Effect of Season on Pregnancy Rates, Milk Progesterone, and Milk Melatonin Profiles in Water Buffalo Reared in Canada

by

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ABSTRACT

EFFECT OF SEASON ON PREGNANCY RATES AND MILK PROGESTERONE AND
MELATONIN PROFILES IN WATER BUFFALO REARED IN CANADA

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Having recently been introduced to Canada, water buffalo products have a growing market demand. While water buffaloes are seasonal breeders in some environments, the impact of season on estrus patterns in Canada is unknown. Pregnancy rates for buffaloes artificially inseminated in different seasons following synchronization or natural estrus were calculated. Milk samples were used to determine variation in progesterone and melatonin during summer (long days) and winter (short days). Milk was collected from randomly selected buffaloes and hormones were measured via enzyme-linked immunosorbent assays (ELISA). Spring and summer pregnancy rates were lower than in fall and winter. Spring and summer samples showed low progesterone concentrations with abrupt rise and fall. Fall and winter samples showed high progesterone with prolonged peaks and 20-22-day estrous cycles. No significant trend was observed in melatonin levels. Results indicate breeding seasons in Canada are fall and winter while low breeding seasons are spring and summer.
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DECLARATION OF WORK PERFORMED

With the exception of the items indicated below, I declare that all work presented in the body of this thesis was performed by me.

Caitlin West, Martin Littkemann, and Lori Smith collected milk samples and recorded fertility data from the buffaloes for use in this project. Edgardo Reyes was responsible for ordering of all materials used. Inayat Gill assisted in measurement of progesterone concentrations in the milk samples.
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ABBREVIATIONS
ACTH- adrenocorticotropin-releasing hormone
CIDR- controlled internal drug releasing device
CL- corpus luteum
CRH- corticotropin-realeasing hormone
ELISA- enzyme-linked immunosorbent assay
FF- Follicular fluid
FSH- follicle stimulation hormone
GC- granulosa cell
GnRH- Gonadotropin Releasing Hormone
HIOMT-hydroxy-indol-methyl-transferase
HPA- hypothalamic-pituitary-adrenal
HPG- hypothalamic-pituitary-gonadal
IU- international unit
IVF- in vitro fertilization
LH- luteinizing hormone
mg- milligram
MGA- melengestrol acetate
mL- milliliter
MT1- melatonin receptor 1
MT2- melatonin receptor 2
MT3- melatonin receptor 3
NAT- n-acetyl transferase
nm- nanometer
ng/ml- nanogram per millilitre
OBSM- out of breeding season mating
PGF2α- prostaglandin-F2 alpha
pg/ml- picogram per millilitre
PMSG- pregnant mare serum gonadotropin
pM- pico molar
PRID- progesterone releasing intravaginal device
RIA- radiolabeled immunoassay
ROS- reactive oxygen species
RZR/ROR- RAR-related orphan receptors
SEM- standard error of the mean
ug- microgram
uM- micro molar
INTRODUCTION

Water buffalo-derived products have long been used for making high quality cheeses and these products have become popular around the world (Barile, 2005). Water buffalo are the second most important contributors to global milk production (De la Cruz-cruz et al., 2014). The water buffalo contributes to meat and milk production around the world, but has been concentrated in Asia (Barile, 2005). As with any animal involved in agriculture, optimization of reproductive efficiency is a key factor and plays a major role in increasing profit. Unfortunately, in contrast to cattle, water buffalo exhibit much less efficient breeding behaviours (Barile, 2005). Several factors contribute to this inefficiency including late puberty, long postpartum anestrus, silent estrus, and seasonal reproduction (Barile, 2005). Studies on reproduction in water buffalo are restricted to Mediterranean and Asian regions (Barile, 2005), as water buffalo have been introduced more recently to the novel Canadian environment.

Although water buffalo are capable of reproducing throughout the year, they show distinct seasonality in reproduction around the world (Singh et al., 2000). As mentioned above, most studies concerning water buffalo reproduction have been performed at lower latitudes (≤41°N) (Barile, 2005) and there is no information regarding reproductive seasonality in Canada. In tropical regions, water buffalo are known to have higher reproductive success in the wet season as compared to the dry season (Singh et al., 2000). In more temperate zones, such as Italy, water buffalo show increased reproductive success in the colder seasons as compared to the hotter seasons (Presicce, 2007). Though this may point to any number of environmental factors as having an influence on seasonality of reproduction, studies have shown that the most influential factor is photoperiod (Barile, 2005).
Changes in photoperiod in an animal’s environment are translated internally by the hormone melatonin (Ikegami and Yoshimura, 2012). Melatonin dictates the circadian rhythm and is also known to be a potent radical scavenger within the body (Tamura et al., 2009). Studies on the variation of melatonin in water buffalo over different seasons are limited but show a diurnal melatonin pattern, which is more pronounced in reproductively seasonal water buffalo (Parmeggiani et al., 1992). Water buffalo exhibiting reproductive seasonality also show increased levels of night time melatonin in the breeding season as compared to the non-breeding season (Parmeggiani et al., 1992). These variations in melatonin related to breeding seasons in Canada have yet to be explored.

Many studies have tried to determine a method for detecting estrous cycle patterns within water buffalo in order to increase breeding efficiency (Hoque et al., 2011). Monitoring estrous cycle patterns can help identify length of estrous within different buffalo and a non-invasive technique would be beneficial for use in the field. Studies have shown that progesterone concentration is a good indicator of ovarian status and can be used to detect pregnancy in water buffalo (Banu et al., 2012). As a hormone that is known to be important in the establishment and maintenance of pregnancy, progesterone plays an important role in fertility by providing the appropriate physiological conditions for a successful pregnancy (Campanile et al., 2013). Therefore, changes in progesterone concentrations were used in this study to monitor estrous cycle patterns over different seasons in the Canadian climate.
REVIEW OF LITERATURE

I. Importance of Livestock Production

Livestock reproduction is a key component of the agricultural economy around the world, providing food, employment, fuel, and transport (FAO, 2011). Livestock contributes to over 50% of the global agricultural output (FAO, 2011). The Food and Agriculture Organization of the United Nations estimated that the livestock population consists of 1.58 billion bovines and 1.95 billion small ruminants (FAO, 2008). Specifically, in the bovine population the number of cattle and buffalo are estimated to be 1.4 billion and 0.18 billion, respectively (FAO, 2008). Livestock contribute to 28% of the world’s protein needs with that number increasing to 48% when looking at developed countries specifically (FAO, 2011). As the population of the world increases, the demand for livestock production will increase with it (FAO, 2011).

The global population is expected to reach 9.6 billion by the year 2050 and there has also been an increase in migration to developed countries, which will contribute to the increased demand of livestock production (Alexandratos and Bruinsma, 2012). It is projected that the world’s demand for milk, meat, and eggs will increase by 30%, 60%, and 80%, respectively by the year 2050, as compared to the demand in 1990 (Alexandratos and Bruinsma, 2012). It is evident that this dramatic increase in world population will require a parallel increase in livestock production (FAO, 2011). Two possible solutions to this problem may be increasing the total number of animals or by increasing the production potential of livestock (FAO, 2011).

II. Water Buffalo (Bubalus bubalis)

Domesticated over 5000 years ago, the water buffalo plays an essential part in the agricultural industry of many countries today (Kumar et al., 2007). The water buffalo is a tropical animal found vastly in Asia, the Mediterranean regions, and South America (Perera,
Close to 95% of the world buffalo population resides in Asia with the total population approximated at 170 million (Presicce, 2007). As a result, this animal is a large part of the agricultural economy in these regions (Banu et al., 2012).

Though the time and place of domestication is still unknown, it is accepted that there are two distinct types of domesticated water buffalo, swamp and river type (Kumar et al., 2007). Swamp type water buffalo have a diploid chromosome content of 48 and are typically used for meat and draught power. River type water buffalo have a diploid chromosome content of 50 and are capable of mating with swamp type buffalo to produce fertile offspring (Di Berardino and Iannuzzi, 1981; Presicce, 2007). River water buffalo are used primarily for milk and meat production rather than draught power and are the main contributor to buffalo milk production (Presicce, 2007). Water buffalo produce milk that is high in fat and protein, resulting in an increased demand for buffalo-derived milk products around the world (NRC, 1981). As a result, from this point onwards in this thesis, only river type water buffalo will be referenced, unless otherwise specified.

After dairy cattle, water buffalo are the second most important source of milk in the world (De La Cruz-Cruz et al., 2014). The water buffalo provides 17% of the world’s total milk production and approximately 1.6 million metric tons of buffalo meat annually (Singh et al., 2000; Patil et al., 2014). Since a majority of the buffalo population is concentrated in Asia, water buffalo contribute to 48% of Asian milk production specifically (Patil et al., 2014). In addition to being a source of food, water buffalo are also used for draught power in developing countries (Singh et al., 2000). In many parts of Southeast Asia, 20% to 30% of farm power is provided by the water buffalo (Singh et al., 2000).

III. Climate and Reproduction
Animals have evolved to adapt to changing environmental conditions and balance costs and benefits of reproduction (Ghosh et al., 2015). Climatic factors such as availability of feed, heat stress, and photoperiod can affect reproduction (Figure 1) (Ghosh et al., 2015). Nutrition and heat stress are important factors influencing reproduction in tropical regions of the world, while photoperiod is an important factor influencing reproduction in the temperate zones (Figure 1) (Ghosh et al., 2015).

In order to understand the influence of the environment on reproduction, the interaction between the hypothalamus-pituitary-adrenal (HPA) and the hypothalamus-pituitary-gonadal (HPG) axes needs to be addressed (Hiller-Sturmhöfel and Bartke, 1998). Any type of stress encountered by an organism activates the HPA axis, causing the release of corticotropin-releasing hormone (CRH) from the hypothalamus (Hiller-Sturmhöfel and Bartke, 1998). CRH then acts on the anterior pituitary to stimulate the release of adrenocorticotropic hormone (ACTH), which goes on to stimulate the release of cortisol from the adrenal gland (Hiller-Sturmhöfel and Bartke, 1998). The HPA axis is regulated by a negative feedback loop which allows cortisol and the other cascade components to repress CRH release (Hiller-Sturmhöfel and Bartke, 1998). This can be summarized as the physiological response to stressful stimuli.

The axis on the reproduction side of the coin is the HPG axis which acts to release sex hormones in both males and females (Hiller-Sturmhöfel and Bartke, 1998). Once again, the cascade begins with a hormone released from the hypothalamus (Hiller-Sturmhöfel and Bartke, 1998). Pulsatile secretion of gonadotropin-releasing hormone (GnRH) causes the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (Hiller-Sturmhöfel and Bartke, 1998). LH and FSH act on the maturing oocyte within the ovary to stimulate production of estradiol (Hiller-Sturmhöfel and Bartke, 1998). After ovulation LH
promotes progesterone production by the corpus luteum (CL) (Hiller-Sturmhöfel and Bartke, 1998). These hormones also act on the hypothalamus through negative feedback for most of the reproductive cycle (Hiller-Sturmhöfel and Bartke, 1998). The exception occurs prior to ovulation, where estradiol promotes an LH surge leading to ovulation (Hiller-Sturmhöfel and Bartke, 1998). Through an understanding of the HPA and HPG axis cascades it can be seen that crosstalk between these two axes can easily occur at the level of the hypothalamus and pituitary in the brain (Hiller-Sturmhöfel and Bartke, 1998).

Gonadotropin releasing hormone (GnRH) is the major hormone that links climatic factors to reproduction (Wade and Jones, 2004). GnRH is under the control of the hypothalamic pituitary adrenal (HPA) and hypothalamic pituitary gonadal (HPG) axes (Zhang et al., 2007). As GnRH is released by the hypothalamus, it is easy to understand why this is where the two axes interact to have an effect of reproduction. Nutrition can change depending on the availability of feed, and malnourishment, diabetes mellitus, and excessive energy expenditure can lead to an activation of the HPA axis (Wade and Jones, 2004). When activated, the HPA releases corticotrophin releasing hormone (CRH) which acts on GnRH neurons to inhibit the release of GnRH (Zhang et al., 2007).

In addition, nutritional deficiency also affects synthesis of steroids by the gonads (Ghosh et al., 2015). In pre-pubertal heifers, caloric restriction causes a suppression of luteinizing hormone (LH) resulting in delayed puberty (Day et al., 1986). In post-pubertal females, although estrogen synthesis is not affected by malnutrition, progesterone synthesis by the corpus luteum (CL) is affected (Apgar et al., 1975). Interestingly, studies on feed-restricted cattle have shown an elevation of LH (Imakawa et al., 1986). This can be attributed to the absence of negative
feedback by progesterone, caused by decreased levels of progesterone from an insufficient corpus luteum (Imakawa et al., 1986).

