocities, morphological defects, and marginally slower increase in scrotal circumference; however, these changes were not sufficiently large to have major clinical impact. Plasma prolactin concentrations were markedly decreased in ergot-fed bulls, indicative of ergot alkaloid intake and toxicity. No long-term or delayed effects were observed. Ergot alkaloid concentrations fed at the upper limit of current Canadian standards did not appear to have significant negative impacts on body weight gain, body temperature (under mild spring weather), scrotal circumference, or sperm parameters in yearling bulls. However, as current Canadian standards for ergot concentrations in feed elicited a prolactin response in males, we concluded that critical review and re-evaluation of these standards are warranted.

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Conflicts of Interest

The authors declare no conflict of interest.

CHAPTER 3:

SUSTAINED LOW-DOSE ERGOT ALKALOIDS MINIMALLY AFFECT POST-THAW SPERM CHARACTERISTICS IN MATURE AND YEARLING ANGUS BULLS

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This chapter was to assess the influence of low-dose, permissible-level consumption of ergot alkaloids from *C. purpurea* for an extended interval, on post-thaw sperm characteristics in yearling and adult bulls. Artificial Insemination is a standard practice in the cattle industry and determination whether bull semen can be safely cryopreserved following ergot exposure is important for farmers and producers.

3.1 Abstract

Our objectives were to determine if feeding mature and yearling Angus bulls ergot alkaloids (from *Claviceps purpurea*) within the Canadian permissible limit (~3 mg/kg) affect post-thaw sperm quality. In Experiment 1, mature Angus bulls consumed ergot alkaloids (~1 and ~2 mg/kg of daily dry matter intake, DMI; n=8 and n=6 bulls, respectively) for 61 d, with semen collected and cryopreserved bi-weekly, from 12 wk pre-exposure to 10 wk post-exposure. In Experiment 2, yearling Angus bulls (12-13 mo, n=7/group) were fed placebo or ergot alkaloids (3.4 mg/kg of DMI) daily for 9 wk, with semen collected and cryopreserved once weekly, from 5 wk before to 9 wk after exposure. All frozen semen was assessed 0 and 2 h post-thaw. In Experiment 1, post-thaw total and progressive sperm motilities decreased ($P \leq 0.05$) from pre-exposure to exposure periods, then rebounded. During exposure, VAP and VSL decreased ($P \leq 0.01$) at 0 h compared to pre-exposure and subsequently rebounded. Live sperm with intact acrosomes at 2 h post-thaw was affected by ergot ($P=0.01$). Medium mitochondrial membrane potential increased ($P \leq 0.01$) during exposure compared to pre-exposure and subsequently decreased. In Experiment 2, total and progressive sperm motilities at 0 and 2 h increased ($P \leq 0.01$) steadily throughout the study. During post-exposure, VCL, VAP and VSL at 0 h increased ($P \leq 0.01$) whereas VSL at 2 h increased ($P \leq 0.01$) from pre-exposure to exposure to post-exposure. Live sperm with intact acrosome increased ($P \leq 0.01$) at both 0 and 2 h during post-exposure. Medium mitochondrial membrane potential increased ($P \leq 0.01$) from pre-exposure to exposure, followed by a slight decrease in post-exposure. Mature Angus bulls partially supported our hypothesis, with only transient effects of ergot on sperm motilities and velocities. Post-thaw sperm characteristics in yearling bulls underwent expected age-related developments, with any effects of ergot alkaloids potentially masked by sexual maturation. Overall, results partially supported our hypotheses that ergot has no detectable adverse effect on post-thaw sperm characteristics in mature and yearling bulls.

Keywords: Bulls; Cryopreservation; Ergot; Flow cytometry; Reproduction; Semen

3.2 Introduction

Claviceps purpurea produces complex ergot alkaloids that are toxic when consumed by humans and livestock (Belser-Ehrlich et al., 2013). Ergot contamination is easily identified by black, kernel-like structures on grains or grass known as sclerotia. Human ergot poisoning is now rare due to strict regulatory controls; however, livestock fed ergot-containing cereals can be affected (Lorenz and Hoseney, 1979). Ergot poisoning in livestock has been well documented (Belser-Ehrlich et al., 2013; Coppock et al., 1989; Fraser and Dorling, 1983), with prevalence and severity heavily influenced by climate conditions year to year. Additionally, ambient temperature, humidity, and various environmental factors predispose animals to ergot toxicosis (Klotz, 2015).

Ergot alkaloid-induced effects vary from animal to animal (Aiken and Strickland, 2013), with symptoms appearing within hours to months (Belser-Ehrlich et al., 2013). Cattle have mainly gangrenous and/or hyperthermic forms of ergotism (Bourke, 2000; Schneider et al., 1996b), whereas the convulsive form is more common in humans. Vasoconstriction of extremities causes lameness and eventual gangrene of the feet, tail and ears in cattle (Mantle, 1969). Effects of ergot alkaloids on suppression of prolactin concentrations in cows are well documented (Browning et al., 1998; Hurley et al., 1980). However, there are limited reports on fescue grass toxicosis on bull reproduction during summer (Looper et al., 2009; Pratt et al., 2015a, 2015b; Schuenemann et al., 2005a, 2005b), with inconsistent effects on semen quality and physiology. Furthermore, there are limited reports regarding effects on cryopreserved sperm. Adding ergot alkaloids semen extender reduced sperm motility (Gallagher and Senger, 1989) and semen from bulls grazing on non-toxic ergot field had greater than 3 folds post-thaw motility (Pratt et al., 2015b). Sperm from bulls consuming ergot alkaloids produced fewer cleaved embryos following in vitro fertilization (Schuenemann et al., 2005a, 2005b). Sperm concentration and percent total and progressive sperm motility from bulls grazing tall fescue with ergot alkaloids was lower post-thaw, with sperm motility affect for at least 56 days following removal of ergot (Burnett et al., 2017), whereas those that grazed on alkaloid-infested KY31 endophyte for 121 d had reductions in total and progressive motility (Looper et al., 2009). Yearling bulls fed ergot alkaloids for 9 wks had subtle negative effects on progressive motility, midpiece defects, and mitochondrial membrane potential (Chohan et al., 2021).

The Canadian Food Inspection Agency (CFIA) allows ≤ 3 parts per million (PPMs) of ergot alkaloids in animal feed (CFIA, 2017). Proportions of the ergot alkaloids in cereal crops (Grusie et al., 2018a) differ from those in fescue grass (Robbins et al., 1986), depending upon farm management strategies. Most tall fescue grass is infected with a fungal endophyte *Neotyphodium coenophialum* that produces ergot alkaloids somewhat related to those from *Claviceps*. Annual beef production losses due to fescue toxicosis in the USA were estimated at $US600M (Hoveland, 1993) and ~$US1B (Strickland et al., 2011). In 2018, Canadian beef cattle semen exports were ~$CDN5 million (Canadian Dairy Information Centre, 2019) and increasing. The Canadian prairies have milder spring and summer and an extreme winter climate; therefore, mechanisms associated with thermoregulation and temperature stresses for cattle may differ from those in the USA and Europe. Furthermore, there are regional differences in crops, crop management and presumably kinds of ergot alkaloids produced. Despite some studies on the effects of ergot alkaloids from tall fescue on reproduction in bulls, to the best of the authors' knowledge, there is no study on the effects of cereal grain ergot on reproduction in bulls.

Our objectives were to determine if feeding mature and yearling Angus bulls ergot alkaloids (from *C. purpurea*) within the Canadian permissible limit (~3 mg/kg) affect post-thaw sperm quality. We tested the null hypothesis that consumption of CFIA limits for ergot do not significantly affect quality of frozen-thawed sperm of mature or yearling beef bulls.

3.3 Materials and Methods

3.3.1 Statement of animal ethics

This study was conducted in accordance with recommendations of the University of Saskatchewan University Committee on Animal Care and Supply (UCACS) and Animal Research Ethics Board (AREB) under Animal Use Protocol #20170032. Throughout the study, bulls were observed daily for health and fitness.

3.3.2 Experiment 1: Effect of ergot feeding on freezing of semen from mature bulls

There was a pre-exposure period (12 wk; no ergot), exposure period (9 wk; ergot in feed), and post-exposure period (10 wk; no ergot). Exposure and post-exposure periods were based on a 61-d spermatogenic cycle in bulls (Amann, 1962a, 1962b; Staub and Johnson, 2018); consequently, potential adverse effects would be reflected in sperm morphology and function and structure. Bulls were housed at the Goodale Research Farm of the University of Saskatchewan Livestock and Forage Centre of Excellence (LFCE) in two open-air pens and fed in groups with *ad libitum* access to water, shelter, and total mineral salt blocks (CO-OP® Saskatoon, SK). Bulls were fed 1.5-2% of body weight (assuming 1000 kg mean body weight). Barley pellets, alfalfa silage, and hay were offered at 5, 6, and 6 kg/head/d, respectively. Fourteen Angus bulls [age 2 to 5 y; body weight range 717 to 1085 kg] were randomly assigned to one of two ergot treatment groups: low- and high-dose groups (n=8 and n=6, respectively). All bull were deemed breeding sound, according to a regional standard (Western Canadian Association of Bovine Practitioners; Barth, 2013).

Current Canadian tolerance levels of 2 to 3 mg/kg (2 to 3 ppm) of ergot (CFIA, 2017) were used as ergot benchmarks. Low and high ergot alkaloid concentrations were 1 and 2 mg/kg, respectively, of total mixed ration (based on the DMI), assuming total dry matter intake 16.5 kg/head (silage dry matter ~35%). Bulls in each ergot treatment group were kept and fed in two separate pens. Bulls were group-fed in the morning daily by placing 5 kg/bull/d feed pellets (combination of ergotized and control pellets) in a long trough. In the low ergot treatment group, each bull was offered 1.1 kg of ergotized pellets and 3.9 kg of control pellets per day (8.8 kg ergot pellets + 31.2 kg control pellets for group feeding of 8 bulls). In the high-dose group, bulls were offered 2.2 kg ergot pellets and 2.8 kg control pellets per day (13.2 kg ergot pellets + 16.8 kg control pellets for group feeding of 6 bulls). Initially, the high end of allowed concentrations (3 mg/kg) was offered; however due to feed refusal, a lower ergot concentration (2 mg/kg) was used. The remaining ration was offered in the afternoon. On sampling days, bulls were allowed 1 h to consume feed prior to semen collection. Control pellets contained barley (74%), oat hulls (15%), canola meal (6%), and molasses (5%). Ergotized pellets were manufactured using concentrated wheat screenings (Table 1) mixed with control pellets at the Canadian Feed Research Centre (North Battleford, SK, Canada).

