ONSET OF PUBERTY AND SEASONAL FERTILITY IN BISON BULLS

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ABSTRACT

Onset of puberty was observed in 12 bison bulls by the collection of semen at monthly intervals for 12 months beginning at 13 months of age. Onset of puberty was defined as the time in development when an ejaculate contained a minimum of $50x10^6$ sperm showing at least 10% progressive motility. At each collection, data were recorded on body weight, semen quality, fecal testosterone concentration, and physical development. Semen was evaluated for gross motility, individual progressive motility, sperm morphology, sperm concentration and volume. From these data bison bulls attained onset of puberty at an average age of 16.5 months and an average body weight of 353 ± 52.8 kg. Age was the greatest determining factor for onset of puberty in this group of bulls.

Monthly abattoir collections of epididymal sperm (n=288) and testicular tissue (n=120) were evaluated to determine if bison bulls undergo seasonal changes in sperm production. Although epididymal sperm morphology did not give any indication of seasonal variation, the histological study of testicular tissue showed greater seminiferous tubule diameter (27.0 ± 4.3 μm) during the breeding months (July, August and September) than during any other seasons. Semen collected at 4 different occasions during the year (June, November, January, and April) from live mature breeding bulls (n=21) was used to verify data collected from abattoir samples. Semen from mature bulls showed a significantly greater proportion of normal sperm in June than in November (73.8 ± 9.1%; 44.1 ± 24.3%), respectively. There was little improvement in sperm morphology at the January sampling but in April morphology improved to a level close to that observed in June. Fecal testosterone concentrations were highest in June (128.6 ± 67.4 ng/g) and lowest in April (48.5 ± 33.3 ng/g). Although there was no clear seasonal trend in sperm morphology from bulls sampled at the abattoir, mature bulls showed slight seasonal variations in semen quality.

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LIST OF ABBREVIATIONS

ADG	Average daily gain
BRADWG	Bison Research and Development Working Group
BS	Breeding season
CP	Crude protein
Cr	Creatine
DMR	Distal midpiece reflex
EtOH	Ethanol
FSH	Follicle stimulating hormone
GEE	Generalized estimating equation
IPM	Individual progressive motility
LH	Luteinizing hormone
MeOH	Methanol
OR	Odds ratio
PBS	Phosphate buffered saline
PdG	Pregnanediol-3-glucuronide
SFT	Society for Theriogenology
USDA	United States Department of Agriculture
WCVM	Western College of Veterinary Medicine

1. INTRODUCTION

The North American bison (*Bison bison*) population has gone through many documented transitions in the last several hundred years. During the early 1800's their numbers have been estimated at 30 million and even as high as 60 million (McHugh 1972). By the end of that century, due to hunting, their numbers plummeted to a few hundred, but bison population numbers have made a miraculous recovery through conservation efforts and commercial production.

In 2002, the bison population in North America was probably just under 500,000 animals. The number of bison residing on private Canadian farms in 2002 was estimated at 169,000 animals (Nixdorf 2003). A 2002 census done by the National Agricultural Statistics Service of the United States Department of Agriculture (USDA) estimated the bison population on private farms in the United States to be 231,950 animals (USDA 2002). As markets for bison products develop and expand these populations are expected to grow. The number of bison, both Plains- (*Bison bison bison*) and Wood bison (*Bison bison athabascae*), residing in public herds in Canada and the United States, is estimated to be 28,145 animals (Delaney 2003).

With the rapid expansion in bison populations and profitable markets for breeding stock there was relatively little interest in conducting research on bison whether it be nutrition, reproduction or animal health. There was no need for efficiency; however, in today's market the commercial population is growing faster than the market demand. As a result the market price for live animals has dropped, and producers are now looking for ways to raise bison at lower costs.