**Figure 1.** Effect of climate change on reproductive efficiency in temperate and tropical regions of the world (adapted from Ghosh et al., 2015)

**IV. Seasonal Reproduction in Mammals**

When the earth first started to warm up, the separate continents we know today were known as one large landmass, Pangea (Bronson, 2009). At this point, portions of Pangea showed extreme seasonal changes in climate (Crowley, 1993). It is hypothesized that even the earliest mammals needed to adapt by adjusting their reproductive behaviour to give birth during the most favourable environmental conditions (Bradshaw and Holzapfel, 2007). Today, over 4000
mammalian species are spread across various habitats and climates, most of whom exhibit
seasonality in reproduction (Bradshaw and Holzapfel, 2007). The two driving factors that lead to
seasonality have been proposed as foraging conditions and seasonal cues (Bronson, 2009).
Foraging conditions influence the balance between energy expenditure and gain, while seasonal
cues, such as photoperiod, can indicate ensuing changes in foraging conditions (Davis and
Levitan, 2005).

Energy balance is a critical indicator of whether or not reproduction is feasible at a given
time for an organism (Bronson, 2009). Negative energy balance occurs when the amount of
calories gained from the energy source is less than the amount of energy spent foraging for the
source (Bronson, 2009). Energy is first directed towards mandatory processes within the body
including homeostasis, thermoregulation, and foraging (Manning and Bronson, 1990).
Reproduction and growth, however, are functions in the body that can be delayed when there is
an extreme negative energy balance (Manning and Bronson, 1990).

Ovulation and puberty are examples of reproductive processes that are delayed when an
organism is low on energy. Negative energy balance influences reproduction by depressing
gonadotropin releasing hormone (GnRH) pulse activity (Wade and Jones, 2004). Experiments in
mice show that food restriction blocks ovulation, as does exposure to cold if increasing food
intake is inhibited (Bronson, 2009). However, if female mice are allowed to increase food intake
to compensate for the energy spent on thermoregulation, ovulation and reproduction can be
maintained at low temperatures (Bronson, 2009). In humans, a delay in puberty has been
observed in young females practicing ballet and ovulation is also known to be blocked in adult
athletes undergoing strenuous training (Wade and Jones, 2004). In both of these cases,
individuals are maintaining the minimal amount of positive energy balance which has a negative impact on their reproductive system and ability to reproduce (Bronson, 2009).

Around the world, the cost of foraging increases with latitude (Bradshaw and Holzapfel, 2007). The extreme temperatures at the poles make it more challenging for food acquisition and decrease the amount of food that is even available (Bronson, 2009). Animals in temperate regions, located at higher latitudes, generally rely on the change in photoperiod to determine the optimal time for breeding (Menassol et al., 2012). In this way, animals ensure that offspring are born at a time when there is maximum availability of naturally-occurring food (Menassol et al., 2012). The food availability can also be influenced by the climate in the preceding season, impacting the growth and reproduction of animals in the following year (Langvatn et al., 1996). Nutrition is strongly linked to reproduction and the age at which animals reach reproductive maturity (Langvatn et al., 1996). In wild and domesticated ungulates, reproductive age is linked to a critical body mass that increases the chances of a female ovulating and becoming pregnant (Langvatn et al., 1996).

V. Estrous Cycle

The estrous cycle is a reproductive cycle in most female mammals excluding higher primates (Austin and Valentine, 1984). The length of the estrous cycle can vary dramatically between species and is largely dependant upon the gestation length (Austin and Valentine, 1984). This cycle can be divided into four phases: proestrus, estrus, metestrus, and diestrus (Ali et al., 2003). Monoestrus animals will come into heat or exhibit male receptivity once annually, while polyestrus animals will have multiple cycles throughout the year and are capable of mating each time estrus occurs (Austin and Valentine, 1984). Some animals may also be seasonally polyestrus, meaning they will only cycle during certain seasons throughout the year but have
more than one estrus cycle during this period (Austin and Valentine, 1984). The period of time
during the year that the animal is not cycling is termed anestrus and is a prolonged period of
sexual inactivity (Austin and Valentine, 1984).

The four phases of the estrous cycle are in reference to when estrus occurs and each
phase can be identified by specific physiological changes in the reproductive tract (Heape, 1990).
Proestrus is the phase of the estrous cycle immediately prior to estrus (Heape, 1990). In this
phase, the body is preparing the endometrium for implantation and the ovarian follicles are
continuing to develop (Heape, 1990). The onset of estrus is characterized by sexual receptivity
and possible swelling of the genitals (Heape, 1990. During estrus, ovulation will take place and if
mating has occurred the oocyte will be accessible to the sperm (Heape, 1990). The next phase of
the estrous cycle is metestrus and during this time the female is no longer sexually receptive and
the ruptured follicle is transforming into a corpus luteum (Heape, 1990). Finally, the last stage of
the estrous cycle is diestrus (Heape, 1990). In this phase, a functional corpus luteum has formed
and is producing progesterone (Mondal et al., 2007). If the animal has become pregnant,
progesterone will help to maintain the pregnancy and prevent another estrous cycle from
occurring before the offspring is born (Campanile et al., 2013). If the animal did not become
pregnant, and is polyestrous, another cycle will begin with proestrus (Austin and Valentine,
1984). Hormone fluctuations throughout the estrous cycle contribute to the physiological and
behavioural changes observed at each phase of the cycle (Terzano et al., 2012).

VI. Mammalian Reproductive Endocrinology

The various phases of the reproductive cycle are dictated by the interaction between a
series of hypothalamic, pituitary, and gonadal hormones (Terzano et al., 2012). Overall this
interaction between the hypothalamus, pituitary, and gonads, is termed the hypothalamic-
pituitary-gonadal axis (HPG) (Terzano et al., 2012). A balance in this axis is required for proper reproductive function, and as a result, problems in reproduction generally originate from HPG malfunction (Terzano et al., 2012).

Progesterone is a steroid hormone that is part of the progestogen family and is produced by the corpus luteum and during pregnancy by the placenta (Terzano et al., 2012). In a cycling buffalo, the levels of progesterone rise and fall with the formation and regression of the corpus luteum, while levels remain elevated during pregnancy until birth (Mondal et al., 2007). Progesterone is known to play key role in the maintenance of pregnancy, especially in the early stages (Campanile et al., 2013). It also has a synergistic effect with estrogens and helps to stimulate growth and development of alveolar tissue in the mammary gland (Terzano et al., 2012). Plasma progesterone level is a good indicator of puberty, and levels higher than 1 ng/ml indicate the onset of puberty (Terzano et al., 2012). Another key hormone associated with reproduction is estradiol. It is part of the group of hormones called estrogens, is produced by the ovary, and exerts its effects on the central nervous system to induce behavioural changes associated with estrus (Terzano et al., 2012). Studies have shown that increased levels of estradiol can be correlated to increasing size of the follicle (Palta et al., 1998).

GnRH stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary (Medhamurthy et al., 2012). LH is secreted in a pulsatile manner, and changes in pulse frequency result in a change in LH concentration within the organism (Medhamurthy et al., 2012). While FSH stimulates follicle development, a surge in LH levels causes the follicular wall to rupture and culminates in ovulation of the oocyte (Barile 2012; Terzano et al., 2012).
VII. Water Buffalo Reproduction

Water buffalo have a gestation period of 10 months and 10 days (Singh et al., 2000). The average length of the estrous cycle in water buffalo is 21 days, with estrus lasting anywhere from 5 to 27 hours (Perera, 2011). Although they are capable of reproducing throughout the year, water buffalo show distinct reproductive seasonality (Singh et al., 2000). In addition, silent estrus is very common in water buffaloes, making it hard for farmers to estimate time of ovulation (Hoque et al., 2011). Manipulation of time of breeding and ovulation through hormone monitoring has become a key objective of research, in order to increase milk production throughout the year to avoid milk shortages (Singh et al., 2000). Climate, temperature and metabolism are factors that affect ovulatory activity (Singh et al., 2000). Hormone concentrations fluctuate differently depending on the season, which contributes to anestrus (Singh et al., 2000). A study in India and Pakistan demonstrated that these animals calved often in December, leading to very scarce milk production in summer months (Singh et al., 2000).

Traditionally, water buffalo are known to be more difficult to breed than cattle due to several factors, including seasonal anestrus, delayed puberty, silent estrus, and prolonged postpartum inactivity (Suthar and Dhami, 2010). The age of puberty for water buffalo is highly variable and can be affected by breed (Perera, 2011). Specifically, river buffalo reach puberty at around 15-18 months, while swamp buffalo reach puberty around 21-24 months (Drost, 2007). It is generally accepted that puberty is reached once the animal is 55-60% of the adult body weight (Perera, 2011). As mentioned previously, water buffalo reach puberty later than cattle but the reproductive lifespan of buffalo is much longer than that of cattle, which accounts for the initial economic loss that may be brought on by lack of milk production prior to reaching puberty (Perera, 2011).
Compared to cattle, ovaries in the buffalo are much smaller and have significantly less primordial follicles (Presicce, 2007). Specifically, post-pubertal water buffalo have approximately 10,000-20,000 primordial follicles (Perera, 2011). In contrast, cattle have 10 times more primordial follicles (Perera, 2011). In addition to the small size of the ovary, the corpus luteum in water buffalo does not protrude from the ovary as it does in cattle (Perera, 2011). This adds to the difficulty in determining the presence of a corpus luteum by palpation or other means (Presicce, 2007).

Silent heat is one of the major contributors to the inefficiency in breeding in the water buffalo product market (Banu et al., 2012). Basal levels of estradiol are quite low in water buffalo and this causes an absence in estrus behaviour (Terzano et al., 2012). Without physical presentation of heat, estrus often goes unnoticed (Drost, 2007). A study conducted with Pakistani river buffalo showed that 51.5% of estrus goes undetected due to silent heat (Qureshi and Ahmad, 2008). In another study, researchers reported an incidence of silent estrus of up to 73% (Shah et al., 1990).

After calving, postpartum anestrus in water buffalo also affects the productivity of this animal (Barile, 2005). While this interval in cattle generally remains consistent, in water buffalo postpartum anestrus depends on season of calving, milk yield, uterine involution, suckling, and nutrition (Barile, 2005). A study was performed to analyze the changes in LH and FSH during and after gestation by treatment of exogenous GnRH (Palta and Madan, 1996). The results showed that the LH and FSH response to GnRH was significantly reduced as gestation progressed and remained low after parturition (Palta and Madan, 1996). This lack of effect of GnRH on LH and FSH is thought to contribute to the long postpartum anestrus seen in water buffalo (Palta and Madan, 1996).
VIII. Pregnancy Diagnosis in Bovine

With the problems in detecting estrus already weighing heavily on reproductive efficiency in buffalo, early pregnancy diagnosis tools can be a very useful (Ingawale et al., 2012). Early diagnosis of pregnancy can allow farmers to determine which animals are still open to rebreed. In this way, early diagnosis of pregnancy can allow for minimizing the calving interval and optimizing productivity (Ingawale et al., 2012).

Although return to estrus is a logical indicator of a cycling buffalo, the difficulty in estrus detection deters farmers from using return to estrus alone for breeding (Pawshe et al., 1994). Milk progesterone levels detected using radio-immunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) have also been used as reliable indicators of non-pregnant buffalo (Singh and Puthiyandy, 1980). Identifying non-pregnant buffalo using these assays has had an accuracy of up to 100% (Singh and Puthiyandy, 1980). The accuracy of detecting pregnant buffaloes using these techniques has been low, with the highest being 75% (Karen et al., 2007). Low progesterone levels can be used as a reliable indicator of non-pregnant buffaloes and can be identified as early as 20-24 days after insemination (Singh and Puthiyandy, 1980). Palpation via rectum is another technique that can be used to detect pregnancy (Karen et al., 2007). The drawbacks to this technique are that there is a risk of fetal mortality and is only reliable to detect pregnancy after 45 days (Karen et al., 2007).

Ultrasound has become the most reliable and safe method for detection of early pregnancy in bovine (Ingawale et al., 2012). Using ultrasound, the conceptus can be identified and embryo viability can also be detected (Tiwari et al., 2002). In 2012, Ingawale et al (2012) examined the efficacy of using ultrasonography for early pregnancy detection in buffaloes at varying time points after artificial insemination. A total of 72 buffaloes were inseminated and
checked using ultrasound at days 21, 28, and 37 (Ingawale et al., 2012). They found the optimal time for detecting pregnancy in buffalo was on day 37 post artificial insemination (Ingawale et al., 2012). Using ultrasonography on day 37 the researchers were able to identify the embryo and compartmentalization, the amniotic vesicle surrounding the embryo, limb buds, and a heartbeat (Ingawale et al., 2012).

A method of measuring fertility within buffalo that has recently become popular is the daughter pregnancy rate (Patil et al., 2014). Daughter pregnancy rate identifies the percentage of non-pregnant buffalo that become pregnant in one 21-day period (Patil et al., 2014). This method of measurement uses a 21-day period to represent the average length of one estrous cycle, and therefore one chance for the animal to become pregnant (Patil et al., 2014). It has been shown that animals with higher daughter pregnancy rates have a lower number of days open which leads to an overall increase in productivity (Norman et al., 2008). Pregnancy rate can also be measured simply by calculating the percentage of inseminated animals that become pregnant. In dairy cattle, studies have used the number of cows pregnant at a fixed time post-artificial insemination divided by the total number of cows inseminated as a measure of pregnancy rate (Pursley et al., 1997).