Semen collection was conducted fortnightly for all bulls. Eight bulls were sampled on Tuesdays (4 bulls from low dose and high dose groups each) and six bulls on Thursdays (4 bulls

from low dose group and 2 bulls from high dose group). Bulls from each pen were walked to the collection barn one-by-one with the order alternating every collection day. Semen was frozen as described below.

Table 3.1 Concentrations (mean ± SD; mg/kg dry matter) of six ergot alkaloids in feed pellets, as determined by LC/MS following solvent extraction.

Ergot alkaloid	Concentration (mg/kg DM)	Proportion of total (%)
Ergocornine	1.571 ± 278	9
Ergocristine	8.465 ± 2203	51
Ergocryptine	3.089 ± 373	18
Ergometrine	0.431 ± 28	3
Ergosine	0.917 ± 105	5
Ergotamine	2.285 ± 377	14
Total (ppm)	17 ± 3	100
*mg/kg = parts per million (ppm)		

3.3.3 Experiment 2: Effect of ergot feeding on freezing of semen from yearling bulls

Fourteen yearling Angus bulls (age 12 to 14 mo; body weight range 417 to 549 kg at the start of experiment) were randomly allocated (n=7) into 2 groups, using body weight and sperm concentration as blocks. All bulls were housed at the Goodale Research Farm, University of Saskatchewan LFCE in an open-air pen with shelters. Bulls had *ad libitum* access to water and trace mineral salt blocks (CO-OP® Agro Centre, Saskatoon, SK, Canada). As yearling bulls were attaining reproductive maturity during the study, semen characteristics were expected to improve progressively; therefore, a sham treatment (Control) group was included. All bulls had an initial 5 wk pre-exposure period without ergot offered. Ergot feeding was done for 9 wk (exposure period) in order to complete 1 full spermatogenic cycle of 61-d (Amann, 1962b, 1962a; Staub and Johnson, 2018). In the ergot group, 2 to 4 gelatin capsule(s) were pre-weighed for each yearling bull to feed 60 µg ergot alkaloids/kg body weight, equivalent to 3.4 mg/kg (3.4 ppm) of dry matter intake (DMI). Bulls in the ergot group were given ergot screenings (467 mg ergot

alkaloids/kg of screening, Canadian Feed Research Centre, North Battleford, SK, Canada) daily (morning) with an oral bolus gun (Balling-Gun Metal Ideal Multi Bolus 17.5 inch; Neogen, Lexington, KY, USA). Measurements of ergot alkaloids in grain screening samples were done by the University of Missouri Veterinary Medical Diagnostic Laboratory on February 13th, 2018 and were: ergocornine 47.1 mg/kg, ergocristine 254.2 mg/kg, ergocryptine 62.2 mg/kg, ergosine 24.1 mg/kg, and ergotamine 79.9 mg/kg. Therefore, ergot group bulls were fed 26.54 to 36.47 mg total ergot alkaloids daily. The dosage of ergot alkaloids was adjusted at the end of wk 3 and 6 (wk 0 = beginning of the ergot feeding) to account for increased bull weight during maturation. During the exposure period, control group bulls were given a single placebo capsule daily containing either cornmeal or oat flour using the oral bolus gun and were not exposed to ergot at any time. This was followed by a post-exposure 9 wk period with no ergot, to determine potential delayed or long-term adverse effects. Capsules were packed with ground ergot screenings in a biosafety cabinet at the Western College of Veterinary Medicine, with researchers wearing disposable gowns and respirators. The daily ration for the yearling bulls was set at 1.75% of body weight, which included a combination of silage (12.7 kg/head/d), hay (3.2 kg/head/d), and barley (1.8 kg/head/d) group-fed in their pen. Semen was collected on Mondays and cryopreserved using a standard protocol, as described below.

3.3.4 Semen collection

Ejaculates were collected from each bull by electroejaculation, using a Pulsator IV console (Lane Manufacturing, Denver, CO, USA). Bulls were restrained in a cattle handling chute by securing the head in a gate. Using an arm covered with a lubricated palpation sleeve, the operator evacuated feces from the rectum and massaged the ampulla, seminal vesicles, and prostate, followed by insertion of a lubricated electroejaculator probe. The voltage was increased progressively with alternating intervals of stimulation and rest, according to the bull's response. Semen samples were collected fortnightly from mature bulls and weekly from yearling bulls. If semen could not be collected on a specified day, semen collection was attempted on an alternate day in the same week.

After semen collection from each bull, the ejaculate was transferred to a prewarmed 15-mL Falcon conical tube and volume recorded. Semen tubes were then placed in a Styrofoam box

containing water at 37° C. After collection of semen from every bull (1 to 2 h), semen tubes were transported to the laboratory (approximately 25-min drive). Semen tubes were placed in a water bath (37° C) prior to semen analysis and freezing.

3.3.5 Semen cryopreservation

After initial evaluation for volume, concentration and motility, semen was diluted in Tris-citric acid (TCA) egg yolk extender to ~ 50 × 10^6 sperm/mL. Diluted semen was cooled slowly (≥2.5 h) to 4 °C in a water bath, then packaged in pre-printed (date, bull and treatment) 0.5-mL polyvinyl French straws and frozen in a programmable cell freezer (IceCube 14S; SY Lab Gerate GmbH, Neupurkdersdof, Austria) from +4 to −80 °C, as reported (Anzar et al., 2010), then plunged into liquid nitrogen and stored until thawed and analyzed.

3.3.6 Post-thaw sperm analyses

Two frozen semen straws per bull were thawed at 37 °C for 1 min and semen contents pooled. Semen samples were incubated at 37 °C in their original extender and sperm motility characteristics, plasma membrane integrity, mitochondrial membrane potential and live sperm-intact acrosomes were determined immediately at 0-h (≤15 min post-thaw) and 2-h post-thaw.

3.3.7 Sperm motion characteristics - Computer Assisted Sperm Analysis (CASA)

Post-thaw sperm motion characteristics were analyzed with computer-assisted sperm analyzer (CASA; Sperm Vision1 3.0, Minitube, Ingersoll, ON, Canada). Semen (2.5 µL each) was loaded onto 4-chamber slides (depth 20 µm; Microtool, Cytonix, Beltsville, MD, USA), with assessment of the following: sperm concentration (x10^6/ml); total sperm motility (% of all moving sperm); progressive sperm motility (% of sperm moving in a straight line, >10 µm radius at >4.5 µm/s); and sperm velocities: average path velocity (VAP, µm/s; the spatial averaged path that eliminates the wobble of the sperm head); curvilinear velocity (VCL, µm/s; total distance travelled by a sperm during the acquisition divided by time taken); and straight-line velocity (VSL, µm/s; the straight-line distance from beginning to the end of track divided by time taken).

3.3.8 Sperm structural characteristics

Plasma membrane integrity, acrosome intactness, live-dead ratio, and mitochondrial membrane potential of each ejaculate were evaluated with flow cytometry; using fluorescent probes propidium iodide (PI), fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) and Mitotracker deep red (MtDR), respectively. Protocols used were previously established in our laboratory (Anzar et al., 2011).

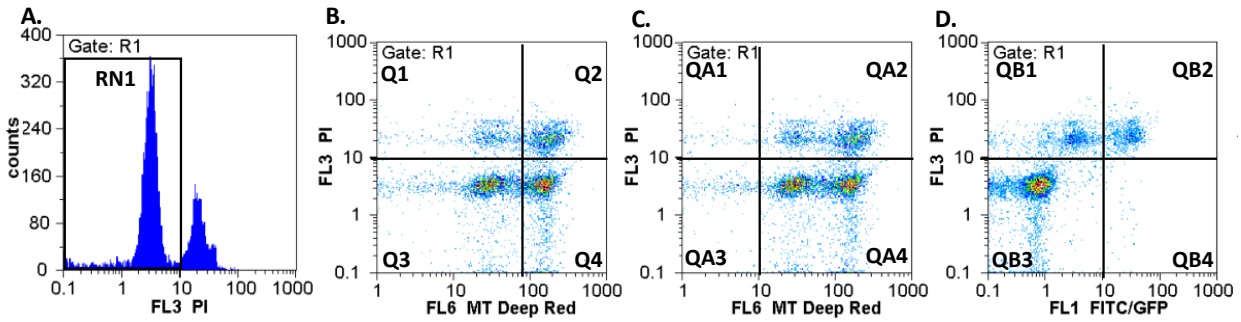
Briefly, fluorescent probes, 1 μL fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA; Sigma Chemicals, St. Louis, MO, USA; 1 mg/mL in PBS), 2 μL Mitotracker deep red (MtDR; Invitrogen, Burlington, ON, USA; 0.1 mM in DMSO), and 3 μL propidium iodide (PI; Invitrogen; 2.4 mM in water) were added to 1 mL of semen and incubated for 10 min at room temperature. After incubation, 20 μL of 10% formalin was added to fix sperm. Samples were analyzed with a CyFlow Space flow cytometer (Partec GmBH, Munster, Germany) and data obtained by FloMax software (Version 2.4, Partec GmBH). Forward and side light scatters were used to identify sperm-specific events and a gate was drawn around them. Fluorescent data were collected from 10,000 sperm on a log scale.

Sperm stained with PI had compromised plasma membranes (dead), whereas unstained sperm were considered to have an uncompromised plasma membrane (live) (RN1 region; Fig. 1, Panel A).

Two-dimensional MtDR/PI histograms revealed percentages of 6 subpopulations of sperm (Fig. 1, Panels B and C). Sperm with compromised plasma membranes (dead) and low membrane potentials (QA1; Fig. 1, Panel C); sperm with compromised plasma membrane (dead) and medium mitochondrial membrane potential (QA2-Q2; Fig. 1, Panel C and B); sperm with compromised plasma membrane (dead) and high mitochondrial membrane potential (Q2; Fig. 1, Panel B); sperm with uncompromised plasma membrane (live) and low mitochondrial membrane potentials (QA3; Fig. 1, Panel C); sperm with uncompromised plasma membrane (live) and medium mitochondrial membrane potential (QA4-Q4; Fig. 1, Panels C and B); and sperm with uncompromised plasma membrane (live) and high mitochondrial membrane potential (Q4; Fig. 1, Panel B).

Two-dimensional FITC-PNA/PI histogram (Fig. 1, Panel D) recorded percentages of 4 sperm subpopulations: sperm with compromised plasma membrane (dead) and intact acrosomes

(QB1), sperm with compromised plasma membrane (dead) and damaged acrosomes (QB2), sperm with uncompromised plasma membrane (live) and intact acrosomes (QB3), and sperm with uncompromised plasma membrane (live) and ruptured acrosomes (QB4).