Individuals such as Dale Lott, Joel Berger and Carol Cunningham, and Tom McHugh have dedicated much of their lives to the study of bison biology and the dynamics of their behaviour. Without these individuals our knowledge of bison would be almost non-existent. Now is the time to build on that knowledge and create a more detailed understanding of reproductive physiology in bison. This is the next step in helping both commercial producers and conservationists to achieve their objective of sustainable and profitable bison populations.

A committee made up of scientist and industry representatives, the Bison Research and Development Working Group (BRADWG), has developed a strategic research plan for the bison

industry. This strategic plan identifies immediate and long-term research goals to aid in the development of the Alberta (and if appropriate, the Canadian) bison industry. Although there is little desire within the industry to over manipulate reproduction in bison, the need to understand basic reproductive function remains (Rutley 2003). Reproduction in bison, specifically male fertility, has been identified as a high-priority for both immediate and long-term consideration.

Research described in this thesis is an initial attempt to characterize reproduction in male bison. Biologists involved in conservation efforts are discovering that the lack of knowledge for reproductive physiology in bison is making their work very challenging. As commercial producers need to increase the efficiency of their bison farms, basic knowledge about reproductive physiology will help them make sound decisions affecting the economic viability of their operations.

2. LITERATURE REVIEW

2.1 Introduction

There is limited published information on reproduction in the North American bison. Much of the available information pertains to their reproductive biology and behaviour; very little deals with physiologic and endocrine regulating mechanisms. Due to the phylogenetic relationship between bison and domestic cattle, in this thesis, comparisons between them will be made where appropriate. This literature review covers material available on the physiological reproductive processes for bison and information on other ruminant species is used where appropriate to describe relevant processes and concepts not yet recorded in bison. Even though bison belong to the subfamily bovinae they will only be referred to as "bison" in this thesis. When the word bovine is used in this thesis it refers to domestic cattle, and "buffalo" will refer to animals of the genus *Bubalus*.

2.2 Sexual maturation

Sexual maturation is the result of an organized pubertal process. During sexual maturation the development of the reproductive hypothalamic-pituitary-gonadal axis, reproductive organs and secondary sex characteristics, and sexual behaviour are simultaneous and inter-dependent. A fully functional endocrine system is necessary for the production of viable gametes and their subsequent transfer through the uro-genital tract for insemination and fertilization (Foster, Ebling 1999). Full development of the sex organs is required to physically accomplish a successful mating and development of libido is needed to initiate copulation. Failure to achieve maturity in any aspect will result in reproductive incapability.

2.2.1 Definition of onset of puberty

In a broad sense puberty is a multifactorial process of physical maturation resulting in reproductive readiness. Puberty is achieved when sexual maturation has progressed to the point where insemination and fertilization can be accomplished.

Puberty in bovine bulls has been defined as "the phase of bodily development during which the gonads secrete hormones in amounts sufficient to cause accelerated growth of the

genital organs and the appearance of secondary sexual characters" (Donovan, van der Werff ten Bosch 1965) or as the age at which the first sperm or motile sperm could be collected by electroejaculation (Wolf, Almquist *et al.* 1965). The accepted definition for onset of puberty in bovine bulls has been further refined to include the age at which they first produce an ejaculate containing at least 50 million sperm, having a minimum of 10% individual progressive motility (Wolf, Almquist *et al.* 1965). This definition can be applied to bison and will be used in this thesis will also refer to the onset of puberty, facilitating comparisons of domestic bovine bulls and bison bulls. In rams, onset of puberty has been defined as the appearance of mature spermatozoa in the seminiferous tubules (Wilson, Lapwood 1979) or ejaculate (Claypool 1985). Lincoln (1971) defined puberty in red deer stags as the period from 9 to 15 months of age; from the onset of testosterone secretion to the completion of spermatogenesis.