IX. Reproductive Hormone Profiles in Water Buffalo

The seasonal differences in concentrations of reproductive hormones have been studied primarily in tropical regions (Mondal et al., 2007). In these regions, the four seasons are characterized by amount of heat and rainfall (Perera, 2011). Estradiol concentrations in plasma were measured over the four seasons and found to be lower in the hot season compared to the cooler season (Rao and Pandey, 1983). Levels of follicle stimulating hormone (FSH) are also affected by climate (Terzano et al., 2012). FSH levels measured at estrus and the luteal phase
show significantly higher concentrations in November to December compared to March to October (Terzano et al., 2012). The ratio of FSH to luteinizing hormone (LH) has also been analyzed in relation to season and shows a higher ratio in the peak breeding season (November to December) (Mondal et al., 2007). LH levels measured over seasons have also shown significantly increased concentrations on the day of estrus in cooler months compared to hotter months (Mondal et al., 2007).

The endocrinology of water buffalo during the estrous cycle is similar to that of cattle in regards to estradiol, luteinizing hormone, follicle stimulating hormone, and progesterone profiles (Terzano et al., 2012). Peak estradiol concentrations of 35.8 pg/ml are observed one day before estrus and gradually decline from the onset of estrus, reaching the lowest values of 5.7 pg/ml two days after estrus (Bachlaus et al., 1979). Minor peaks throughout the estrous cycle, at approximately 5 and 10 days after estrus, have been observed (Figure 2) (Bachlaus et al., 1979). Peripheral plasma concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH) are highest on the day of estrus (Figure 2) (Heranjal et al., 1979). FSH peak values range from 52.9 to 57.9 ng/ml (Heranjal et al., 1979; Galhotra et al., 1985). The peak concentration range for LH is much broader ranging anywhere from 20-40 ng/ml (Heranjal et al., 1979). After this peak, LH levels fall abruptly while FSH levels gradually decline to basal levels (Mondal et al., 2007). Basal levels of FSH range from 8-16 ng/ml and LH basal values are much lower from 0.72-3 ng/ml (Galhotra et al., 1985; Heranjal et al., 1979).
Figure 2. Relative levels of oestradiol, luteinizing hormone, follicle stimulating hormone, and progesterone during estrous cycle of water buffalo. (Adapted from Perera, 2011)

X. Water Buffalo Synchronization and Breeding

It has been established that artificial insemination is a useful tool in breeding water buffalo (De Rensis and López-Gatius, 2007). Insemination, however, must take place at the appropriate time after ovulation in order to result in pregnancy. Yet again, estrus detection is a key factor as ovulation occurs approximately 2 days after estrus (De Rensis and López-Gatius, 2007). To overcome this barrier and increase the efficiency of artificial insemination in water buffalo, hormonal events of the estrous cycle can be manipulated (Driancourt, 2001). Regression of the corpus luteum, follicular growth, and timing of ovulation can be manipulated using treatment with exogenous hormones (Driancourt, 2001).

Levels of peripheral progesterone are regulated by inducing premature luteolysis or stimulating CL function (Barile, 2012). The corpus luteum is a structure formed on the ovary after ovulation, which produces progesterone (Campanile et al., 2013). Elevated levels of progesterone, in turn, inhibit resumption of the estrous cycle (Campanile et al., 2013). This is
useful in maintaining the corpus luteum until the formation of the placenta, which is also able to produce progesterone (Campanile et al., 2013). Luteolysis, on the other hand, is the process of degradation of the corpus luteum, which causes a decrease in progesterone levels (Barile, 2012). Decreasing progesterone results in increased levels of gonadotropins and estradiol (Hiller-Sturmhöfel and Bartke, 1998). This allows the animal to enter into the next estrous cycle. Both CL function and luteolysis can be manipulated using synthetic compounds to synchronize ovulation in buffalo (Barile, 2012).

Prostaglandin-F2α is a naturally-occurring compound responsible for the degradation of the CL (Barile, 2012). In the event that implantation does not occur, PGF2α is secreted by the uterus during the luteal phase (Barile, 2012). PGF2α derivatives have been produced for use in synchronization protocols (Barile, 2012). Treatment with PGF2α or its derivatives causes regression of the CL within 24-72 hours, and ovulation 2-3 days after that (Dhaliwal et al., 1987). The key factor in application of this compound is that the CL is only responsive to PGF2α during the mid-luteal phase, days 5-17 of the estrous cycle (Barile, 2012). Treatment outside of this range will not result in degradation of the CL (Barile, 2012). Two protocols exist for application, where either one or two injections of PGF2α are given (De Rensis and López-Gatius, 2007). The one shot method applies only to animals that have a functional CL, which can be detected with palpation or ultrasound (Barile, 2012). The drawback here is that there needs to be confirmation of a functional CL before PGF2α administration (Barile, 2012). With the two shot method, animals with unknown CL status can all be treated at once on day 0 and again on day 11 (Barile, 2012). Animals in the mid-luteal phase during the first shot will respond to PGF2α while animals outside this range (days 18-4) will not (Barile, 2012). After 11 days, all of
the animals will be in the mid-luteal phase and will respond to treatment with PGF2α (Barile, 2012).

The advantage of the two shot method is that a group of animals can be synchronized without knowing what stage of the estrous cycle each one is in (Barile, 2012). This overcomes the cost of an ultrasound that would be necessary to detect a functional CL in the one shot method (Barile, 2012). Generally, after PGF2α treatment either observed estrus or timed insemination, insemination after a certain amount of time for all buffalo, can be applied (De Rensis and López-Gatius, 2007). Using PGF2α and timed artificial insemination alone has proven to be inefficient (Chohan et al., 1993). In addition, application of luteolytic compounds causes decreased expression of estrus which makes observing estrus more difficult (Sahasrabudhe and Pandit, 1997). In addition, large variation in the duration of estrus after treatment with PGF2α has been reported (Baruselli, 2001a). Studies have found that depending on when during the cycle PGF2α is given, the interval to ovulation can also show large variability (Baruselli, 2001a).

In contrast to luteolytic agents, progestins or progesterone derivatives can be used to stimulate existing CL function (Barile, 2012). Though they use a different mechanism of action than PGF2α, application of progestins is also used for resumption of the estrous cycle (Barile, 2012). The theory behind using progestins is that elevated levels of progesterone can be controlled (Barile, 2012). The level of progesterone can remain elevated for the duration of a natural luteal phase (16 days) or for a shorter amount of time (Barile, 2012). Removing the progestin source causes a rapid decrease in progesterone levels leading to the start of the next estrous cycle (Barile, 2012). Exposing buffalo to progestins for a long period of time (16 days) may lead to deleterious effects on the uterine environment and cause a reduction in conception.
rates (Barile, 2012). The reason for keeping progesterone levels elevated this long is to make sure that any naturally-occurring CL does not outlive the progestin treatment (Barile, 2012). To overcome the reduction in conception rate and the possibility of a natural CL remaining in tact, synchronization protocols commonly use a shorter duration of progestin application in conjunction with PGF2α treatment (De Rensis and López-Gatius, 2007). There are three types of progestins that are generally used, melengestrol acetate (MGA), Norgestomet, or an intravaginal device (Barile, 2012). Melengestrol acetate is delivered orally, through addition to feed (Barile, 2012). The drawback here is that the dosage is hard to control and uniformity of dosage across the herd cannot be achieved (Barile, 2012). Norgestomet is a subcutaneous implant that is placed under the skin of the ear (Barile, 2012). The two main intravaginal devices that can be used are the progesterone releasing intravaginal device (PRID) and the controlled internal drug releasing device (CIDR), which also releases progesterone (Yotov et al., 2012).

In addition, follicular growth and the timing of ovulation can be affected through the use of exogenous hormones. Gonadotropin-releasing hormone can be used to stimulate release of luteinizing hormone from the anterior pituitary which allows for ovulation of the dominant follicle (Yotov et al., 2012). LH can also cause luteinisation or atresia of a pre-dominant follicle (Yotov et al., 2012). Application of only prostaglandin and GnRH for synchronization has been termed the Ovsynch protocol and is ideal for use during breeding seasons (Barile et al., 2004). Adding a progsynch device to the protocol has been recommended for use during the low or non-breeding seasons (Barile et al., 2004).

XI. Reproductive Seasonality in Water Buffalo

Seasonal reproductive patterns in water buffalo are evident from studies performed at lower latitudes around the world (Singh et al., 2000) A comparison of reproductive seasonality in
Pakistan was made between buffaloes and Zebu cows by housing both groups on the same farm and exposing them to the same environment (Shah et al., 1989). The breeding frequency in the buffalo was highest during winter (December-February) and lowest in the summer (June-September) (Shah et al., 1989). The opposite effect on breeding frequency was observed in Zebu cattle, with highest breeding frequency in the summer and lowest in the winter (Shah et al., 1989). Length of postpartum anestrus is also affected by season (Singh et al., 2000). Buffaloes that calved from February to May showed a significantly longer period of postpartum anestrus than buffaloes that calved during the rest of the year (Singh and Nanda, 1993). Ovarian activity in buffaloes that calved from late winter to spring was delayed 116-148 days compared to inactivity for 38-64 days in buffaloes that calved during the rest of the year (Singh and Nanda, 1993). The conception rates are also much lower between February and August than in other seasons (Madan, 1988). A study performed in Punjab showed that 77% of the buffaloes that are not pregnant during the summer become anestrus (Singh et al., 1989). Reduced reproductive function in the summer has been seen through shorter duration of estrus, a longer estrus to ovulation interval, and fewer ovulatory cycles (Singh et al., 2000). A study calculated the incidence of anestrus by observing the absence of large follicles and CL in the ovaries (Singh and Singh, 1985). They found that incidence of anestrus was 78% in July and 14% in November (Singh and Singh, 1985).

There are multiple environmental factors that affect reproductive outcome in the water buffalo. To discriminate between the effects of photoperiod and other environmental factors, a two-year long study was conducted examining the interval from parturition to first estrus during different seasons (Singh and Nanda, 1993). Of all the detected incidences of estrus, only 23% were recorded during the hotter months (Singh and Nanda, 1993). Interestingly, the highest
recorded temperature during the two-year period was in June, while the lowest incidence of estrus was in July (Singh and Nanda, 1993). In addition, the percentage of estrus was 15% in September compared to 5% in March while similar temperatures were recorded during both months (Singh and Nanda, 1993). Relative humidity was found to have no distinct association with ovarian activity (Singh and Nanda, 1993).

A strong correlation exists between air temperature, day length, and level of reproductive hormones in water buffalo (Zicarelli, 2010). High temperature can affect ovarian function, which explains why less buffaloes are pregnant in summer months (Zicarelli, 2010). Previous evidence shows more buffaloes being in estrus during shorter day lengths (winter), than longer day lengths (summer) (Zicarelli, 1997). Moreover, progesterone has been found to be lower after ovulation in non-breeding seasons compared to breeding seasons (Roy and Prakash, 2007).

As a consequence of seasonal breeding, water buffalo milk yield is negatively impacted during the low or non-breeding season. For example, in Italy the highest market demand is during the period of the year when calving is less frequent (Presicce, 2007). To overcome the seasonal effect, the out of breeding season mating (OBSM) technique is employed (Presicce, 2007). OBSM is implemented by removing the bulls from the herd in October, and reintroducing them gradually between March and September (Presicce, 2007). In this way, the majority of calves are born between January and August (Presicce, 2007). OBSM is implemented gradually over the years and results in the selection of animals that are less sensitive to daylight variation (Presicce, 2007). This technique has been implemented by Italian buffalo farmers for over 20 years and the percentage of spring calvings has changed dramatically over the years (Barile, 2005). In 2002, spring calves made up 44.12% of the total calves for that year, while in 1981 only 28.42% of total calvings occurred in spring (Barile, 2005).
XII. Progesterone

Progesterone is the first biologically active compound in the steroid biosynthesis pathway (Qureshi et al., 2000). In female mammals, the level of progesterone is a reliable indicator of the reproductive status (Qureshi et al., 2000). Progesterone is also a good indicator of ovulation and can be used to diagnose pregnancy in bovine animals (O’Connor, 1998). In domesticated ruminants, progesterone can be used to determine the functional status of the corpus luteum and ovarian activity (Banu et al., 2012).

The plasma progesterone profile of water buffalo has been shown to be very similar to that of cattle (Mondal et al., 2007). In a cycling buffalo, the corpus luteum (CL) is the source of progesterone, and as a result, the progesterone profile shows the rise and fall of progesterone levels in parallel with the emergence and regression of the CL (Mondal et al., 2007). The progesterone peak stabilizes for approximately 10 days and then falls, which signals the beginning of the next period of estrus, while a prolonged progesterone peak is an indicator of pregnancy in buffaloes (Hoque et al., 2011).