Live sperm with uncompromised plasma membranes and intact acrosomes = QB3
Live sperm with high mitochondrial membrane potential (MtDr++) = Q4
Live sperm with medium mitochondrial membrane potential (MtDr+) = QA4-Q4
Live sperm with low mitochondrial membrane potential (MtDr-) = QA3

Figure 3.1 Flow cytometry graphs of bull sperm. Panel A: One-dimensional histogram of PI fluorescence intensity (x-axis) and number of sperm-specific events (y-axis). Left peak (RN1) represents sperm with an uncompromised plasma membrane. Panels B and C: Two-dimensional MtDR vs PI dot plots divided into quadrants. Data are in percentages and each quadrant represents: Q1 (PI+/MtDR- and MtDR+), sperm with compromised plasma membranes and low+medium mitochondrial membrane potentials; QA1 (PI+/MtDR-), sperm with compromised plasma membrane potentials and low mitochondrial membrane potentials; Q2 (PI+/MtDR++), sperm with compromised plasma membranes and high mitochondrial membrane potential; QA2 (PI+/MtDR+ and MtDR++) sperm with compromised plasma membranes and medium+high mitochondrial membrane potentials; Q3 (PI-/MtDR- and MtDR+), sperm with uncompromised plasma membranes and low+medium mitochondrial membrane potentials; QA3 (PI-/MtDR-) sperm with uncompromised plasma membrane potentials and low mitochondrial membrane potentials; Q4 (PI-/MtDR++), sperm with uncompromised plasma membranes and high mitochondrial membrane potential; QA4 (PI-/MtDR+ and MtDR++) sperm with uncompromised plasma membranes and medium+high mitochondrial membrane potentials. Panel D: Two-dimensional dot plot divided into quadrants representing: QB1 (PI+/FITC-PNA-), sperm with compromised plasma membranes and intact acrosomes; QB2 (PI+/FITC-PNA+), sperm with compromised plasma membranes and damaged acrosomes; QB3 (PI-/FITC-PNA-), sperm with uncompromised plasma membrane and intact acrosomes; QB4 (PI-/FITC-PNA+), sperm with uncompromised plasma membranes and damaged acrosomes. Abbreviations: PI, propidium iodide; FITC-PNA, fluorescein isothiocyanate conjugated peanut agglutinin; MtDR, Mitotracker deep red.

3.3.9 Statistical analyses

Statistical Analysis Software (SAS) version 9.4 with Enterprise Guide 6.1 (SAS Institute, Cary, NC, USA) was used for all statistical analyses. A repeated-measures Mixed procedure was used to determine effects of treatment (high versus low ergot for mature bulls and control versus ergot treatment for yearling bulls), experimental period (pre-exposure versus exposure versus post-exposure), and their interactions (complete syntax provided in supplementary section; on-line version only). Outcome variables analyzed included total sperm motility, progressive sperm motility, sperm velocities, live sperm acrosome integrity (intact vs damaged), and live sperm high and medium mitochondrial membrane potentials at 2-wk (mature bulls) or 1-wk (yearling bulls) intervals.

Experimental week was used as a repeated variable in the model. The best fit model was selected based on the smallest Akaike information criteria (AICC) value from the 0 tested covariance structures (simple, compound symmetry, heterogeneous compound symmetry, Toeplitz, banded Toeplitz, Huynh-Feldt, autoregressive, heterogeneous autoregressive and ante-dependence). Statistical significance was set at $P \leq 0.05$. If P-value of main effects or interaction term was ≤ 0.05 (Type 3 Test of Fixed Effects), final analysis of the data included post-hoc multiple comparisons using least square means. Group descriptive values (arithmetic mean ± SEM) of each outcome variable for a given period (pre-exposure, exposure and post-exposure) were obtained using SAS command and presented in Fig. 2 to 5.

Differences between 0 and 2 h analyses were determined by subtracting individual values for each bull/endpoint at 2 h from 0 h. The same repeated measures Mixed procedure was used to determine effects of treatment (high versus low ergot for mature bulls and control versus ergot treatment for yearling bulls), experimental period (pre-exposure versus exposure versus post-exposure), and their interactions.

3.4 Results

3.4.1 Experiment 1 - Effect of ergot feeding on frozen-thawed semen from mature bulls

Sperm motion (CASA) and structural characteristics (flow cytometry) are shown (Fig. 3.2 and 3.3, respectively).

3.4.1.1 Sperm motion characteristics

For post-thaw total sperm motility and progressive motility at 0 h (Fig. 3.2A and B, left column) and 2 h (Fig. 3.2A and B, middle column), and their decline over time, there was no difference ($P \geq 0.27$) between high and low ergot groups; therefore, data were combined over ergot groups. However, period affected ($P \leq 0.05$) all sperm motion characteristics and their decline over 2 h post-thaw.

Both total and progressive motilities (averaged over ergot group) decreased from pre-exposure (25.0 ± 1.43 and 15.0 ± 1.50 at 0 h; and 17.1 ± 1.40 and 5.9 ± 1.52 at 2 h, respectively) to exposure (18.3 ± 1.43 and 9.7 ± 1.50 at 0 h; and 14.5 ± 1.29 and 3.7 ± 1.64 at 2 h). Both motilities increased equivalent) to the pre-exposure level during the post-exposure period (27.2 ± 1.40 and 17.0 ± 1.48 at 0 h; and 17.8 ± 1.48 and 6.8 ± 1.65 at 2 h, $P > 0.05$). Likewise, the decline in all sperm motion characteristics from 0 h to 2 h was influenced by period; it was the smallest during exposure period compared to pre-exposure and exposure periods ($P \leq 0.05$) (Fig. 3.2A and B, right column).

Sperm curvilinear velocity (VCL), average path velocity (VAP), and straight-line velocity (VSL) were analyzed at 0 and 2 h (Fig. 3.2C, D, and E). Sperm VCL did not change due to ergot treatment, period, or treatment*period interaction ($P \geq 0.05$) at 0 h. Sperm VAP and VSL at 0 h decreased during exposure ($P \leq 0.01$) compared to pre-exposure, and both velocities increased during post-exposure, equivalent to pre-exposure ($P \geq 0.05$). At 2 h, none of the sperm velocities (VCL, VAP and VSL) changed due to period ($P \geq 0.25$). The relative decline in VAP and VSL between 0 and 2 h was smaller ($P \leq 0.01$) during exposure compared to pre- and post-exposure periods. Relative decline of VSL was not significant due to period ($P \geq 0.15$).

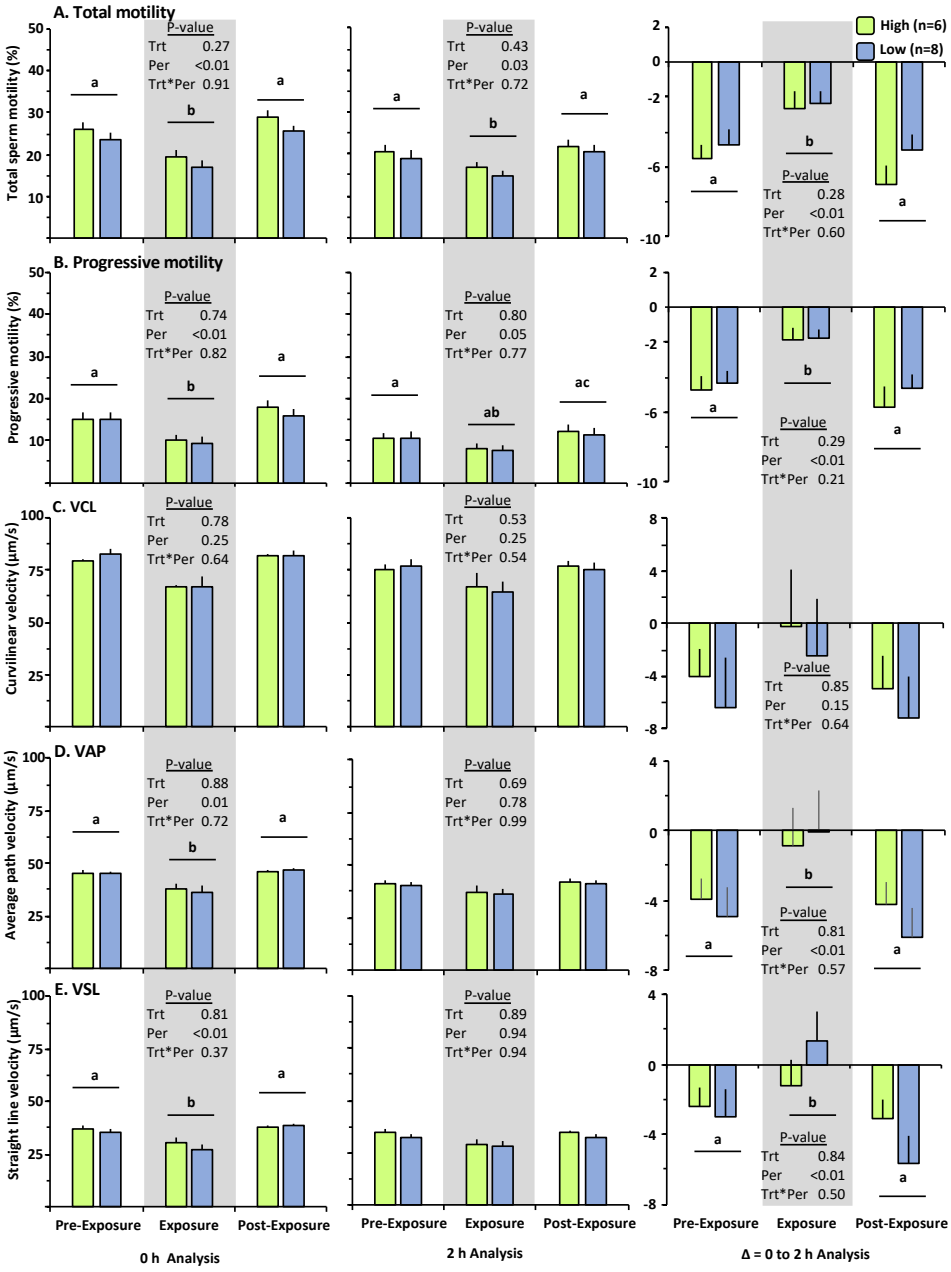


Figure 3.2 Effects of ergot alkaloid consumption between High (n=6) and Low (n=8) treatment groups of mature Angus bulls presented as mean (± SEM). Effect of treatment (ergot feeding), period and their interaction on sperm total motility (%; Panel A), progressive motility (%; Panel B), curvilinear velocity (VCL, µm/s; Panel C), average path velocity (VAP, µm/s; Panel D) and straight-line velocity (VSL, µm/s; Panel E) during pre-exposure, exposure and post-exposure periods. Ergot exposure period is shaded. Sperm were analyzed at 0 and 2 h and their difference (Δ) was calculated. [a-c]Within an endpoint, means without a common superscript differed (P≤0.05).