2.2.2 Development of the testes and establishment of spermatogenesis

There is no published information on testicular growth and development in bison. Using a single observation on one bison bull, Peters *et al* (1957) suggested that as yearlings, bison do not produce an adequate quality of semen for normal fertility. No observations were made on testes growth and development and no subsequent semen samples were collected to determine if and when any criteria for fertility were finally met. However, the same study concluded that bison bulls reach puberty later than domestic Hereford bulls. Haugen (1974) reported that at 18 months of age 3 of 6 plains bison had sperm in the epididymis and at 29 months of age 5 of 6 bulls had sperm in the epididymis. No observations on morphology or motility of the sperm at 29 months were reported as the study was done after the animals were slaughtered.

A more recent study of 234 bison between the ages of 28 and 30 months found that 65.3% of bulls at that age passed a breeding soundness evaluation based on the criteria of the Society for Theriogenology for domestic cattle (Keen, Rupp *et al.* 1999). This work did not include information on testicular development, age of pubertal onset, or age when semen quality first reached an adequate quality for reproduction.

In contrast, the literature on sexual maturation in male bovine calves and lambs is extensive. Testicular growth is slow for the first 6 months of life in bull calves and 4 months in lambs (Coulter 1986; Curtis, Amann 1981; Evans, Davis *et al.* 1995; McCarthy, Hafs *et al.* 1979; Howles, Webster *et al.* 1980; Lincoln 1998; Yarney, Sanford 1989). After this period of slow

development, rapid testicular growth occurs. An increase in the amount of testicular parenchyma, mainly the length and diameter of seminiferous tubules, results in an increase in testicular size and weight (Curtis, Amann 1981). Once puberty has been achieved at about 11 months of age in the bovine bull calf, testicular growth continues at a slower but steady rate through to sexual maturity (Coulter 1986; Evans, Davis *et al.* 1995). In other species, such as the Mouflon ram (*Ovis musimon*), additional testicular growth is influenced by seasonal changes in photoperiod (Lincoln 1998).

The development of spermatogenesis in bovine bull calves, described by Coulter (1986), occurs in phases. These phases, closely linked to testicular growth and development (Curtis, Amann 1981) are described as infantile, proliferation, pre-pubertal and pubertal phases. In the infantile phase, at birth and for a short period afterwards, the sex cords in the testicular parenchyma are devoid of any lumen and are primarily composed of gonocytes and undifferentiated supporting cells. The proliferation phase is marked by the increase in number of undifferentiated supporting cells. The pre-pubertal phase follows the early initial rise of luteinizing hormone (LH) and follicle stimulating hormone (FSH) described by Evans *et al.* (1995) and is marked by the appearance of primary spermatocytes and their subsequent increase in numbers (Coulter 1986; Curtis, Amann 1981). The final (pubertal) phase extends to a time at which spermatogonia reach adult levels and all undifferentiated cells have matured to Sertoli cells (Coulter 1986). Although not having reached adult levels of quantity and quality, mature sperm make their appearance during the pubertal phase.

After the onset of puberty there is a steady increase in the quantity and quality of semen as the animal approaches sexual maturity. Motility and concentration of sperm per ejaculate increase steadily in bovine calves from about 4 weeks prior to onset of puberty (Evans, Davis *et al.* 1995). Rapid increases are observed in the proportion of live sperm, of which increasing numbers exhibit progressive motility and normal morphology (Evans, Davis *et al.* 1995; Lunstra, Echternkamp 1982). At the same time there is a consistent decrease in the incidence of sperm defects such as proximal cytoplasmic droplets and knobbed acrosomes (Evans, Davis *et al.* 1995; Lunstra, Echternkamp 1982). In cattle, this maturation process occurs during the 6 to 8 weeks (Evans, Davis *et al.* 1995; Lunstra, Echternkamp 1982) following the onset of puberty resulting in sperm characteristics similar to that of adult bulls with good fertility. A similar progressive improvement of semen quality has been described in Suffolk ram lambs where puberty was

attained at 16-20 weeks (Skinner, Booth *et al.* 1968).