Recently, measurement of progesterone concentrations in milk has become more favourable for detecting pregnancy and ovarian cyclicity in the field (Banu et al., 2012). Often rectal palpation is used in cattle to determine ovarian status but this can be difficult in buffaloes as the corpus luteum is generally embedded in the stroma of the ovary (Banu et al., 2012). To overcome this, ultrasonography is another option for detection of pregnancy, although it does result in much higher costs than measurement of progesterone concentrations in milk (Banu et
Plasma progesterone concentrations may also be helpful in determining ovarian status but this is a significantly more invasive approach and not practical in field (Qureshi et al., 2000). Changes in progesterone over different seasons has also been observed in buffalo. In warmer months, progesterone levels have shown to be depressed as compared to cooler months (Mondal et al., 2007). Literature review shows that the post-partum interval before estrus was affected by changing progesterone levels in different seasons (Singh et al., 2000). An study on water buffalo in India revealed that there was a longer time period between estrus and ovulation in animals during summer and more ovulatory cycles were observed in winter as compared to summer months, due to small follicles and CL (Singh et al., 2000).

I. Melatonin

Melatonin is another hormone that may contribute to the reproductive seasonality of water buffalo. Melatonin is a small lipophilic indolamine, produced and stored within the pineal gland (Kumar et al., 2014). It is a hormone that regulates sleep by reducing arousal and increasing sleep proclivity (Asher et al., 2015). Serotonin serves as a precursor for melatonin production within the pineal gland (Asher et al., 2015). During the day, the pineal gland produces serotonin and in the absence of light, serotonin is converted to melatonin (Asher et al., 2015). Melatonin production within the pineal gland is stimulated by darkness and inhibited by short wavelength illumination (Asher et al., 2015). Melatonin is a conserved molecule and is present in all organisms from prokaryotes to humans (Kumar et al., 2014) As a result, its role as a regulator of the circadian rhythm across species has long been studied.

The other major function of melatonin is thought to be as an extremely potent free radical scavenger (Kumar et al., 2014). Melatonin is capable of quenching both reactive oxygen species as well as reactive nitrogen species (Kumar et al., 2014). In addition, these interactions produce
metabolites that are themselves excellent scavengers (Kumar et al., 2014). Reactive oxygen species (ROS) are known to be harmful to maturing oocytes and have a large impact on oocyte quality (Tamura et al., 2009). ROS cause breakdown of lipids, damage DNA, accelerate apoptosis, induce two-cell block, and inhibit fertilization (Tamura et al., 2009). In women with unexplained infertility, low levels of antioxidant enzymes in follicular fluid have been reported (Tamura et al., 2009). Relative to unfragmented embryos, fragmented embryos also show higher levels of H$_2$O$_2$, an oxidant present within the body (Tamura et al., 2009). Balancing ROS and antioxidants, such as melatonin, is a key factor in oocyte maturation and fertilization (Tamura et al., 2009). Melatonin has also been found to have an effect on the reproductive, neuroendocrine, immunological, and cardiovascular systems.

Of interest to this study was the impact of melatonin on the reproductive function within animals. Free radicals can affect the microenvironments of oocytes by their presence in follicular fluid (Tamura et al., 2009). Changes in these environments can affect follicular development, ovulation, quality of oocytes, interaction between sperm and oocytes, implantation, and early embryonic development (Tamura et al., 2009). Levels of melatonin in follicular fluid are almost three times higher than the concentration found in serum (Tamura et al., 2009). In photoperiodic animals, the circadian rhythm dictated by melatonin, is responsible for the synchronization of reproductive behaviour to the appropriate environmental conditions (Tamura et al., 2009). The presence of receptors within the reproductive organs indicates a role for melatonin within the reproductive system and in ruminants specifically, its role has been studied in inducing ovarian cyclicity in seasonal animals like goats and sheep (Chemineau et al., 1992).

There are two types of receptors that melatonin can act through, either membrane receptors or nuclear binding sites (Tamura et al., 2009). The nuclear binding sites are part of the
nuclear receptor RZR/ROR superfamily (Tamura et al., 2009). Less is known about the nuclear
binding sites than the membrane receptors MT1, MT2, and MT3 (Tamura et al., 2009).
Melatonin binding sites have been detected in the membrane fraction of human granulosa cells
(Niles et al., 1999) and luteal (Woo et al., 2001) cells as well as in rat ovaries (Soares et al.,
2003a).

Melatonin has a direct effect on ovarian function and alters granulosa cell steroidogenesis
and follicular function in hen (Murayama et al., 1997), hamster (Tamura et al., 1998), and
humans (Woo et al., 2001) Folliculogenesis starts with a limited number of primordial follicles
which are required to grow to primary, preantral, and antral stages before ovulation can occur to
release an oocyte (Tamura et al., 2009). The developing follicle relies on circulating levels of
FSH as well as hormones within the follicular fluid produced by the granulosa cells (Tamura et
al., 2009). As discussed earlier, high levels of melatonin have been found in follicular fluid
(Tamura et al., 2009). In addition, the precursors of melatonin and the two melatonin
synthesizing enzymes NAT and HIOMT have been found in human ovarian extracts (Itoh et al.,
1999). This may indicate that the ovary is capable of synthesizing melatonin in situ but it is
generally believed that most of the melatonin in follicular fluid comes from circulation (Tamura
et al., 2009) As stated earlier, growing follicles depend on FSH in the earlier part of their growth
and later shift to LH dependence (Tamura et al., 2009). Treatment with melatonin increases
expression of mRNA for LH receptors in granulosa cells but not of FSH receptors (Tamura et al.,
2009).

Melatonin has also been implicated in affecting production of sex steroids at different
preantral follicles with melatonin for 12 days and observed a corresponding increase in
production of progesterone and androstenedione (Adriaens et al., 2006). The same observation was noted in porcine antral follicles but there was no change in the level of estradiol (Tanavde and Maitra, 2003). Pinealectomy studies have also shown that removal of the pineal gland in rats results in an increase in estradiol levels and reduced progesterone (Soares et al., 2003b). Analysis of follicular fluid in human preovulatory follicles has also shown a positive correlation between levels of melatonin and progesterone (Nakamura et al., 2003). The direct relationship of melatonin and follicular steroidogenesis is complex and depends on cell type, duration of treatment, experimental model, species, and dose (Tamura et al., 2009).

On a larger scale, pinealectomized rats also showed an increase in the number of atretic follicles within the ovary (Soares et al., 2003b). Intrafollicular concentrations of a biomarker for DNA damage, showed significantly higher levels in women with poor oocyte quality (Tamura et al., 2008). Treatment of melatonin in women with fertilization rates lower than 50% during their first IVF cycle improved fertilization rates after treatment in the next cycle (Tamura et al., 2008). In the mouse model, melatonin supports fertilization as well as early embryonic development in vitro (McElhinny et al., 1996). In the porcine model, melatonin causes increased embryo cleavage rates and blastocyst total cell numbers (Rodriguez-Osorio et al., 2007). No negative effects of melatonin have been observed throughout application of a range of doses from 1pM-100µM (McElhinny et al., 1996) in mice, including application during pregnancy in rats (Jahnke et al., 1999).

II. Melatonin in Seasonal Breeders

As explained above, some animals exhibit seasonal anestrus and as a result are only capable of breeding during certain times of the year. Some ruminants that have been determined to be seasonally reproductive are goat, sheep, and horses (Chemineau et al., 1992; Guerin et al.,
1995). The link between seasonality in reproduction and variation in melatonin has also been studied in these animals in an attempt to uncover a method to overcome seasonality.

In goats and sheep, seasonal anestrus occurs from spring to late summer and the estrous cycle resumes during fall and winter (Blaszczyk et al., 2004). For this reason, they are considered to be short-day breeders (Blaszczyk et al., 2004). Studies examining plasma concentration of melatonin found that the light/dark cycle influenced the circadian melatonin rhythm during both breeding and non-breeding seasons, with high levels of melatonin at night compared to low levels during the day (Chemineau et al., 1992). The duration of melatonin secretion however is what contributes to the effects of photoperiod on reproductive seasonality in goat and sheep (Chemineau et al., 1992). Due to the nightly increases in melatonin secretion, the duration of melatonin secretion in short days is longer than secretion during long days (Chemineau et al., 1992). Studies on pinealectomized sheep exposed to variable duration of melatonin treatments have reproduced the effects of season on reproductive behaviour (Bittman et al., 1983). Sheep lacking the pineal gland have constant levels of melatonin in plasma and as a result cannot differentiate between changing day lengths (Bittman et al., 1983).

Horses are also known to be seasonal breeders but exhibit anestrus during fall and winter and resume sexual activity in spring and summer (Guerin et al., 1995). Horses do exhibit a diurnal rhythm of melatonin secretion, with elevated levels at night and decreased levels during the day (Guerin et al., 1995). Levels of melatonin remain elevated from sunset until sunrise (Guerin et al., 1995). The duration of melatonin secretion rather than the level of melatonin itself differs between seasons (Guerin et al., 1995).

Research on seasonal breeders has established that exposure to short days or long days alone does not contribute to presence or absence of sexual activity (Chemineau et al., 1992). It
has been found that the difference between day lengths allows the animal to resume or
discontinue sexual activity for that period, which is evident from the studies conducted on
pinealectomized goats (Bittman et al., 1983). Long days not only inhibit sexual activity, but they
also allow the animals to become sensitive to short days (Chemineau et al., 1992). As a result,
when short days resume the animal is sensitive to this change in day length and has a
corresponding return to sexual activity (Chemineau et al., 1992).
RATIONALE

Data on water buffalo fertility is mostly restricted to animals raised in tropical environments (Shafie, 1990), thus the present study is the first to examine variation in pregnancy rates and milk progesterone and melatonin profiles caused by changing seasons with the continental climate in Canada. With over 95% of the world buffalo population concentrated in Asia, information regarding the estrous cycle patterns of this species in different climate conditions is scarce (Presicce, 2007). It has been demonstrated that water buffalo show a distinct seasonality in reproduction and photoperiod is a major contributor to this seasonality (Zicarelli, 1997). As melatonin is a key regulator of the circadian rhythm, photoperiodic species rely on changes in melatonin levels to determine the optimal time for reproduction (Chemineau et al., 1992). Reproductive seasonality contributes to variation in milk production throughout the year. The market for water buffalo milk-derived products, first established in Italy, is starting to expand around the globe (Presicce, 2007). As the Canadian market for water buffalo products grows, buffalo farms are being established in the Canadian environment. By studying reproduction parameters of these animals in Canadian climates, we can better understand the conditions in which they can thrive and breed. This knowledge can then be applied to manipulate hormones to overcome milk scarceness in low breeding seasons. The aim of the present study was to better understand the variation pregnancy rates over seasons and develop a non-invasive method of detecting hormone changes that occur between seasons, in the Canadian climate.
HYPOTHESIS AND OBJECTIVES

Pregnancy rates differ over seasons in water buffalo reared in the Canadian climate and progesterone and melatonin profiles in milk reflect the seasonality in reproduction.

To test this hypothesis, the following objectives were addressed:

1. To analyze pregnancy rates over fall, winter, spring, and summer.
2. To determine buffalo milk progesterone profiles during fall, winter, spring, and summer.
3. To analyze variation in buffalo milk melatonin levels between winter and summer.
CHAPTER 1: Seasonal Changes in Pregnancy Rates and Milk Progesterone Profiles of Water Buffalo in Canada

INTRODUCTION

Water buffalo have long been an important agricultural resource in South America, Asia, China, Europe and Australia (Perera, 2011). Of the two subtypes of water buffalo, river and swamp type, river buffalo contribute mainly to meat and milk production (Kumar et al., 2007). The Food and Agricultural Organization estimates that there are 0.18 billion buffalo contributing to agricultural production as of 2008 (FAO, 2008). The milk produced by river buffalo is high in fat and protein content making it appealing for use in dairy products (Senosy and Husein, 2013). As a result, the demand for water buffalo products is beginning to grow around the world and most recently in North America.

Unfortunately, from the production standpoint, water buffalo show low reproductive efficiency in comparison to cattle (Perera, 2011). Several factors contribute to this problem including, silent estrus, seasonal breeding, long postpartum anestrus, and delayed puberty (Perera, 2011). Although water buffalo in heat express behaviours, such as mounting, this is rare and varies between individuals (Drost, 2007). Physical signs such as mucous discharge, vulvar swelling, and bellowing are present but are also much less obvious than in cattle (Singh et al., 2000). Water buffalo also exhibit both delayed puberty and long postpartum anestrus which in turn hinders productivity (Perera, 2011). Seasonal breeding also has a major impact on productivity throughout the year in water buffalo (Singh et al., 2000). This study focuses on analyzing reproductive seasonality in water buffalo in the latitude and climate of Southern
Canada. Specifically, we aim to identify changes in pregnancy rates and milk progesterone profiles in water buffalo over different seasons.