3.4.1.2 Sperm structural characteristics

Flow cytometric analysis revealed no effect of treatment (P=0.15), period (P=0.32) nor their interaction (P=0.34) on the proportion of live sperm with intact acrosomes (PI negative and FITC-PNA negative) at 0 h (Fig. 3.3A). However, there was an effect of ergot treatment on live sperm with intact acrosomes at 2 h (P=0.01, Fig. 3.3A, middle column). There was no effect of treatment (P=0.87), period (P=0.15), or their interaction (P=0.50) on the decline in live sperm with intact acrosomes between 0 and 2 h (Fig. 3A).

The proportion of live sperm (PI negative) with high mitochondrial membrane potential at 0 h was negligible and did not change (Fig. 3.3B, left column) due to ergot treatment (P≥0.24), period (P≥0.14) or their interaction (P≥0.36). The proportion of live sperm with medium mitochondrial membrane potential at 0 h differed due to period (P≤0.01, (Fig. 3.3C); this population was the highest during exposure versus pre- and post-exposure. Furthermore, it increased from 10.65 ± 0.85% during pre-exposure to 23.56 ± 1.24% (P<0.01) during exposure, followed by a subsequent decrease during post-exposure (7.94 ± 1.03%), equivalent to pre-exposure (P>0.05). There was no recorded difference in the proportional distribution of live sperm with medium or high mitochondrial membrane potential at 2 h (Fig. 3.3B and C, middle column) due to treatment groups (P≥0.66) or periods (P≥0.14). The relative decline in live sperm with medium mitochondrial membrane potential between 0 h and 2 h post-thaw (Fig. 3.3C, right column) was greater (P≤0.01) during exposure compared to pre- and post-exposure.

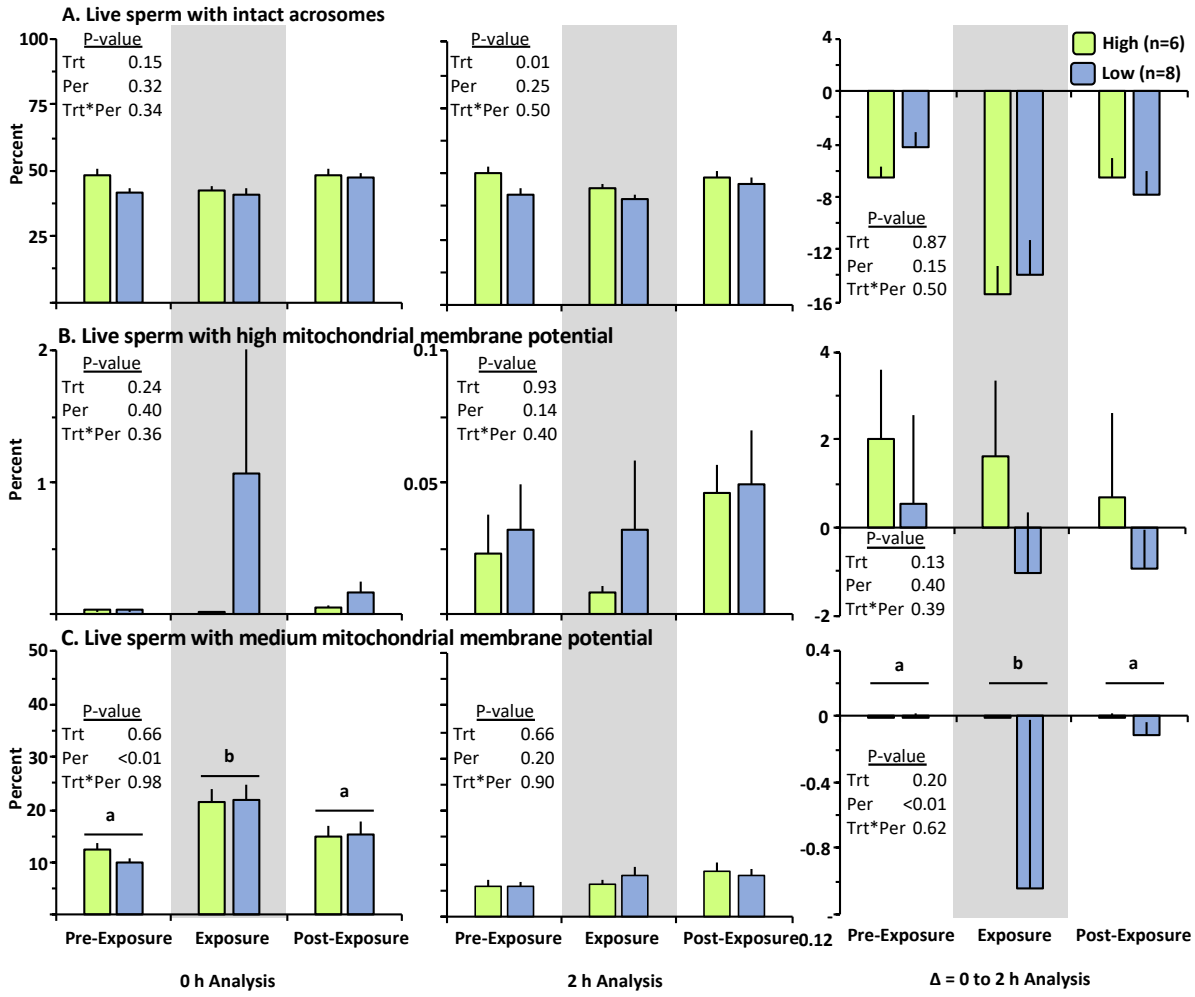


Figure 3.3 Effects of ergot alkaloid consumption between High (*n*=6) and Low (*n*=8) treatment groups of mature Angus bulls presented as mean (± SEM). Effect of ergot feeding on live sperm with intact acrosomes (%; Panel A), live sperm with high mitochondrial membrane potential (%; Panel B) and live sperm with medium mitochondrial membrane potential (%; Panel C). Ergot exposure period is shaded. Sperm were analyzed at 0 and 2 h and their difference (Δ) was calculated. [a,b]Within an endpoint, means without a common superscript differed ($P \leq 0.05$).

3.4.2 Experiment 2 - Effect of ergot feeding on frozen-thawed sperm from yearling bulls

Sperm motion (CASA) and structural characteristics (flow cytometry) are shown (Fig. 3.4 and 3.5, respectively).

3.4.2.1 Sperm motion characteristics

Total sperm motility at both 0 and 2 h post-thaw (Fig. 3.4A, left and middle columns), increased from pre-exposure to exposure to post-exposure period ($P \leq 0.01$). Sperm progressive motility at 0 h increased ($P \leq 0.01$) from pre-exposure to exposure to post-exposure (Fig. 3.4B, left column). Likewise, post-thaw progressive motility increased from exposure to post-exposure period ($P \leq 0.01$) but did not differ between pre- and exposure periods (Fig. 3.4B, middle column). There was no effect of ergot treatment ($P \geq 0.36$) or treatment*period interaction ($P \geq 0.56$) on total sperm motility or progressive motility at 0 or 2 h post-thaw. The relative decrease in sperm motility and progressive motility between 0 and 2 h analyses changed due to period ($P \leq 0.02$), with no effect of treatment ($P \geq 0.25$) nor their interaction ($P \geq 0.13$; Fig 3.4A and B, right column). Both sperm motilities increased from pre-exposure to exposure and post-exposure periods ($P < 0.05$).

There was no effect of treatment ($P \geq 0.23$) or treatment*period interaction ($P \geq 0.57$) on sperm velocities (VCL, VAP, VSL) at 0 h or 2 h post-thaw CASA analyses. Sperm VCL, VAP and VSL (averaged over control and ergot groups) at 0 h did not differ between pre-exposure and exposure but increased ($P < 0.01$) between exposure and post-exposure (Fig. 3.4C, D, and E, left column). Sperm VCL and VAP values did not differ ($P \geq 0.31$), but VSL increased due to period ($P \leq 0.01$) at 2 h (Fig. 3.4E, middle column). Relative change in VCL and VAP between 0 and 2 h did not differ due to treatment ($P \geq 0.15$) or period ($P \geq 0.17$) (Fig. 3.4C and D, right column). Sperm VSL (Fig. 3.4E, right column) had a greater relative decrease (Period $P = 0.01$) during post-exposure, whereas pre- and exposure periods did not differ.