Water buffalo are capable of reproducing throughout the year, but it has been noted that they show distinct seasonality in reproduction (Ghuman et al., 2010). As a result, production of water buffalo products such as dairy and meat fluctuates throughout the year. In countries such as Italy, market demand is highest during spring and summer months, which corresponds to the low breeding seasons for water buffalo and results in economic loss (Presicce, 2007). Research in Asia has shown that buffaloes show seasonal variation in estrus, conception rate, and calving rate (Singh et al., 2000). The observed trend is that increasing daylight hours correspond to decreased fertility in the water buffalo (Singh et al., 2000).

Hormonal analysis can provide insight about the effects of the environment within the body. Progesterone is one such hormone, which can inform us about the status of the corpus luteum as well as pregnancy (Campanile et al., 2013). In ruminants, progesterone has been implicated in the maintenance of pregnancy and levels of progesterone remain elevated from conception until birth (Campanile et al., 2013). In accordance with this, increased progesterone levels represent the presence of a functional corpus luteum and sustained elevated levels have been linked to pregnancy (Campanile et al., 2013). Association of progesterone level and seasonality in water buffalo is controversial (Singh et al., 2000). Some studies looking at progesterone concentration over different seasons found higher concentrations in winter as compared to summer (Rao and Pandey, 1983). While others found the opposite effect (Shafie et al., 1982). As with the data on seasonality, these studies have focussed on buffaloes living in lower latitudes.
Currently, the mechanism by which reproductive seasonality occurs in water buffalo is
unknown, and investigation on reproductive seasonality has been limited to lower latitudes.
Seasonal changes in progesterone levels may help explain the seasonality in reproduction. Water
buffalo are mainly found in tropical and subtropical regions of the world and as a result research
in water buffalo has focussed on this part of the world. Seasonality in reproduction in water
buffalo within newly established Canadian farms is yet to be explored. This study is the first to
address seasonal changes in pregnancy rates and milk progesterone concentrations in water
buffalo in the Canadian climate, specifically in Southern Ontario.

MATERIALS AND METHODS

Analysis of Fertility Data

Animals

This study was conducted over two years, from May 2014- January 2016. All water
buffalo included in this study are housed at the Water Buffalo Company located in Stirling,
Ontario (44.3667 N, 77.5917 W). All buffaloes from which data was collected were in good
general and reproductive health, according to routine veterinary check-ups.

Synchronization of Ovulation

Buffaloes were either allowed to ovulate naturally or were synchronized for ovulation by
implementing one of two synchronization protocols outlined below.

Protocol A

On day 0, animals were administered 2 mg of estradiol benzoate and an intravaginal
progesterone releasing device (CIDR®) containing 1.38 g of progesterone. The progesterone
releasing device was removed on day 9. On the same day, 375 µg of Estrumate® (prostaglandin)
and 780 IU of pregnant mare serum gonadotropin (PMSG) were administered to each buffalo.
On day 10, 50 µg of Cystorelin® (Gonadotropin-releasing hormone) was administered. Artificial insemination was performed 12 hours after Cystorelin® (Gonadotropin-releasing hormone) administration.

Protocol B

Animals were administered 375 µg of Estrumate® (prostaglandin) in the afternoon on Day 0. Artificial insemination was performed three days after Estrumate® (prostaglandin) administration.

Data Collection

Data on the pregnancy rates of each animal was obtained from the Ontario Water Buffalo Company in Stirling, Ontario. Animals that did not respond to the synchronization protocols were not included in the calculation of pregnancy rates. Data from animals was first divided based on synchronization protocol: A (N=73), B (N=83), and natural estrus (N=26). Data from four seasons was compared based on the date of artificial insemination. Inseminations were grouped into Fall (September, October, November), Winter (December, January, February), Spring (March, April, May), and Summer (June, July, August). Pregnancy rates were calculated as the number of pregnancies confirmed after artificial insemination, using ultrasound. Pregnancy rates in Fall/Winter were compared against pregnancy rates in Spring/Summer.

Statistical Analysis

Results are expressed as mean percentages over each season and the SEM represents the variation in percentages between different months. Differences among percentages were assessed by chi-square test. Statistical analysis was performed using IBM SPSS Statistics 23 software (IBM corp., USA).

Progesterone Profile Analysis

Animals
Milk samples for analysis were collected over one year from May 2014 to March 2015. Samples from water buffalo housed at the Water Buffalo Company in Stirling, Ontario were obtained. All buffaloes from which samples were collected were in good general and reproductive health.

Sample Collection

Water Buffalo Company located in Stirling, Ontario, (44.3667 N, 77.5917 W) collected milk every day at approximately 0730h between May 21/2014 - June 11/2014, August 28/2014 - October 16/2014, November 25/2014 - January 13/2015 and February 20/2015 - March 20/2015. Average daylight hours for May 21/2014-June 11/2014, August 28/2014 - October 16/2014, November 25/2014 - January 13/2015 and February 20/2015 - March 20/2015 were 15 hours, 12 hours, 9 hours, and 11.5 hours, respectively. The average temperatures in this region for the time periods May 21/2014-June 11/2014, August 28/2014 - October 16/2014, November 25/2014 - January 13/2015 and February 20/2015 - March 20/2015 were, 17ºC, 16ºC, 0ºC, 2ºC, respectively. Milk samples were provided from five different randomly selected non-pregnant buffaloes for each season (summer, fall, winter, spring). During each round of collection, whole milk was collected in 50 mL tubes, and several 1ml aliquots were prepared as needed in Eppendorf tubes and stored in the freezer at -20ºC until further use.

Progesterone Measurement by ELISA

Progesterone was measured per buffalo from milk samples taken every other day because the stage of estrous cycle in the individual cows was not known at the time of milk collection. Samples were thawed at room temperature (~ 23ºC) and vortexed to homogenize. The Ovuchek Milk Competitive ELISA Kit (Biovet, Quebec City, Canada) was used to determine progesterone concentrations in each sample. In triplicates, hormone quantities in whole milk samples were
measured as per manufacturer’s instructions (Biovet, Quebec City, Canada). The four progesterone standards (1 ng/ml, 5 ng/ml, 10 ng/ml, 20 ng/ml) provided and the samples were loaded (10 µl per well) onto a 96-well plate. 200 µl of the provided conjugate was added to each well and the plate was incubated in the dark at room temperature (~23°C) for 30 minutes. The plate was washed using de-ionized water three times using a multi-channel pipette. 200 µl of the substrate provided was added to each well and the plate was incubated in the dark at room temperature for 30 minutes. 100 µl of the provided stop solution was added to each well. The assay plate was read by an automatic microplate reader (KCjunior, Bio-Tek Instruments, Inc.) at a wavelength of 405 nm that gave optical density values based on sample colour change for each of the standards and samples. The colour change was negatively correlated to the concentration of progesterone in the corresponding sample.

**Calculation of Progesterone Concentration**

Using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, California, 2015) progesterone quantities were calculated using the optical density values generated by the microplate reader. The software generated a standard curve graph using the 1, 5, 10 and 20 ng/mL known standards. From the standard curve graph, hormone concentrations of each of the unknown samples were calculated. Progesterone concentrations are reported in ng/ml.
RESULTS

Effect of Season on Pregnancy Rate

The total inseminations in Protocol A, B, and natural estrus were 73, 83, and 26, respectively. Comparison of pregnancy rates between Spring/Summer and Fall/Winter for buffaloes undergoing Protocol A showed no significant difference in pregnancy rates (Figure 3). The average pregnancy rate in Fall/Winter and Spring/Summer for protocol A were calculated as 50.00±15.94% and 48.83±12.13%, respectively (Figure 3). A Chi-square test showed that there was no significant difference (p=0.888) between the average pregnancy rates for protocol A in Fall/Winter as compared to Spring/Summer (X^2(1, N=87) =0.020).
Figure 3. Comparison of pregnancy rates between Fall/Winter (n=29) and Spring/Summer (n=44) in water buffalo undergoing synchronization protocol A using the progesterone implant (n=73). Data is reported as mean percentage of pregnancies ± standard error of the mean (SEM). Animals that did not respond to the synchronization treatment were not included. No significant differences were observed in pregnancy rates.
Comparison of pregnancy rates over summer/spring and fall/winter showed a significantly lower proportion of pregnancies observed in the summer/spring group compared to the fall/winter group in Protocol B and natural estrus (Figures 4 and 5). The average pregnancy rate in Fall/Winter for protocol B was 64.94±9.09% while the average pregnancy rate in Spring/Summer for protocol B decreased to 21.73±7.67% (Figure 4). Chi-square test revealed that there was a significant difference (p<0.01) between the average pregnancy rates for protocol B in Fall/Winter as compared to Spring/Summer ($\chi^2(1, N=91) = 37.616$).

The average pregnancy rate in Fall/Winter for animals in natural estrus was the highest, at 81.67±10.67%, while the average pregnancy rate in Spring/Summer for animals in natural estrus was 6.67±6.67% (Figure 5). A Chi-square test showed that there was a significant difference (p<0.01) between the average pregnancy rates for animals bred during natural estrus in Fall/Winter as compared to Spring/Summer ($\chi^2(1, N=26) = 113.878$).
Figure 4. Comparison of pregnancy rates between Fall/Winter and Spring/Summer in water buffalo undergoing synchronization protocol B using Estrumate administration alone (n=83). Data is reported as mean percentage of pregnancies ± standard error of the mean (SEM). Animals that did not respond to the synchronization treatment were not included. Significant difference, indicated by the asterisk (*), was observed in pregnancy rates between Fall/Winter (n=47) and Spring/Summer (n=36).
**Figure 5.** Comparison of pregnancy rates between Fall/Winter and Spring/Summer in water buffalo that did not receive any synchronization treatment (n=26). Data is reported as mean percentage of pregnancies ± standard error of the mean (SEM). A significant difference was observed in pregnancy rates between Fall/Winter (n=14) and Spring/Summer (n=12).
Effect of Season on Milk Progesterone Profiles

Fall milk samples showed more stable progesterone levels with a consistent estrous cycle length of 20-22 days among all buffaloes (Figure 6). The peak stability varied from ~5 to 8 days depending on the buffalo with peaks as high as 20 ng/ml. In the winter, stabilized progesterone levels were observed with consistent 10-day elevated levels and 20-day long estrous cycle (Figure 7). As seen in Figure 7, levels increased to 30 ng/ml with progesterone peaks fluctuating around 15-25 ng/mL. Progesterone concentrations in the spring showed no peak as well as no estrous cyclic pattern (Figure 8), where progesterone levels fluctuated rapidly and did not follow any pattern and ranged anywhere between 0-16 ng/ml (Figure 8). Summer months showed erratic changes in progesterone levels with short intervals and no progesterone peak (Figure 9). During the summer months there was no consistent estrous cyclic pattern among all buffaloes tested and progesterone levels remained low, with peaks as low as 1 ng/mL and as high as 8 ng/mL (Figure 9). Average progesterone concentrations for all the buffalo sampled in the fall, winter, spring, and summer are shown in Figure 10. Average progesterone concentrations reached as high as 23.69±1.17 ng/mL in winter, 18.07±0.81 ng/mL in fall, and 9.93±2.29 ng/mL in spring. Summer season progesterone levels were the lowest with the average peak reaching 4.22±0.93 ng/ml.
Figure 6. Milk progesterone levels every other day in the fall season (August 28-October 16), as measured by a competitive ELISA. Typical cycling of progesterone is observed among all buffaloes (n=5) showing day 0 as the lowest observed concentration. Each colour and symbol represents different buffalo.
Figure 7. Milk progesterone profiles every other day during the winter season (November 25-January 13) as measured by a competitive ELISA. Regular cycling of progesterone and stable progesterone peaks are maintained during winter months among all buffaloes (n=5), showing day 0 as the lowest observed concentration. Each colour and symbol represents a different buffalo.
Figure 8. Milk progesterone profiles every other day during the spring season (February 20-March 20) as measured by a competitive ELISA. Rapid fluctuations in progesterone levels with absence of a regular cycle is seen among all buffalo (n=5) during spring months, showing day 0 as the lowest observed concentration. Each colour and symbol represents a different buffalo.
Figure 9. Milk progesterone profiles every other day during the summer season (May 21-June 11) as measured by a competitive ELISA. Abrupt rise and fall in progesterone levels, with absence of regular cycling is seen among all buffalo (n=5) during summer months, showing day 0 as the lowest observed concentration. Each colour and symbol represents a different buffalo.
Figure 10. Average milk progesterone concentrations among all buffaloes examined in summer (May 21 – June 10) (n=5), fall (August 28 – October 14) (n=5), winter (November 25 – January 12) (n=5) and spring (February 20 – March 20) (n=5) months. Each season is represented by a different colour and symbol.
DISCUSSION

There is a large amount of literature available on the effects of season on water buffalo reproduction. However, all of these studies were conducted in the lower latitudes, where the water buffalo originated. This is the first report concerning the effects of season on pregnancy rates and milk progesterone profiles of water buffalo reared in Canada in different seasons. Our data demonstrated seasonal changes in pregnancy rates in water buffalo bred on a Canadian farm in both natural estrus and after using estrus synchronization prior to timed artificial insemination. Increased pregnancy rates were observed in fall and winter as compared to rates in spring and summer. Significantly higher pregnancy rates were observed in fall and winter when buffaloes were synchronized using Estrumate® alone, or inseminated during natural estrus. Interestingly, milk progesterone profiles also showed distinct seasonal differences. Milk progesterone profiles in winter and fall samples showed cyclical rise and fall in progesterone levels. In contrast, spring and summer milk progesterone profiles showed an absence of a cyclic pattern and random peaks. These results indicate that fall and winter are the optimal breeding seasons for water buffalo in Canada.

Reproductive seasonality in water buffalo has been reported in tropical and sub-tropical regions of the world (Singh et al., 2000). As these regions are closer to the equator than Canada, changes in rainfall and nutrition throughout the year affect reproduction more significantly than changing daylight hours (Perera, 2011). Baruselli et al (2001b), studied the effects of different latitudes on conception rates during spring and summer in Brazil (Baruselli et al., 2001b). Conception rates were calculated based on 16,487 births during Spring-Summer according to the different latitudes of Brazil: 44% on 0-8°S; 18% on 8-16°S; 9% on 16-24°S; 7% on 24-32°S (Baruselli et al., 2001b). It is evident that moving further away from the equator has an effect on
reproduction in water buffalo, even with the minor changes in latitude seen in study done by Baruselli et al (2001b).

Studies in sub-tropical regions have shown distinct seasonality in display of estrus pattern, conception rate, and calving rate (Singh et al., 2000). A study in Pakistan found that the breeding frequency in water buffalo was highest in winter, lower in fall and spring, and lowest in the summer (Shah et al., 1989). Studies in India also found consistent results regarding breeding frequency and also found that 64-75% of buffaloes exhibited estrus during September to January (Singh and Nanda, 1993). In Punjab, suppression of reproductive function has been observed in the summer, characterized by shorter duration of estrus, longer time from estrus to ovulation, and fewer ovulatory cycles (Singh et al., 2000). A study in India, looking at the incidence of anestrus noted 78% anestrus buffaloes in July (summer) as compared to 14% in November (winter) (Singh et al., 1989). Although the studies described are restricted to lower latitudes (20-30°N) in comparison to Canada (~44°N), the results of this study support what has been found previously regarding seasonality, showing increased pregnancy rates in winter as compared to summer.

Studies in temperate regions, such as Italy, have also reported distinct seasonality in buffalo reproduction (Perera, 2011). In these regions, nutrition is constant throughout the year and season is primarily affected by changing day length (Perera, 2011). Studies in Italy report highest reproductive efficiency during September to October (Campanile et al., 2013). Being further from the equator, there is a greater variation in day length over the year which is similar to that seen in Canada. These current study supports the results found previously, as higher pregnancy rates were observed in fall and winter, which are characterized by decreasing daylight hours. Research in water buffalo reproduction at latitudes higher than Italy (40.5-41.5N) has been scarce (Campanile et al., 2013). This study analyzed seasonal variation in pregnancy rates...
and milk progesterone profiles in water buffalo exposed to the Canadian environment (~44N) for the first time. Our data supports previous studies and indicates that seasonality is preserved from the domesticated regions.

Progesterone is known to be a reliable indicator of pregnancy, stage of the reproductive cycle, and ovarian function (Qureshi et al., 2000). Fluctuations in progesterone concentrations in buffalo are similar to that of cattle but average levels are known to be lower in water buffalo (Perera, 2011). In a cycling buffalo, levels of progesterone rise and fall with the formation and regression of the corpus luteum (Mondal et al., 2007). Progesterone concentrations are lowest around 3 days before estrus and remain low until after ovulation (Mondal et al., 2007). Levels of progesterone continue to rise after ovulation and reach their peak around day 13 to 15 (Mondal et al., 2007). In a cycling buffalo, progesterone levels will begin to decline if pregnancy does not occur and reach basal levels 3 days before the next estrus (Mondal et al., 2007). Progesterone profiles in winter and fall in this study are consistent with this typical cycling pattern described in the results. In contrast, spring and summer samples show atypical profiles without peaks or troughs lasting for the expected amount of time. Milk progesterone profiling has been identified as an accurate method of assessing typical and atypical ovarian function (Qureshi et al., 2000).

Progesterone has been implicated in the maintenance of pregnancy (Tamura et al., 2009). As progesterone is produced by the corpus luteum and by the placenta in pregnancy it can be used as a measure for ovarian status (Campanile et al., 2013). In later stages of the pregnancy the placenta will begin to produce progesterone and contribute to increased levels within the animal (Campanile et al., 2013). In the context of reproductive physiology, milk progesterone profile can be used as a non invasive tool to assess ovarian function and pregnancy status in water buffalo. As this was a blinded study the pregnancy status of the buffaloes was not known during
milk progesterone measurement. Interestingly, the milk progesterone concentrations for one buffalo (#63) remained elevated for greater than the typical 10 days. After referring to the farm records, this buffalo (#63) was confirmed to be pregnant during the sampling period. None of the other buffaloes showed elevated progesterone for longer than 10 days and were subsequently confirmed as not pregnant. As indicated in literature, elevated progesterone levels for longer than the typical 10 days indicates pregnancy in water buffalo (Banu et al., 2012). Though only one pregnant buffalo was sampled, this finding highlights the sensitivity of this test for detecting pregnancy in water buffalo.

There have been varying results found in terms of the seasonality of progesterone profiles within water buffalo (Singh et al., 2000). Research on progesterone levels in hot months compared to cool months has shown significant differences in the progesterone levels. The result of such studies they have found that progesterone levels at estrus and the mid-luteal phase are much lower in the hotter months as compared to the cooler months (Rao and Pandey, 1983). Still others have found that progesterone concentrations are significantly higher in summer compared to winter months (Mondal et al., 2007).

In cattle, it has been found that low levels of progesterone during the pre-ovulatory period can have negative effects on several reproductive processes (Carvahlo et al., 2014). These processes are associated with oocyte quality and establishment of pregnancy as they affect uterine morphology and hormone secretions (Carvahlo et al., 2014). Premature regression of the corpus luteum can occur as a result low levels of progesterone (Carvahlo et al., 2014). The current study shows that average progesterone concentrations are greater in winter and fall months as compared to spring and summer months. The reduced levels of progesterone in spring and summer may be indicative of a dysfunctional corpus luteum. The low levels of progesterone
may be insufficient to maintain pregnancies, leading to the observed reduction in pregnancy rates in the spring and summer seasons.

The milk progesterone profiles observed in this study show higher concentrations of progesterone than seen in literature. This may be due to the use of whole milk in our study. Previous studies measuring progesterone concentrations have mainly used plasma progesterone concentrations. Studies examining milk progesterone report defatting the milk prior to measuring progesterone concentrations (Qureshi et al., 2000). Progesterone is a steroid hormone and is more likely to associate with the lipid content within milk samples. As a result, the whole milk samples used in this study contribute to the higher overall concentration in progesterone observed. Although milk progesterone levels are higher than levels in plasma, milk progesterone level is a reliable non-invasive indicator of ovarian function (Qureshi et al., 2000). In previous studies the average concentration of progesterone in buffalo milk was found to be 0.5 ng/ml at estrus, which increased to 18 ng/ml on day 15 (Mondal et al., 2007). Therefore, using whole milk is a convenient and non-invasive technique for evaluating ovarian function in water buffalo. This may prove to be a convenient and practical method for use in the field.

The findings of this study show that both fertility and progesterone profiles are affected by season in water buffalo. The typical progesterone profile, indicating the presence of a functional corpus luteum corresponds to winter and fall, along with a higher pregnancy rate. Pregnancy is supported by continued progesterone production by the CL and later the placenta (Campanile et al., 2013) as we observed with samples obtained from the pregnant buffalo in this study. Seasonality in reproduction in water buffalo may be due to the differential production of progesterone throughout the year and many other endocrine factors may change due to environmental stimuli.
Seasonality in reproduction in water buffalo affects the productivity of the water buffalo industry. The gestation period for water buffaloes is approximately 10 months (Patil et al., 2014) and when breeding is restricted to a particular time during the year, such as October-December, calving will occur August-October. The peak demand for buffalo-derived products occurs during the spring and summer seasons which is well before the time of calving indicated above (Presicce, 2007). This results in differential production of water buffalo milk throughout the year. This study can help the Canadian buffalo industry to gain better insight into the newly introduced livestock. Further investigation will assist in optimizing the productivity of the buffalo farms and to overcome the seasonal effect by implementing reproductive manipulation.

The objective of this study was to analyze the variation in pregnancy rates and buffalo milk progesterone profiles in water buffalo housed in the Canadian climate. The findings suggest that the breeding season for water buffalo in Canada includes fall and winter (September-February). According to the results, the low breeding season for water buffalo in Canada occurs during spring and summer (March-August). Water buffalo milk progesterone profiles show the anticipated pattern in concentration during winter and fall months and irregular progesterone profiles were observed during spring and summer months. Further research is necessary to understand the connection between the variation in progesterone profiles and pregnancy rates in water buffalo between winter and summer months in Canada.
CHAPTER 2: Analysis of Seasonal Variations in Buffalo Milk Melatonin Concentrations of Water Buffalo in Canada

INTRODUCTION

Variation in photoperiod is relayed to an organism’s body through changes in levels of melatonin (Bronson, 2009). This is an important environmental cue that allows the animal to prepare for the changing environment and in turn the changing availability of resources (Lacasse et al., 2014). In photoperiodic species, the circadian rhythm dictated by melatonin is important for the synchronization of the reproductive response to favorable environmental conditions (Bronson, 2009). Though changing day length is not as important near the equator, at higher latitudes variation in day length is much more significant and has a larger impact on the animal’s environment (Bronson, 2009).

It was mentioned that buffaloes are polyestrous and capable of breeding throughout the year, but they show distinct seasonality in their reproductive behaviour. It has been found that this fluctuating reproductive efficiency is not necessarily dependent on food availability, diet, or metabolic status (Barile, 2005). In fact, climate and photoperiod are known to have a greater impact on the reproduction of water buffalo throughout the year (Barile, 2005). This is further substantiated by the fact that breeding seasons are reversed in opposite hemispheres (Barile, 2005). This is important because day length increases in one hemisphere as it decreases in the other. Looking at these findings in conjunction, it seems that changing day length has an impact on reproductive efficiency in buffalo. To understand how changes in photoperiod are reflected in the changes in reproduction, variations in melatonin over summer and winter were analyzed in this study.

Melatonin is a neuro-hormone synthesized from tryptophan and secreted from the pineal gland (Aparicio et al., 2007). Serotonin is the precursor of melatonin and during light hours, the
pineal gland secretes serotonin (Asher et al., 2015). In the dark, serotonin within the pineal gland is metabolized to melatonin (Asher et al., 2015). As a result, levels of melatonin rise to peak concentrations in the dark and fall to minimal levels during daylight hours (Asher et al., 2015). Melatonin is known to be involved in the synchronization of the circadian rhythm (Fernando and Rombauts, 2014) and the duration of melatonin secretion is inversely proportional to the day length (Revel et al., 2009). In this way, melatonin relays information about changing photoperiod in the environment to the body.

The other major function of melatonin is as an antioxidant, as it is a very potent free radical scavenger (Fernando and Rombauts, 2014). Melatonin is known to be a broad spectrum anti-oxidant important in reducing oxidative stress within an organism (Tamura et al., 2009). In addition, the balance between reactive oxygen species production and scavenging capabilities within the organism are important for oocyte maturation and fertilization (Tamura et al., 2009). High concentrations of melatonin have been found in human follicular fluid and melatonin receptors have also been identified on granulosa cells (Tamura et al., 2009), suggesting that melatonin may function to protect the oocyte from the detrimental effects of ROS.

The effect of photoperiod on reproductive seasonality in water buffalo coupled with the relationship between melatonin and reproduction, leads to the question of how levels of melatonin in water buffalo may change over the seasons. Thus, this study was designed to determine the variation in melatonin levels in water buffalo milk between winter and summer seasons in Canada.
MATERIALS AND METHODS

Melatonin Analysis

Animals

Milk samples were collected once per week for four weeks in both summer and winter seasons from May 2014 to June 2014 and December 2015. Milk samples were obtained from water buffalo housed at the Water Buffalo Company in Stirling, Ontario. All buffaloes from which samples were collected were in good general and reproductive health.