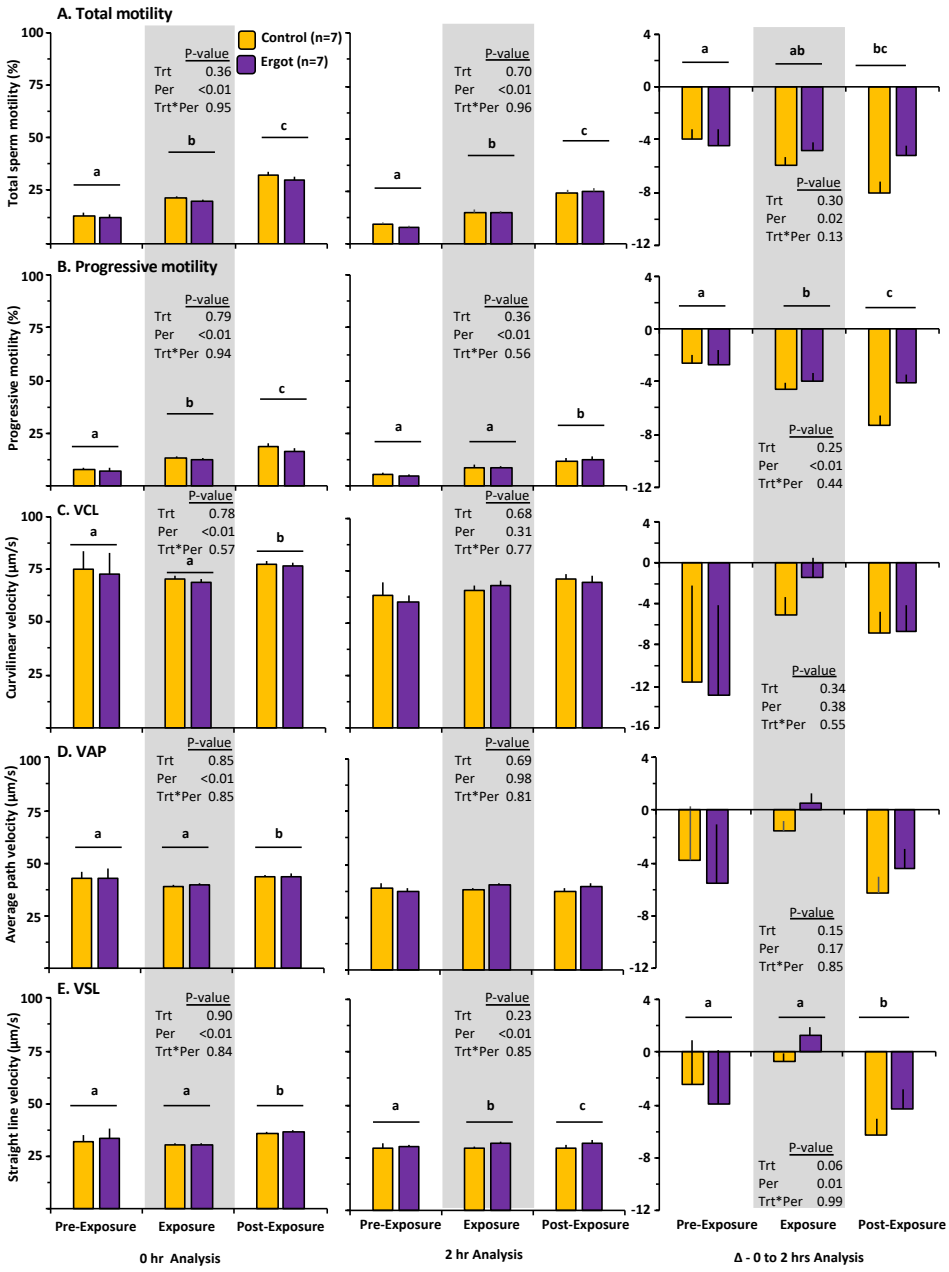


Figure 3.4 Effects of ergot alkaloid feeding between ergot treatment (n=7) and control (n=7) groups of yearling Angus bulls presented as mean (± SEM). Effects of treatment (ergot feeding), period and their interaction on sperm total motility (%; Panel A), progressive motility (%; Panel B), curvilinear velocity (VCL, µm/s; Panel C), average path velocity (VAP, µm/s; Panel D) and straight-line velocity (VSL, µm/s; Panel E) during pre-exposure, exposure and post-exposure periods. Ergot exposure period is shaded. Sperm were analyzed at 0 and 2 h and their difference (Δ) was calculated. [a-c]Within an endpoint, means without a common superscript differed ($P \leq 0.05$).

3.4.2.2 Sperm structural characteristics

Live sperm with an intact acrosome from exposure to post-exposure periods increased at both 0 and 2 h (Fig. 3.5A, left and middle columns); however, there was no effect of treatment or treatment*period interaction ($P \geq 0.35$). The relative decline in percent of live sperm with intact acrosomes between 0 and 2 h post-thaw (Fig. 3.5A, right column) tended to be greater ($P=0.06$) for the post-exposure period when compared to pre-exposure and exposure periods.

The proportion of live sperm with high mitochondrial membrane potential was not affected due to period, treatment or treatment*period interaction at 0 or 2 h (Fig. 3.5B, left and middle columns). The relative decrease in proportion of live sperm with high mitochondrial membrane potential between 0 and 2 h post-thaw (Fig. 3.5B, right column) was influenced by treatment*period interaction $P=0.02$) and was highest in ergot-fed yearling bulls during exposure. The proportion of live sperm with medium mitochondrial membrane potential at 0 and 2 h (Fig. 3.5C, left and middle columns) was highest during exposure ($P<0.01$) and it increased from pre-exposure to exposure, followed by a post-exposure decrease. Treatment groups did not differ ($P \geq 0.55$) at either 0 or 2 h post-thaw analysis. The relative decline in proportion of live sperm with medium mitochondrial potential between 0 and 2 h (Fig. 3.5C, right column) was the lowest for pre-exposure, ($P<0.01$) and it differed from exposure and post-exposure values.

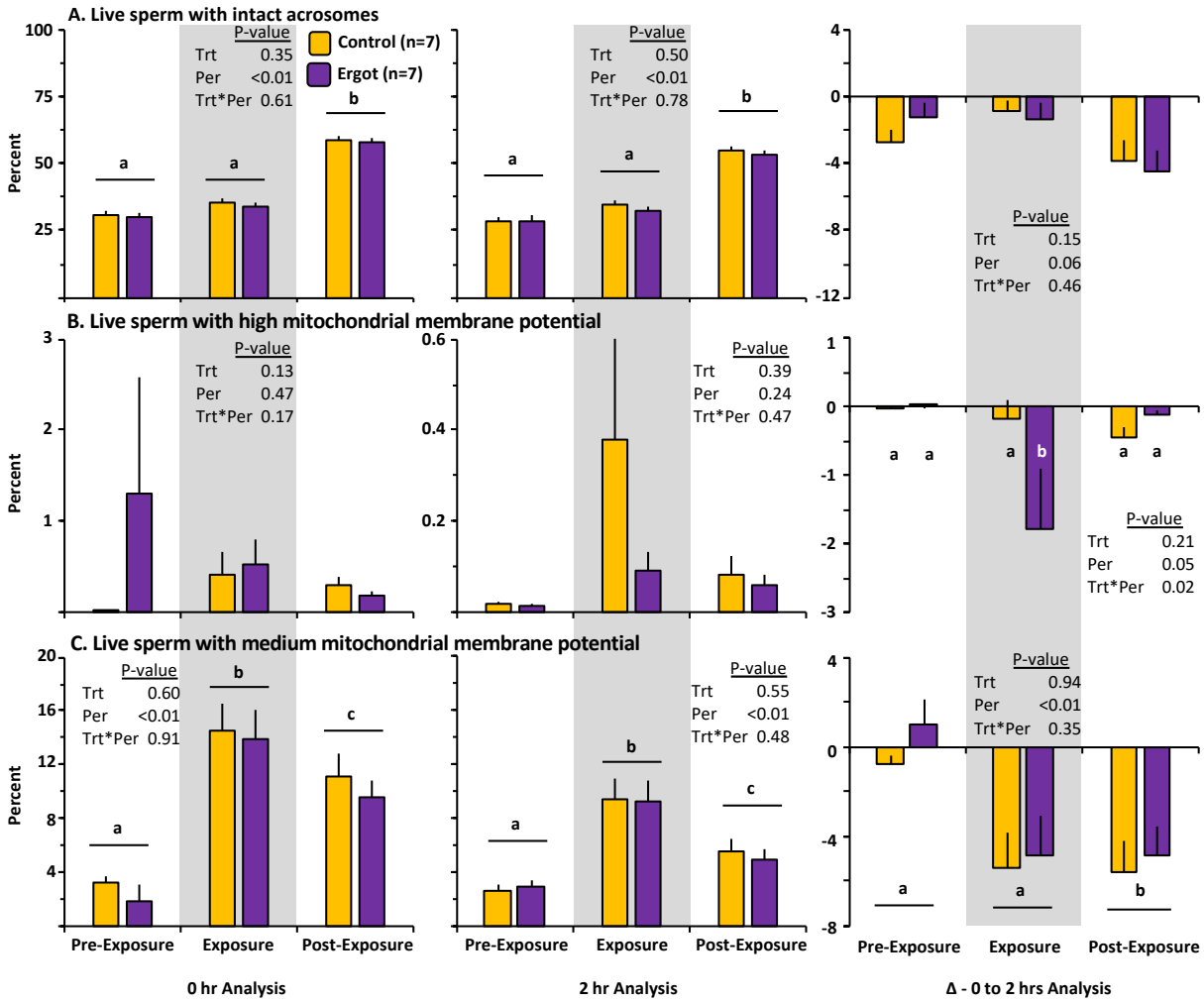


Figure 3.5 Effects of ergot alkaloid feeding between ergot treatment (n=7) and control (n=7) groups of yearling Angus bulls presented as mean (± SEM). Effect of ergot feeding on live sperm with intact acrosomes (%; Panel A), live sperm with high mitochondrial membrane potential (%; Panel B) and live sperm with medium mitochondrial membrane potential (%; Panel C). Ergot exposure period is shaded. Sperm were analyzed at 0 and 2 h and their difference (Δ) was calculated. $^{a\text{-}c}$Within an endpoint, means without a common superscript differed (P≤0.05).

3.5 Discussion

This study investigated the influence of low-dose ergot alkaloids from *C. purpurea* on post-thaw sperm motion and structural characteristics in mature and yearling bulls, exposed over 10 and 9 wk, respectively, within Canadian (CFIA) limits. In this study, ergot *per se* had no major adverse effect on post-thaw semen quality of beef bulls. However, post-thaw quality varied among pre-exposure, exposure and post-exposure periods. Mature and yearling bulls yielded similar results. In mature bulls, total sperm and progressive motility decreased during exposure, but regained pre-exposure values during post-exposure. There was a gradual increase in total sperm and progressive motilities and velocities. Additionally, live sperm with intact acrosomes markedly increased in control bulls 2 h post-thaw during experimental periods. Live sperm with medium mitochondrial membrane potential increased significantly from pre-treatment to treatment, followed by a subsequent decline to pre-exposure values. In yearling bulls, total sperm and progressive motilities increased throughout the entire study period. Similarly, velocities also increased in the post-exposure period. Additionally, live sperm with intact acrosomes markedly increased during post-exposure. Medium mitochondrial potential also increased from pre-exposure to exposure. Our model was robust enough to detect differences during sexual maturation in yearling bulls; increases in post-thaw sperm quality were expected in yearling bulls. Overall, results supported our hypotheses that ergot has no detectable adverse effect on post-thaw semen parameters in mature and yearling bulls during or after low dose-ergot exposure. Furthermore, to our knowledge, this was the first study on effects of ergot alkaloids that included a very comprehensive assessment of structural post-thaw sperm characteristics.

The method of exposure to ergot alkaloids differed between mature and yearling bulls, due to an earlier pilot study conducted in our lab (unpublished). Mature bulls expressed feed refusal when we attempted individual ergot feeding; however, they readily consumed an ergot-free ration, implying ergot palatability, rather than lack of hunger. This observation, along with the aggressiveness of the mature bulls, required us to attempt group feeding for ergot exposure, whereas yearling bulls were more cooperative and fed ergot in gelatin capsules.

Sperm motion characteristics of mature bulls were decreased during ergot exposure versus non-ergot experimental periods. Similarly, in previous semen cryopreservation studies, incubating motile sperm with ergot alkaloids reduced sperm motility and intact acrosomes

compared to non-exposed sperm (Gallagher and Senger, 1989; Wang et al., 2009), with a >3 fold decrease in post-thaw motility in ergot-grazed bulls versus control bulls (Pratt et al., 2015b). In yet another study, sperm from bulls grazing ergot alkaloids had decreased motility and progressive motility when compared to bulls grazing non-ergot alkaloid-producing fescue pasture (Burnett et al., 2017). In the present study, decreased sperm motilities and velocities during exposure, followed by recovery during post-exposure, implied that whereas ergot alkaloids may have a negative effect on post-thaw sperm, the effect was transient. The decrease from pre-exposure to exposure in mature bulls may also have been compounded by the study being conducted in cooler weather, along with possible handling stress. In the current study, post-thaw total sperm and progressive motilities in yearling bulls continued to improve throughout the study. In a fresh semen experiment in our lab (Chohan et al., 2021), there was a similar trend, indicating the effect was likely due to sexual maturity rather than ergot toxicity.

To our best knowledge, this was the first report using flow cytometry to detect changes in post-thaw sperm structural characteristics resulting from bulls consuming ergot alkaloids in Canadian cereal grains. During pre-exposure, there was an effect of treatment on live sperm with intact acrosomes in mature bulls at 2 h post-thaw. The number of post-thaw live sperm with a high mitochondrial membrane potential was exceptionally low (<5%). However, for live sperm with medium mitochondrial membrane potential, there was a subtle shift to increased proportions during exposure, followed by a decrease post-exposure, indicating a decrease in low mitochondrial membrane potential to medium mitochondrial membrane potential. Perhaps ergot affected mitochondrial cryotolerance. Further, there was a sharp decline in the proportion of live sperm with medium mitochondrial membrane potential between 0 and 2 h during exposure, possibly negating observed effects and sperm at 2 h did not have differences between exposure periods. However, the effect at 0 h was likely transient and may not be functionally important. Ergot alkaloids are vasoconstrictive in the testicular artery of yearling bulls (Aiken et al., 2015) which may have compromised testicular thermoregulation, leading to low mitochondrial membrane potentials in mature bulls.

In mature bulls, post-thaw sperm motilities were lower during ergot exposure, but overall had a consistent level, building on results from prior studies with fescue (Burnett et al., 2017; Pratt et al., 2015b). Prolonged high-dose ergot alkaloids did not cause any detected effects in yearling bulls. Although our experimental model detected potential gradual sperm maturation in

yearling bulls, ongoing changes due to sexual maturation may have masked potential changes due to ergot alkaloids. Sperm velocities (VCL, VAP, VSL) for yearling bulls had marked increases between exposure and post-exposure periods that likely resulted from sexual maturation. Differences in VCL and VAP disappeared at 2 h post-thaw analysis, perhaps due to low cryotolerance as yearling bulls matured.

Live sperm with intact acrosomes in yearling bulls had a similar increase from exposure to post-exposure periods, consistent with ongoing sexual maturation. Live sperm with high and medium mitochondrial membrane potential in yearling bulls was also low, similar to mature bulls. Live sperm with medium mitochondrial membrane potentials expressed a similar change as in mature bulls. A sharp increase during exposure, as in mature bulls, followed by a decrease back to pre-exposure values, indicated a possible effect of ergot alkaloids on mitochondria, subtly lowering mitochondrial membrane potential proportions towards medium and low membrane potentials. This unexpected result should be confirmed in future studies.

A distinctive characteristic of this study was individually feeding yearling bulls ergot (via capsules). A consistent amount of ergot alkaloids (60 µg ergot alkaloids/kg body weight/d; 3.4 mg/kg DMI) was ingested in a capsule(s) form at regular intervals. In previous ergot toxicity studies, the total amounts of ergot alkaloids being consumed by each individual bull or cow were not specifically known and day-to-day variations in consumption may have occurred due to variations in feeding or grazing conditions, although concentrations of ergot alkaloids were measured in fresh or frozen-thawed sperm (Aiken et al., 2013; Burnett et al., 2017; Cowan et al., 2019, 2018; Grusie et al., 2018b; Schuenemann et al., 2005b). The current study assessed, with considerable certainty, effects of ergot alkaloids in yearling bulls. Furthermore, this was apparently the first study in yearling cattle to relate ergot alkaloid concentrations with body weight. Although mature bulls were not able to be fed with such precision on a daily basis, results from group feeding extended prior studies of ergot alkaloids ingested by grazing tall fescue.

According to the Society of Theriogenology, bull semen should have >30% sperm progressive motility to achieve acceptable fertility (Barth, 2007). Adverse effects of freeze-thawing sperm are well established and approximately ~50% sperm lose their viability during freezing and thawing (Watson, 1995). During freezing, sperm undergo chemical, osmotic, thermal and mechanical shocks, with the freeze-thawing stage causing greater damage to sperm

than dilution or cooling (Hussain et al., 2011; Rasul et al., 2001). Overall post-thaw motility was low relative to earlier reports from our laboratory (Anzar et al., 2019, 2011). The same protocol was used in this study, suggesting low post-thaw semen quality could have been due to breed, bull, season, and operator differences. Ergot alkaloids may also have affected cryotolerance of sperm membranes, leading to diminished sperm characteristics in frozen-thawed sperm in this study. Although every mature bull was deemed breeding sound, expected post-thaw motilities were only slightly lower than expected. Yearling bulls did not undergo a bull breeding soundness evaluation prior to the study; regardless, we inferred that sexual immaturity and not effects of ergot alkaloids were most likely responsible for the lowered post-thaw sperm characteristics.

Overall, there were no major effects of ergot alkaloids on post-thaw structural characteristics of sperm from mature or yearling bulls, although the former may have had a transient effect of treatment on sperm motility, with return to pre-treatment values soon after termination of ergot treatment. In mature bulls, group feeding precluded assessing whether each bull received the same amount of ergot alkaloids on a daily basis, making it difficult to compare results to yearling bulls. Regardless, the overall lack of major adverse effects of ergot alkaloids in mature or yearling bulls post-thaw sperm parameters was likely multifactorial and a strong indicator that the Canadian government limits of ergot alkaloids in feed are within an acceptable range for beef bulls.

3.6 Conclusions

Despite some fluctuations in frozen-thawed semen of mature and yearling bulls, there were no major effects of ergot treatment. Although effects of ergot alkaloids cannot be definitively excluded, we attributed these fluctuations to mature bulls being group fed and therefore not consistently receiving consistent confirmable daily doses of ergot alkaloids. In the yearling bulls, despite higher and consistent ergot exposure, improvements in sperm quality throughout the study were attributed to sexual maturation, precluding detection of any effects of ergot alkaloids. Based on the results of our study, feed containing up to ~2 mg/kg total ergot alkaloids may affect total sperm and progressive sperm motilities and mitochondrial membrane potential in mature bulls. In yearling bulls, feeding ~3.4 mg/kg may negatively affect mitochondrial membrane potentials. However, there was largely no functional effect of ergot

alkaloids on sperm motion and structural characteristics. Based on our results, the changes in all assessed endpoints were mild with any clinical applications for bull testing of ergot exposure limited. Therefore, semen evaluation cannot be used to detect moderate to low concentrations ergot alkaloid exposure. Overall, results partially supported our hypotheses that ergot has no detectable adverse effect on post-thaw sperm characteristics in mature and yearling bulls.

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Conflicts of Interest

The authors declare no conflict of interest.

CHAPTER 4
GENERAL DISCUSSION AND FUTURE DIRECTIONS

4.1 General discussion

The overall objective of this thesis was to determine the effects of dietary consumption of ergot, at or under current Canadian CFIA standards, on reproductive end points in yearling and adult Angus bulls.

Tall fescue grass is widely used in livestock production systems in the United States. It has a wide range of adaptation and is tolerant of poor management. Unfortunately, consumption of toxic fescue infested with alkaloids causes fescue toxicosis in cattle. However, there has been some research on the effects of this toxic fescue on bull reproduction. Further, the variability between studies and varying reports makes it difficult to pinpoint the reproductive end points affected. Although there are some studies on fescue toxicosis and bull reproduction from the United States, Canada has different crops and climate. We do, however, have ergot alkaloid infestations in cereals and grains from *Claviceps purpurea*. This differs from the ergot alkaloids from tall fescue and as such, there are no studies on effects of ergot alkaloid on bull reproductive parameters. Regulatory agencies and producers require this information for policy development and herd management. The research conducted and described in this thesis was intended to add to the growing need for scientific evidence with Canadian relevance on ergot alkaloid exposure in bulls.

In Study 1 (Chapter 2), we investigated effects of feeding yearling bulls the higher permissible limit of ergot alkaloids for 9 wk on sperm quality, motility, sperm structural characteristics, and prolactin concentrations of. As expected, plasma prolactin concentrations decreased during treatment and subsequently rebounded. Alarmingly however, there was a 4-fold decrease in plasma prolactin during the ergot feeding period. Ergot alkaloids are similar in structure to dopamine, which acts on the D_2 receptors on anterior pituitary and suppresses prolactin secretion; the ergot alkaloids act in a similar manner, thereby reducing prolactin secretion (Bouilly et al., 2012; Goffin et al., 2002; Klotz, 2015). As prolactin is an important clinical marker for ergot toxicity, this may be a factor that needs to be reflected in government guidelines. While prolactin is a good clinical marker for initial diagnosis of ergot toxicity, there may be challenges under field setting to obtain blood samples from bulls and may also increase cost for a farmer or producer. Furthermore, although sperm concentration, total ejaculate sperm

count, sperm morphology, sperm motilities, sperm velocities, and sperm structural parameters were not significantly affected by ergot treatment, there were age-related improvements. A possible stress factor in yearling bulls may be the bolus gun feeding and collection of semen on the same day. We attempted to decrease this stress factor by coating the balling gun in molasses to increase palatability. The bulls accepted the balling gun within a week once molasses was introduced. A second stress or confounding factor may be electroejaculation procedure itself. Semen collection with an artificial vagina may have provided a more realistic ejaculate volume and sperm concentration estimates. However, we alleviated this confounding factor by using electroejaculation on all bulls and electroejaculation equipment was operated by a single operator throughout the study.