Sample Collection

Water Buffalo Company in Stirling, Ontario, (44.3667 N, 77.5917 W) collected milk once per week between May 21/2014 - June 11/2014 and December 9/2015-December 30/2015. Average daylight hours for May 21/2014-June 11/2014 and December 9/2015-Dec 30/2015 were 15 hours and 9 hours, respectively. The average temperatures in this region for the time periods May 21/2014-June 11/2014 and December 9/2015-Dec 30/2015 were, 17ºC and -4ºC, respectively. Milk samples were provided from four different buffaloes for each season (summer and winter). All milk samples were collected at approximately 0730h in both summer and winter. Milk samples collected for comparison of melatonin during different times of the day were collected from one buffalo during the winter season at 0730h and 1630h. During each round of collection, whole milk was collected in 50 mL tubes, and several 1ml aliquots were prepared as needed in Eppendorf tubes and stored in the freezer at -20ºC until further use.

Melatonin Measurement by ELISA

Milk melatonin was measured per buffalo from milk samples taken once per week in summer (May 21/2014-June 11/2014) and winter (December 9/2015-December 30/2015). Samples were thawed at room temperature (~ 23°C) and vortexed to homogenize. The Salimetrics Competitive ELISA Kit (Salimetrics LLC, Pennsylvania, USA) was used to
determine melatonin concentrations in each sample. In duplicates, hormone quantities in whole milk samples were measured as per manufacturer’s instructions (Salimetrics LLC, Pennsylvania, USA). Standards (25 pg/ml, 12.5 pg/ml, 6.25 pg/ml, 3.13 pg/ml, 1.56 pg/ml, and 0.78 pg/ml) were prepared by serial dilutions using the assay diluent provided starting with the 50 pg/ml melatonin standard provided. 100 µL of each standard and sample was added in duplicate to a 96-well plate. The enzyme conjugate provided was diluted 1:500 using the assay diluent and 50 µL of the diluted conjugate was added to each well. The plate was placed on a plate rotator at 500 rpm for 3 hours at 4°C. The plate was washed four times with de-ionized water using a multi-channel pipette. 100 µL of the substrate provided was added to each well and the plate was placed on a plate rotator at 500 rpm in the dark at room temperature (~23°C) for 30 minutes. 50µL of the provided stop solution was added to each well. The assay plate was read by an automatic microplate reader (KCjunior, Bio-Tek Instruments, Inc.) at a wavelength of 450 nm that gave optical density values based on sample colour change for each of the standards and samples. The colour change was negatively correlated to the concentration of melatonin in the corresponding sample.

Calculation of Melatonin Concentration

Using MyAssays Ltd. online analysis tool, melatonin quantities were calculated using the optical density values generated by the microplate reader. The software generated a standard curve graph using the 0, 0.78, 1.56, 3.13, 6.25, 12.5, 25 and 50 pg/mL known standards. From the standard curve graph, hormone concentrations (pg/ml) of each of the unknown samples were calculated.

Statistical Analysis
Differences among average milk melatonin between summer and winter were assessed by independent samples t-test. Statistical analysis was performed using IBM SPSS Statistics 23 software (IBM corp., USA).
RESULTS

Melatonin concentrations in milk samples collected in the summer (May 21/2014-June 11/2014) range from 5.361-15.65 pg/ml (Figure 11). A trend of increasing melatonin over the four-week period in summer was observed for all four buffalo sampled (Figure 11). Buffalo #562 had the highest milk melatonin out of all four buffaloes sampled, throughout the four-week period (Figure 11).
Figure 11. Milk melatonin concentrations of four randomly selected water buffalo over four weeks during the summer season (May 21/2014-June 11/2014), as measured by a competitive ELISA.
Melatonin concentrations in milk samples collected in the winter (December 9/2015-December 30/2015) ranged between 2.856-13.37 pg/ml (Figure 12). Milk melatonin levels showed a declining trend over the four-week period in winter for all four buffaloes sampled (Figure 12). Three (#60, #802, and #562) out of the four buffaloes showed a dip in melatonin levels on December 22/2015, before rising again on December 30/2015 (Figure 12).
Figure 12. Milk melatonin concentrations of four randomly selected water buffalo over four weeks during the winter season (December 9/2015-December 30/2015), as measured by a competitive ELISA.
Average milk melatonin concentrations in summer and winter, for each of the four randomly selected water buffalo showed increased levels of melatonin in summer samples as compared to winter in three out of the four buffaloes (#547, #562, and #802) (Figure 13). Using the independent samples t-test, it was determined that there was no significant difference between summer and winter milk melatonin (Figure 13).
Figure 13. Average milk melatonin concentrations for each of the four randomly selected water buffaloes for four time points in summer (May 21/2014-June 11/2014) and winter (December 9/2015-December 30/2015), as measured by a competitive ELISA. Independent t-test revealed no significant difference (p>0.05) between summer and winter melatonin levels for all four buffalo sampled.
Variation in milk melatonin between morning and afternoon milking was also determined for milk samples obtained in the winter season (December 16- December 30). Milk samples were obtained from one buffalo during the two milking times (0730h and 1630h) of the day. No consistent trend was found between morning (AM) and afternoon (PM) milk melatonin (Figure 14).
Figure 14. Milk melatonin concentration from morning milking (0730-AM) and evening milking (1630-PM) for one buffalo (#63) in the winter, as measure by a competitive ELISA.
DISCUSSION

This is the first study to look at variations in melatonin levels in buffalo milk from cycling water buffalo reared in the Canadian climate (~44°N). This study examined the variation in milk melatonin in summer and winter, as well as between morning and afternoon milking in the month of December. Average milk melatonin was measured for a four-week period in the summer (May-June) and winter (December). No significant difference (p > 0.05) in melatonin concentrations between the seasons were found, but a trend of higher melatonin levels in the summer was observed. In addition, no consistent trends were observed between melatonin levels in morning and afternoon milk in December.

Though there have been many studies addressing the issue of reproductive seasonality in water buffalo at low latitudes (≤41°N), the link between changing seasonality and changing photoperiod still remains to be found. As melatonin is the key hormone implicated in signalling an organism of photoperiodic changes in the environment (Revel et al., 2009), we were curious to see how levels of melatonin changed in water buffalo between summer (long days) and winter (short days). It is known that water buffalo exhibit a diurnal melatonin rhythm (Parmeggiani et al., 1992), which is why we chose to look at how melatonin changes with large changes in day length between seasons.

Not only does melatonin change with day length, which addresses seasonality, but melatonin is also known to interact with parameters of reproduction. For instance, data on melatonin changes corresponding to the menstrual cycling show that melatonin levels peak at the luteal phase and remain low during the preovulatory phase (Tang et al., 1998). This indicates that melatonin may have varying effects depending on the stage of the reproductive cycle (Tang et al., 1998). Another study done in humans, associating melatonin and reproduction, observed the
dysregulation of the menstrual cycle in shift workers (Attarchi et al., 2013). It is no surprise that shift workers experience a disruption in their circadian rhythm, but it is interesting to note that deviation also has an impact on their menstrual cycle (Attarchi et al., 2013). Once again, melatonin seems to play a pivotal role in regulating reproduction in addition to the circadian rhythm.

Studies conducted in climate regions similar to Canada, such as Italy (lat. ~40°N), have found variations in serum melatonin levels between seasons (Parmeggiani et al., 1992). These studies have confirmed that a diurnal rhythm of melatonin exists within water buffalo (Parmeggiani et al., 1992). It has been found that levels of melatonin increase dramatically during nighttime and remain low during the day (Parmeggiani et al., 1992), however, in this study, melatonin levels measured between morning and afternoon milk did not show a consistent pattern. This may be due to the fact that nighttime melatonin in previous studies was measured a few hours after sunset, while afternoon milk in this study was collected at 5 pm (Parmeggiani et al., 1992). At a latitude of 44°N during December 2015, sunset would have occurred around 4:30 pm which may not have left sufficient time for the melatonin levels to rise and enter the milk prior to milk collection.

As the day length changes, the persistence of these high levels of nighttime melatonin changes as well (Parmeggiani et al., 1992). As expected, longer nights result in longer periods of increased melatonin secretion (Parmeggiani et al., 1992). Night levels of melatonin have also been compared between summer and winter. Highest night time levels of melatonin have been recorded in December, lower levels from March-April, and lowest in June (Parmeggiani et al., 1992). Average melatonin levels between summer and winter in this study did not differ significantly. Studies looking at seasonal differences in melatonin compared nighttime serum
melatonin levels and our study compared morning milk melatonin levels. Comparing morning milk samples as opposed to night milk samples may have contributed to the lack of difference seen between summer and winter melatonin levels. As it has been shown that melatonin levels begin to rise shortly after sunset and fall once again after sunrise, the levels detected in our study may only indicate basal melatonin levels.

Although differential melatonin concentrations have been found between seasons, this finding has not been consistent across all water buffalo. One study analyzed the variation in melatonin between buffalo that showed distinct reproductive seasonality compared to those that did not and found that significant increases in night time melatonin only occurred in water buffalo that showed seasonality in reproduction (Parmeggiani et al., 1993). Buffaloes that were reproductively active consistently throughout the year did not show the significant increase in night time melatonin (Parmeggiani et al., 1993). According to the results of the current study, the consistent levels of melatonin may point to reduced seasonality in the buffaloes sampled.

In true seasonally polyestrous animals, such as horses, sheep, and goats, melatonin has been studied to help shift the breeding season (Guerin et al., 1995; Chemineau et al., 1992). In these animals, there is a period of the year in which they are anestrous, meaning they are unable to reproduce. Estrous is resumed at the beginning of the breeding season. In order to minimize economic loss brought on by the period of reproductive inactivity, researchers have tried to use melatonin to overcome anestrous. In sheep and goats, administering melatonin in a way that mimics long and short day profiles causes a change in reproduction that corresponds to the photoperiod (Chemineau et al., 1992). In horses, the breeding season in the northern hemisphere extends from April to September indicating that horses become reproductively active as the days grow longer (Murphy et al., 2014). Again, altering light exposure can change the reproductive
activity in this seasonal breeder and has become standard practice (Murphy et al., 2014). Through the use of light masks, studies have shown that the breeding season in horses can be shifted by exposing animals to light for longer periods (Murphy et al., 2014). Understanding the link between melatonin and reproductive seasonality in water buffalo may help alleviate the economic burden brought on by reduced reproductive efficiency during spring and summer.

Though little work has been performed on levels of melatonin in water buffalo milk, hormone profiles in milk have been used as a less invasive approach in comparison to plasma. One of the factors that may have influenced the lack of difference between melatonin concentrations in morning and afternoon could be the use of milk. To compare differences in hormone profiles in milk and plasma, studies looking at progesterone, estradiol-17β, and prolactin levels in plasma and milk from water buffalo have been conducted (Pahwa and Pandey, 1983). Research has shown that milk and plasma progesterone profiles are parallel except that the concentrations are 2-4 times higher in milk (Pahwa and Pandey, 1983). The profiles of estradiol-17β and prolactin, however, vary depending on time of collection relative to the calving (Pahwa and Pandey, 1983). The relationship between melatonin levels in plasma and milk have been explored in cows and also show a variation in melatonin depending on time from calving and analysis of melatonin during the beginning of lactation in May did not show a correlation between plasma and milk melatonin levels (Eriksson et al., 1997). However, there was a correlation seen in plasma and milk melatonin levels during a later stage of a lactation period in February (Eriksson et al., 1997). The results from the study in cows showed that milk melatonin levels in cattle reflect blood concentrations of melatonin, although there is a slight delay in timing from plasma to milk (Eriksson et al., 1997). As we did not have control over which buffalo we received samples from, the time of calving might have been different and affected the
levels of melatonin in the milk collected. This could explain why there was no difference observed between melatonin levels in summer and winter samples. We were also unable to see a difference in milk melatonin levels between morning and afternoon milk samples. As the samples in the afternoon were collected within 30 minutes after sunset, the rise in plasma melatonin may not be reflected in milk that early. As seen in cattle, buffalo milk melatonin levels may also show a delay in reflecting levels of melatonin from plasma in water buffalo.

Individual differences in seasonality need to be considered when analyzing melatonin variations in the buffaloes in this study. As discussed above, the buffalo in this study may not be seasonal and, as a result, the melatonin levels do not show any distinct differences between seasons or time of day. These findings may help to explain the lack of pattern seen between seasons in melatonin levels measured in this study. To strengthen the findings in this study, milk samples collected 2 hours after sunset should be analyzed to determine whether a diurnal melatonin rhythm can be detected. In addition, the sample size for the average melatonin levels during summer and winter was quite small (n=4). This may contribute to the discrepancies seen between our results showing no differences and the seasonal variation reported in literature.
GENERAL DISCUSSION

The buffalo farm industry is very new to Canada and the buffalo dairy and meat product market is growing. Currently there is no research on the breeding of water buffalo in the Canadian environment for buffalo farmers to refer to and importing these animals is very expensive. Fundamental research is needed to characterize water buffalo reproduction in the Canadian climate. This study is the first of its kind to initiate a physiological based analysis to gain better insight into the reproduction and breeding of this species in a novel environment. We designed a research plan to test seasonality of reproduction in water buffalo reared in Canada. In order to do this in a simple manner, we analyzed the pregnancy rate, ovarian function and seasonality related hormones using a non-invasive method. Milk progesterone profile was chosen to evaluate estrous cycle pattern and melatonin was chosen to determine the effects of photoperiod.