The objective of the second study (Chapter 3) was to assess the influence of low-dose, permissible-level consumption of ergot alkaloids from *C. purpurea* for an extended interval, on post-thaw sperm characteristics in yearling and adult bulls. In Experiment 1, we group-fed adult Angus bulls 1 or 2 mg ergot alkaloids per kg of daily DMI for 10 weeks. In Experiment 2, we compared control group yearling Angus bulls to those individually fed ~3.4 mg ergot alkaloids per kg of daily DMI (highest levels of ergot allowed) for 9-wk during reproductive maturation – this amount was equivalent to feeding 60µg ergot alkaloids daily for every kg of body weight. In adult bulls, 2 mg/kg concentration of ergot alkaloids was based on a small pilot study (unpublished) where the adult bulls refused ergotized pellets at 3 mg/kg. Adult bulls were difficult to handle, so for safety reasons, we used group feeding method for the adult bulls whereas in yearling bulls we used capsules to introduce ergot therefore feed refusal was not a factor. There were multiple points of stress ranging from ergot palatability in adult bulls, to movement from pen to barn chute system for feeding and sperm collection. While we did not measure hormonal markers for stress, we tried to mitigate these stress factors by building a routine for the animals with consistent feeding, movement, and sperm collection times. The yearling bulls, fed with an exact mount via bolus, provided an exact amount of ergot to each individual bull, however the amount of ergot alkaloids per bull in adults cannot be ascertained due to the group feeding nature of the experiment. One potential alternative fo adult bulls could have been to feed animals in individual pens, however, the daily movement would have increased stress on the animals and increased safety concerns for researchers. A hydraulic chute system may have further alleviated the stress levels in bulls, especially adults, helping to restrain

the bulls completely. For both experiments, we tested the null hypothesis that ergot will not significantly affect sperm motion or structural characteristics of cryopreserved semen during and after the feeding period and found only partial support for our hypothesis. Ergot alkaloids did not appear to have major long-term impacts on sperm, only transient effects while being treated with ergot, whereas yearling bulls continued to have age-related improvements, as observed with the fresh sperm study. Given the decrease in sperm motility, farmer or producers may be hesitant to cryopreserve semen from bulls fed ergot, however, we would speculate that if the option of a spermatogenic cycle off ergot is not possible, cryopreservation is still a viable option. The use of such cryopreserved sperm could be mitigated by inseminating using a larger quantity of frozen sperm than industry standard.

Prolactin data was in agreement with previous reports on tall fescue (Looper et al., 2009; Pratt et al., 2015b; Schuenemann et al., 2005b; Stowe et al., 2013) and supports use of prolactin as an indicator of ergot exposure in yearling bulls. This effect was detectable around ~ 3 ppm of ergot alkaloids in DMI after ~ 2 weeks after exposure to ergot. The bulls on ergot alkaloids did not show a complete ablation of prolactin making it difficult to speculate whether the reduced prolactin concentration would negatively impact male fertility. Nevertheless, it is not problematic to suggest that some detrimental effects on male reproduction may occur from the augmentation of effects from ergot alkaloids. Recently the presence of prolactin receptor has been established in bull testes (Pratt et al., 2015a); this in combination with decreased prolactin due to ergot may cause decreased male fertility. Prolactin concentration is consistently negatively affected due to ergot toxicity and may be a good marker for ergot toxicity in bulls. Whereas prolactin can be used as a marker for ergot toxicity based on current and previous results, the implications of lowered prolactin on male fertility require further study. Most likely however, we can say with certainty any observed differences in the biological parameters evaluated were most likely due to ergot toxicosis or perhaps toxicosis by environment interaction such as ambient temperatures and stress.

In yearling bulls, there was only transient but apparently not permanent negative effects on sperm. Sperm motility seems to be a consistent factor, in other studies and ours, that had changes with ergot alkaloid exposure. However, motility differs from animal to animal and collection to collection. The inconsistent reporting and no effect of treatment observed in our study would indicate that it is not a reliable factor as a clinical marker for ergot toxicity in

individual animals. Progressive motility in the Ergot group remained almost unchanged whereas it increased in the control group; the effect of ergot on progressive motility is likely due to the continued sexual maturation of the bulls in the control group. Sperm concentration and total ejaculate sperm in yearlings were not affected by ergot alkaloids, although they continued to increase due to sexual maturation throughout the study periods. The continued sexual maturation in ergot-treated bulls indicated that this level of ergot in feed did not affect growth. Furthermore, based on our experiments, none of the sperm end points assessed were reliable markers for a clinical diagnosis of ergot toxicity at the current allowable ergot limits.

The overall results of our studies suggest that male reproduction is largely not affected by feeding ergot for 9 weeks at the upper limit of permissible level in Canada. Previous studies with fescue toxicosis report contradictory results, some report no effects of ergot (Schuenemann et al., 2005a, 2005b) while others report reduced sperm concentration, reduced motility, and a higher proportion of abnormal sperm (Looper et al., 2009; Pratt et al., 2015b). The results of our studies detected a negative effect on certain sperm parameters however, clinically there is likely no adverse effect. An interesting symptom of fescue toxicosis is "summer slump" (Schmidt and Osborn, 1993), the cattle appear hot with difficult respiration and excessive salivation. The cattle avoid grazing during the day and seek shade to find respite from heat. This is a phenomenon not seen in Canada due to the relatively cooler summer temperatures. Although our bulls did not undergo post-study breeding soundness evaluations, the concern of reported studies is bulls passing the BSE regardless of having ergot feed or clean feed. This would have implications in clinical settings where any effects of ergot toxicity may not be detected using a standard BSE. The most important characteristic of our study was the distinctive feeding process for yearling bulls. Whereas previous studies used various pen or grazing conditions, we had complete control of the precise amount of ergot alkaloids ingested by the bulls which allowed us to more confidently make conclusions.

Sperm motility appears to the parameter most commonly affected from ergot alkaloid exposure albeit without consistency. The exact mechanism by which ergot alkaloids may disrupt sperm motility is unknown and needs further study. Overall, the clinical sperm parameters are not good markers for ergot toxicity due to the variable results reported. We can speculate that while clinical sperm parameters are not useful for diagnosis of ergot toxicity, there may be changes at the cellular level or potential for breach of the blood-testes barrier causing disruption

to male reproduction. It has been documented that ergocristinine accumulation leads to weakened blood-brain barrier (Mulac et al., 2012). A similar effect is also feasible on the blood-testes barrier and requires further study.

Our studies showed that there are few, if any, long-term detrimental effects to the sperm parameters. While ergot alkaloid consumption did decrease select parameters during the exposure periods, there was a subsequent rebound to pre-ergot levels, both for fresh and post-thaw sperm. This along with the rebound of prolactin concentration implied that a bull's reproductive system can recover from low-level ergot exposure for extended period.

An important factor to consider is food toxicity. However, it appears that cattle can recover from ergot toxicity therefore, while important to have checks on ergot levels in feed, ergot may not pose a long-term food safety concern for humans. The half-life values of ergot alkaloids have been reported to be between minutes to a few hours (Bony et al., 2001; Ibraheem et al., 1983). Therefore, there appears to be minimal to no risk for the public consuming animals or animal products, especially once the animals have been taken off of ergot contaminated feed.

Previous studies have intimated that ergot alkaloids decrease nutrient intake and increase feed refusal in cattle which corresponds to decreased weight gain (Burke et al., 2005; Matthews et al., 2005; Pratt et al., 2015b). The bulls in our studies steadily gained weight throughout the study, especially the yearling bulls which had a reference group. We can confidently say that ergot exposure at 3mg/kg of DMI likely does not affect the overall growth of bulls.

Post-thaw sperm with decreased motility was similar to studies using tall fescue both *in vitro* and *in vivo* (Burnett et al., 2017; Gallagher and Senger, 1989; Pratt et al., 2015b). Our results along with those reported previously suggest that ergot alkaloids may present a challenge for sperm cryopreservation. Although, our results in yearling bulls did not have a significant decrease in post-thaw motility. This may indicate that maturing bulls are not affected by ergot alkaloids for sperm cryopreservation, or the maturing sperm are able to counter the effects of ergot alkaloids. However, the maturing bulls post-thaw sperm motilities were lower during ergot consumption period but rebounded during the post-exposure period. Again, this implies a transient effect of ergot alkaloids on sperm. These effects may be countered in a clinical setting by delaying sperm collection for cryopreservation from mature bulls exposed to ergot alkaloids for at least one spermatogenic cycle. We did not test the bulls by a field fertility trial after

exposure to ergot, which may have given a clearer understanding whether any negative effects were transient or not.

Many ergot alkaloid are lipid soluble and may translocate across sperm membranes to directly interact with intracellular signaling molecules or via membrane alpha adrenergic receptors (Wang et al., 2009). This may partly explain mitochondrial membrane potential changes in our studies. Mitochondrial membrane potential changes could co-relate to decreased motility during ergot exposure given the energy function of mitochondria and as such offer a secondary pathway for ergot alkaloids to disrupt male fertility.

Overall, the changes in our studies to sperm parameters may not indicate a negative effect on cattle reproduction; however, in conjunction with the known severe adverse effects of ergot alkaloids on the female reproductive system the fertility may be decreased as reviewed by Poole and Poole (2019). Ultimately, fertilization studies need to be conducted to determine any effects on embryo development.

These studies would be of importance to regional producers in the Canadian prairie provinces as well as the CFIA. The current quantity of allowed ergot alkaloids in livestock feed established by the CFIA can be confidently kept in place with male reproduction as the basis. Previous work on male reproduction along with our work on bulls does not lead to a conclusion to decrease the maximum allowable amount of ergot alkaloids in feed, however further studies which include field fertility are necessary before making any firm conclusions. Regional producers of the prairie provinces should continue to monitor and take precautions to prevent the prevalence of ergot infestations in the grazing fields and feed. Care should be taken with moisture level in the storage of feed for livestock. While there is speculation that ergot may degrade over time, in cooler temperatures ergot can still germinate (Mitchell and Cooke, 1968). Producers should consider annual climate conditions and whether a higher risk is present in certain years. While pelleting is a low-cost method for livestock feed, producers should consider that the pelleting process involving flooring, rolling, and grinding along with high temperatures may increase the likelihood of ergot alkaloid toxicity (Coufal-Majewski et al., 2016) in the ergot-endemic regions. Factors to consider for producers are crop management, using crops from the autumn seasons for springtime feeding, and potential genetic engineering for ergot resistant crops. To the best of our knowledge, currently there are no genetically engineered ergot resistant wheat, barley, or other crops in the commercial market.

The Canadian Food Inspection Agency is considering lowering the ergot alkaloid concentration standards from the current 2-3 mg/kg. Although this may be appropriate for cows, given the evidence of negative effects on the female reproductive system (Poole and Poole, 2019), the current standards seem to be appropriate for Angus bulls, based on the current results. There is a large difference between the ergot alkaloid concentration standards between Canada and the United States without much publicly available information about the reason. There should be better cooperation and public availability of the data for the respective countries standards. Prior studies from our group analyzed vascular effects on arteries at < 2 mg/kg found a decreased diameter of the caudal and medial sacral arteries (Cowan et al., 2018, 2019). We did not study the arterial responses to ergot alkaloids in bulls and this should be a consideration in future bull studies.

4.2 Future directions

There are many possibilities to further examine effects of ergot toxicity in cattle. Primarily, we do not yet have a clear picture of the pathways in which ergot alkaloids might disrupt sperm quality. Our study was conducted in the spring; however, a winter study, including effects on peripheral vasculature should provide important information for government agencies and producers. To the author's knowledge, this is not widely available in literature. An increase in the dosage of ergot in feed may be needed to determine the lowest concentration needed to produce a permanent negative response in sperm quality. Similarly, studies involving a larger herd of bulls with more prolonged ergot exposure, multiple ergot alkaloid concentration groups, and a minimum of 2-3 spermatogenic cycles would provide valuable insights. Based on the research presented in this thesis and previous studies, sperm motilities and velocities may be potential avenues for detecting ergot toxicity in bulls and potential permanent effects on sperm. To better understand the effect of time and ergot concentration/half-life effects, a second blood collection in the evening along with mornings would be effective. In order to better characterize prolactin concentrations and determine any effects of stress, daily collections throughout a study should be considered. Perhaps testing for stress hormone levels, like cortisol, in addition to prolactin. Another possible avenue for an ergot toxicity study is concurrent studies with yearling and mature bulls with the same feeding patterns. Bulls in the studies presented in this thesis were

fed via different methods and patterns. Group feeding of mature bulls made it difficult to ascertain whether each bull had the same level of ergot alkaloid intake. The studies in this thesis should have major implications in the beef industry as it relates to ergot alkaloid-induced bull reproduction, although more research must be performed to establish which mechanisms of sperm are altered by ergot alkaloids. Sperm parameters are evaluated *in vitro*, however, farmers and livestock producers require field and *in vivo* results. Field fertility trial experiments following ergot exposure should be a focus of future experiments. In the same manner, artificial insemination trials using the cryopreserved sperm from ergot fed bulls would provide insight into whether the decreased sperm motility requires addition of extra sperm in the frozen straws for optimal fertility. In animals with external testes, the scrotum is maintained 2-6 ºC below core body temperature thus allowing normal thermoregulation of the testes (Kastelic et al., 1997). We did not observe an effect of treatment on rectal temperatures in our bulls. The cooler springtime temperatures likely contributed to normal bull temperature regulation and played a positive effect in masking effects of ergot. In future studies scrotal temperatures could be recorded throughout the study. A relatively recent aspect of ergot alkaloid study has focused on epimers of ergot alkaloids (Cherewyk et al., 2020; Krska and Crews, 2008; Pierri et al., 1982). Few studies are available in the literature on research differentiating the effects of (*S*)- or (*R*)-epimers. Historically, (*R*)-epimers were considered the active epimer and research focused primarily focused on (*R*)-epimers with little notice to (*S*)-epimers. Our studies estimate of the ergot alkaloid concentrations were based on the analysis for (R)-epimers only; this provides a pathway for future studies to include (*S*)-epimers to determine which or both epimers are the cause of changes to sperm parameters.

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APPENDICES

Appendix A. *Syntax for Statistical Model for Chapter 2*

For comparisons by experimental period and treatment*:*

```
ods graphics on;
Proc mixed covtest cl plots=all;
class ID Trt period OWeek;
model !! = Trt | period / DDFM=kr htype=3;
repeated OWeek /subject=ID type=##
lsmeans Trt | period /pdiff=all adjust=tukey;
run;
ods graphics off;
```

For comparisons by week age and treatment:

```
ods graphics on;
Proc mixed covtest cl plots=all;
class ID Trt period OWeek week_age;
model !! = Trt | week_age / DDFM=kr htype=3;
 repeated OWeek /subject=ID type=##;
lsmeans Trt | week_age /pdiff=all adjust=tukey;
run;
ods graphics off;
```

*: "!!" in the syntax was replaced with the variable being analyzed

**: "##" in the syntax was replaced with the name of the covariance structure for model comparisons.

Appendix B. ***Ergot Feeding Video Demonstration***

Link: https://ars.els-cdn.com/content/image/1-s2.0-S0093691X20306300-mmc1.mp4

Appendix C. ***Syntax for Statistical Model for Chapter 3***

- For comparisons by period and treatment for yearling bulls:

```
ods graphics on;
Proc mixed covtest cl plots=all;
class ID Trt period OWeek;
model !! = Trt | period / DDFM=kr htype=3;
repeated OWeek /subject=ID type=##;
lsmeans Trt | period /pdiff=all adjust=tukey;
run;
ods graphics off;
```

**: "!!" in the syntax was replaced with the variable being analyzed*

***: "##" in the syntax was replaced with the name of the covariance structure for model comparisons.*

- For comparisons by period and treatment for mature bulls:

```
ods graphics on;
Proc mixed covtest cl plots=all;
class ID Trt period OWeek;
model !! = Trt | period / DDFM=kr htype=3;
repeated OWeek /subject=ID type=##;
lsmeans Trt | period /pdiff=all adjust=tukey;
run;
ods graphics off;
```

**: "!!" in the syntax was replaced with the variable being analyzed*

***: "##" in the syntax was replaced with the name of the covariance structure for model comparisons*

Appendix D. ***Yearling Bulls Date of Birth***

Bull ID	Date of Birth
20 E	1/17/17
29 E	1/21/17
43 E	1/26/17
67 E	1/30/17
94 E	2/4/17
117 E	2/16/17
169 E	3/1/17
170 E	3/1/17
761 E	3/6/17
774 E	1/19/17
779 E	2/16/17
782 E	2/15/17
786 E	2/13/17
794 E	2/7/17

Appendix E. ***Yearling Bulls Ergot in Capsule Calculations***

- **Week 1-3**

Bull ID	Bull body weight (lb)	Bull Body weight (kg)	Intended Alkaloid Conc. (microgram/kg) of BW	Total Alkaloid Needed (mg)	Total Screening (467mg alakloid/kg of screening) Needed (g)	Conc. (mg/kg) based on Dry Matter Intake (DM) = 2.4% of BWt	Capsules Needed	Group
20E	1215	551.11	60	33.07	70.81	3.4	3.54	Control
29E	1220	553.38	60	33.20	71.10	3.4	3.55	Control
43 E	1180	535.24	60	32.11	68.77	3.4	3.44	Treatment
67 E	1245	564.72	60	33.88	72.56	3.4	3.63	Treatment
94 E	1040	471.74	60	28.30	60.61	3.4	3.03	Control
117 E	1100	498.95	60	29.94	64.11	3.4	3.21	Treatment
169 E	1030	467.20	60	28.03	60.03	3.4	3.00	Treatment
170 E	1095	496.68	60	29.80	63.81	3.4	3.19	Control
761 E	1130	512.56	60	30.75	65.85	3.4	3.29	Treatment
774 E	980	444.52	60	26.67	57.11	3.4	2.86	Control
779 E	1080	489.88	60	29.39	62.94	3.4	3.15	Control
782 E	975	442.25	60	26.54	56.82	3.4	2.84	Treatment
786 E	1215	551.11	60	33.07	70.81	3.4	3.54	Treatment
794 E	1220	553.38	60	33.20	71.10	3.4	3.55	Control

- **Week 4-6**

Bull ID	Bull body weight (lb)	Bull Body weight (kg)	Intended Alkaloid Conc. (microgram/kg) of BW	Total Alkaloid Needed (mg)	Total Screening (467mg alakloid/kg of screening) Needed (g)	Conc. (mg/kg) based on Dry Matter Intake (DM) = 2.4% of BWt	Capsules Needed	Group
20E	1310	594.21	60	35.65	76.34	3.4	3.82	Control
29E	1305	591.94	60	35.52	76.05	3.4	3.80	Control
43 E	1200	544.31	60	32.66	69.93	3.4	3.50	Treatment
67 E	1275	578.33	60	34.70	74.30	3.4	3.72	Treatment
94 E	1105	501.22	60	30.07	64.40	3.4	3.22	Control
117 E	1155	523.90	60	31.43	67.31	3.4	3.37	Treatment
169 E	1065	483.08	60	28.98	62.07	3.4	3.10	Treatment
170 E	1185	537.51	60	32.25	69.06	3.4	3.45	Control
761 E	1155	523.90	60	31.43	67.31	3.4	3.37	Treatment
774 E	1010	458.13	60	27.49	58.86	3.4	2.94	Control
779 E	1115	505.76	60	30.35	64.98	3.4	3.25	Control
782 E	960	435.45	60	26.13	55.95	3.4	2.80	Treatment
786 E	1210	548.85	60	32.93	70.52	3.4	3.53	Treatment
794 E	1260	571.53	60	34.29	73.43	3.4	3.67	Control

- **Week 7-9**

Bull ID	Bull body weight (lb)	Bull Body weight (kg)	Intended Alkaloid Conc. (microgram/kg) of BW	Total Alkaloid Needed (mg)	Total Screening (467mg alakloid/kg of screening) Needed (g)	Conc. (mg/kg) based on Dry Matter Intake (DM) = 2.4% of BWt	Capsules Needed	Group
20E	1305	591.94	60	35.52	76.05	3.4	3.80	Control
29E	1340	607.81	60	36.47	78.09	3.4	3.90	Control
43 E	1240	562.45	60	33.75	72.26	3.4	3.61	Treatment
67 E	1280	580.60	60	34.84	74.60	3.4	3.73	Treatment
94 E	1180	535.24	60	32.11	68.77	3.4	3.44	Control
117 E	1155	523.90	60	31.43	67.31	3.4	3.37	Treatment
169 E	1125	510.29	60	30.62	65.56	3.4	3.28	Treatment
170 E	1220	553.38	60	33.20	71.10	3.4	3.55	Control
761 E	1185	537.51	60	32.25	69.06	3.4	3.45	Treatment
774 E	1060	480.81	60	28.85	61.77	3.4	3.09	Control
779 E	1160	526.17	60	31.57	67.60	3.4	3.38	Control
782 E	1025	464.93	60	27.90	59.73	3.4	2.99	Treatment
786 E	1280	580.60	60	34.84	74.60	3.4	3.73	Treatment
794 E	1255	569.26	60	34.16	73.14	3.4	3.66	Control