The main objective of this thesis was to determine the effects of the different seasons in the Canadian climate on reproduction in water buffalo. Although many studies have identified the role that changing photoperiod plays on reproduction in water buffalo in latitudes closer to the equator (Baruselli et al., 2001b; Campanile et al., 2013; Shah et al., 1990), this topic has not been explored in the Canadian environment (~44°N). By calculating pregnancy rates and measuring the variation in milk progesterone concentrations over different seasons, the reproductive pattern of water buffalo in Canada has been identified.

Seasonal reproduction in animals has evolved over time, in order to optimize survival of offspring at the time of birth (Bronson, 2009). Depending on habitat and availability of resources, parturition during a limited period during the year (when resources are optimal) gives animals the best chance of survival (Bronson, 2009). As a result, animals can fall into one of
many reproductive groups, seasonally polyestrous, seasonally monoestrous, polyestrous, or monoestrous (Austin and Short, 1984). Water buffalo are polyestrous and capable of reproducing throughout the year, but they are known to show distinct seasonality in reproduction (Singh et al., 2000). Though they are not strictly seasonal, meaning anestrous is not definite, reproductive efficiency is known to decline during long days (Drost, 2007). The pregnancy rates calculated in this study are consistent with the literature, showing lower pregnancy rates in the spring and summer seasons (long days) as compared to fall and winter seasons (short days).

Pregnancy rates after synchronization as well as after natural estrus showed a difference between winter and summer. The protocol the progesterone insert did not show a significant difference in pregnancy rates between seasons but the alternate protocol using prostaglandin alone as well as natural estrus both showed significant differences in pregnancy rates between summer and winter. Greater pregnancy rates were observed in winter months as compared to summer months, which was consistent with the findings of the progesterone profiles. After seeing the distinct differences in pregnancy rates between seasons, we decided to look at variation in progesterone between seasons. It was evident that even progesterone profiles showed a distinct seasonality, with typical cycles observed in winter and fall and abnormal cycles observed in spring and summer. This also explains why there was no difference observed in pregnancy rates when using the progesterone releasing device. The progesterone profiles indicate that summer levels of progesterone are insufficient to maintain pregnancy. Correcting for this using the progesterone releasing device, completely removes the difference in pregnancy rates seen between seasons. Observing the progesterone profiles in conjunction with the pregnancy rates, it can be concluded that optimal breeding seasons in Canada are fall and winter, while low breeding seasons are spring and summer.
As a classic example of a seasonally polyestrous animal, the effect of season on the reproductive performance in sheep has been extensively studied. Sheep exhibit anestrous during summer months and resume estrous as the days become shorter (Chemineau et al., 1992). As melatonin is known to mediate the effects of changing photoperiod within seasonal animals, studies on the effects of melatonin administration in sheep have been performed (Chemineau et al., 1992). These studies have been performed in pinealectomized sheep in order to understand how changing the melatonin rhythm affects reproduction (Chemineau et al., 1992). Mimicking long and short day profiles of melatonin in these animals replicates the effects of photoperiod on reproduction (Abecia et al., 2012). Application of melatonin in non-breeding seasons for sheep has been useful in alleviating the economic burden of reproductive seasonality (Abecia et al., 2012). Though water buffalo are not strictly seasonal breeders, knowledge of how melatonin affects reproduction in water buffalo may help to determine whether melatonin can be administered during the low breeding season to increase reproductive efficiency.

In an attempt to understand the variation in melatonin in relation to reproductive seasonality in water buffalo, a study in Italy compared melatonin levels in seasonal and non-seasonal water buffalo (Parmeggiani et al., 1993). Though a diurnal rhythm was confirmed, melatonin levels increased significantly at night in seasonal buffalo, while the change was not significant in non-seasonal buffalo (Parmeggiani et al., 1993). In the current study, melatonin showed no distinct pattern in summer or winter months or time of day. According to the results from the Italian study, the animals sampled in this study were not seasonal (Parmeggiani et al., 1993).

The interaction of melatonin and progesterone has also been studied in sheep to understand the mechanism behind the effects of melatonin on reproduction. Melatonin
administration in sheep has been used to advance the breeding season (Ghuman et al., 2010). Melatonin acts on the hypothalamus to stimulate GnRH and subsequently LH release to exert its effects on ovarian activity (Ghuman et al., 2010). The response time to melatonin differs depending on whether it is administered in the breeding or the non-breeding season. In the non-breeding season, GnRH release from the hypothalamus is under strong negative feedback via estradiol, which leads to a delay of about 40-60 days for the administered melatonin to take effect (Ghuman et al., 2010). In the breeding season, administered melatonin takes effect in a few hours because the hypothalamus is no longer under the negative feedback control of estradiol (Ghuman et al., 2010).

Melatonin is known to exert its effects on the levels of GnRH and gonadotropins in water buffalo by acting on the hypothalamus and pituitary gland (Ramadan et al., 2014). Research on melatonin in water buffalo has shown that melatonin has differential effects depending on whether or not the buffalo is cycling (Ramadan et al., 2014). In a cycling buffalo, the presence of estradiol causes melatonin to play a stimulatory role in the release of LH (Ramadan et al., 2014). In the summer, estradiol concentrations are lower and this causes melatonin to play an inhibitory role in LH secretion (Ramadan et al., 2014). The variable effect that melatonin can have on LH secretion impacts ovulation as well as progesterone secretion by the CL (Ramadan et al., 2014). This may explain the lack of pattern that was observed between melatonin and progesterone in buffaloes sampled in the summer months in this study (See Appendix I-Figure 17).

To understand the role of changing photoperiod on reproduction, we combined the results from Chapter 1, regarding progesterone, and Chapter 2, regarding melatonin to examine variations in melatonin and progesterone levels over summer and winter in Canada. There was no significant relationship between changing seasons and level of melatonin observed. To look at
the effect of season on both the hormones studied, a comparison of melatonin and progesterone concentrations in milk samples was also performed (See Appendix I-Figure 15, 16, 17, and 18).

Average concentrations of progesterone and melatonin showed similar patterns in the individual buffalo sampled in the summer, remaining fairly constant (See Appendix I-Figure 15), while a trend of an inverse relationship of progesterone and melatonin in the individual buffalo during the winter was observed (See Appendix I-Figure 16).

When comparing individual buffalo at the various time points, there did seem to be a trend between variations in melatonin and variations in progesterone within individual buffalo for winter (See Appendix I-Figure 18) but not for summer (See Appendix I-Figure 17). In two out of the three buffalo sampled in the winter (#33 and #63), the levels of melatonin and progesterone showed some association, with increasing melatonin corresponding to increasing progesterone (See Appendix I-Figure 18). This is consistent with studies that have shown a positive correlation between follicular progesterone and melatonin concentrations (Tamura et al., 2009). Research on melatonin in water buffalo has also shown that, in cycling buffalo, melatonin has a stimulatory effect on LH secretion, which in turn stimulates progesterone production by the CL (Ramadan et al., 2014). The remaining three out of five buffalo (#34, #62, and #65) did not show any specific trend between variation in melatonin and progesterone concentrations (See Appendix I-Figure 18). Overall, there was a lack of pattern in melatonin secretion between winter and summer, which suggests that the buffaloes in this study are not seasonally reproductive.

In order to determine whether melatonin levels changed with stage of estrous cycle, melatonin variation was compared to the variation in progesterone in the same buffaloes on the same days during four-week periods in summer and winter. No distinct increasing or decreasing
trend was observed in either progesterone or melatonin over the four time points analyzed (See Appendix I-Figure 17 and 18). The average melatonin and progesterone concentrations between buffaloes during winter and summer were also compared. Average melatonin concentrations increased with average progesterone concentrations in the summer (See Appendix I-Figure 15), while no trend was seen in winter samples (See Appendix I-Figure 16). As discussed above, the effect of melatonin on the ovarian function of the buffaloes studied may vary depending on their sensitivity to changing day length. In addition, the buffaloes that showed a synergistic trend between progesterone and melatonin during the winter confirm the findings from previous studies indicating that melatonin stimulates progesterone production in cycling buffalo.

The absence of pattern in melatonin levels as well as the apparent lack of association between melatonin and progesterone may have to do with the origin of the buffaloes that were used in this study. The buffaloes used in this study originated from Italian fathers via artificial insemination. It is known that over the years Italian buffalo farmers have adopted the OBSM (out of breeding season mating) technique to minimize reproductive seasonality in the herd (Presicce, 2007). The selection of less seasonal buffalo may have an impact on the seasonality seen in the buffalo in this study.

To better understand the impact of season on melatonin levels, individual buffalo would need to be monitored for both pregnancy rates as well as melatonin concentrations. This would help to identify which specific animals exhibit reproductive seasonality and whether this has an impact on the variation in melatonin levels observed. Additionally, optimization of the melatonin ELISA for buffalo milk would be beneficial in increasing the precision of measuring levels of melatonin. The mechanism for action of melatonin on the HPG axis in water buffalo is yet to be determined. Future research to elucidate the impact of melatonin in water buffalo reproduction
can help to determine whether melatonin can be used as a tool to alleviate reproductive seasonality.

Silent estrus and seasonal reproduction are major contributors to economic loss within the buffalo dairy industry. Inefficient estrus detection causes unsuccessful breeding due to incorrect timing of artificial insemination. The variation in fertility in water buffalo throughout the year also causes a fluctuation in production of buffalo products. The results of this study show that water buffalo in Canada do exhibit seasonality in reproduction. From the results of the pregnancy rates and progesterone data, it can be seen that fall and winter are the breeding seasons while spring and summer are the low breeding seasons. The melatonin results do not show a definite pattern between seasons and indicate that these buffalo are not seasonally reproductive. Use of melatonin or progesterone administration to shift the breeding season may be used following further research.
SUMMARY AND CONCLUSIONS

Overall, the pregnancy rates calculated for the water buffalo in this study show that the optimal breeding seasons in Canada are fall and winter while the low breeding seasons are spring and summer. The milk progesterone profiles show a typical cycle during fall and winter seasons with average cycle length of approximately 20 days. In the spring and summer seasons, however, the milk progesterone profiles show abrupt rise and fall in progesterone levels with no stability in peaks or regularity in cycle. This indicates a dysfunction in the corpus luteum in spring and summer, as progesterone is secreted by this transient structure. Melatonin data is inconclusive in terms of determining seasonality in the buffalo in this study. No significant trends were observed in melatonin levels between winter and summer or morning and afternoon milking. Additionally, no significant association between progesterone and melatonin levels was observed.

As the market demand for buffalo milk is at its maximum during spring and summer, it would be beneficial to use the information gained from this study to adjust the breeding season in Canada accordingly. Synchronization protocols using progesterone could be used in the summer rather than winter in order to optimize on natural estrus during breeding seasons, while intervening when natural progesterone secretion is deficient. Monitoring progesterone profiles in the milk of individual buffalo can also be used as a non-invasive technique to determine whether synchronization intervention is necessary for that animal. This would cut down on costs by reducing unnecessary application of synchronization when a buffalo is already cycling and coming into heat on her own.

Future studies on melatonin in water buffalo in Canada need to be conducted in order to determine the trend in melatonin between different seasons. Knowing that intrinsic seasonality in the individual buffalo can affect the difference in melatonin pattern, indicates that studies need to
group buffalo with prior knowledge of seasonality. Using the origin of the buffalo may help in determining seasonality. It is known that today, a majority of buffalo from Italy have been bred using the out-of-breeding-season mating technique which, over the years, has selected for buffalo that are less sensitive to changing seasons. Additionally, this technique may be applied to buffalo herds in Canada that exhibit seasonality in reproduction. A longitudinal study looking at the change in seasonality over the years after applying this technique may also determine the genetic influence of reproductive seasonality. Further research on the link between melatonin and progesterone in water buffalo needs to be conducted in order to determine the association. This may also help elucidate the mechanism through which melatonin affects reproductive seasonality in water buffalo.
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Figure 15. Comparison of average melatonin and average progesterone concentrations in buffaloes (n=4) during the summer season (May-June 2014) as measured by competitive ELISA. Different colours represent levels of melatonin (green) and levels of progesterone (blue).
Figure 16. Comparison of average melatonin and average progesterone concentrations in buffaloes (n=5) during the winter season (December 2015), as measured by competitive ELISA. The different colours represent levels of melatonin (green) and levels of progesterone (blue).
Figure 17. Milk melatonin and progesterone variations in summer for four randomly selected water buffalo as measured by competitive ELISA. Different colours represent the three time points in summer when samples were collected.
Figure 18. Milk melatonin and progesterone variations in winter for five randomly selected water buffalo as measured by competitive ELISA. Different colours represent the three time points in winter when the milk samples were collected.