Sexual maturation is generally thought to occur later in bison bulls than in cattle. However, the only publication on this subject noted a relative lack of sperm in bison semen sampled at 12 months of age (Peters, Newbound 1957). There is no published information on the onset of puberty and sexual maturation in bison bulls and it is not clear when this process is complete. Breeding soundness evaluations performed on 234 bison bulls between the ages of 28 and 30 months over a period of five years revealed that 65% had attained at least 30% motility with 70% normal morphology (Keen, Rupp *et al.* 1999). In another study, 72% of bison bulls between the ages of 18 and 24 months subjected to a breeding soundness evaluation were rated as satisfactory breeders, based on Western Canadian Association of Bovine Practitioners (WCABP) standards (Haigh, Berezowski *et al.* 2001). It is presently assumed that bison bulls reach adequate levels of semen quality when rated as satisfactory for breeding purposes by 26 months of age. However, the age at which male bison enter pubertal development and the duration of these developmental processes is still unknown.

2.2.3 Male reproductive behaviour as an indicator of sexual maturity

Male sexual behaviour includes the willingness and desire to mount and copulate with a female in estrus (libido) (Chenoweth 1983). Mounting behaviour starts well before puberty in most ungulates (Fraser 1968) and is often misinterpreted as evidence of the onset of puberty. Mounting accompanied by copulation and ejaculation is not achieved until the pubertal process is nearly complete.

A male capable of breeding a female in estrus must first be able to identify the receptive female. Identifying behaviour has been described for cattle, sheep (Chenoweth 1981) and bison (Berger, Cunningham 1994; Lott 1971; Lott 1981; McHugh 1958). Males perform regular investigations of females in the herd using visual, taste and olfactory cues(Chenoweth 1981). Flehmen is commonly displayed by bison bulls investigating females and is believed to aid in the identification of estrus females (Berger, Cunningham 1994; Lott 1981).

Dominance and social standing are strong determinants of the reproductive success of male bison. The strong dominance behaviour of older males (8 to 11 years) prevents many younger bulls from reaching significant breeding status under free-ranging conditions until they are about six years old, when they are able to successfully compete for estrus females (Berger,

Cunningham 1994; Lott 1971). The social structure of bison during the rut prevents young bulls from actively taking part in the breeding at any significant level, but the actual development of sexual behaviour in bison appears to take place between 1 and 2 years of age (Rothstein, Griswold 1991). In domestic cattle, older bulls dominate younger ones, thereby hindering the development of libido in younger bulls as they are prevented from taking part in breeding activity (Chenoweth 1983).

Development of sexual behaviour and libido are influenced by the genetic makeup of the animal and the environment in which the animal is reared (Chenoweth 1983). Rothstein *et al* (1991) investigated the age, sex preferences, and type of interaction with partners of male bison as calves and as yearlings. They observed that as calves, bison bulls showed no gender preference, but tended to seek out herd mates of the same age for initiation of interactions. Observations on yearling bulls determined that they too preferred initiating interactions with their same age class, but differed in the type of interactions between male and female partners. When interacting with yearling females more olfactory interactions were initiated, whereas with other yearling males, bulls tended to initiate mainly aggressive and agonistic interactions. These observations indicated that as yearlings, bison bulls exhibited behaviours similar to those of adults and were developing behaviours required for successful reproduction. Others have observed that, if given the chance, by 2 years of age bison bulls would mount and copulate with an estrus female (Berger, Cunningham 1994; McHugh 1958). Under commercial farming, many herd sires have demonstrated their ability to reproduce successfully at 26 months age (Grinde 2003).

2.3 Seasonal reproduction

2.3.1 Seasonal changes in male reproductive parameters

Domestic cattle are able to breed successfully at any time of the year and therefore, they are not considered to be seasonal breeders in the strictest sense. However, research into the effects of season on bovine bull fertility indicated that there were slight seasonal changes in gonadotropin secretion, gonadal hormone production, volume and total number of sperm produced and sperm morphology (Stumph, Wolfe *et al.* 1993; Everett, Bean *et al.* 1978; Barth, Waldner 2002). Barth *et al* (2002) found that beef bulls were more likely to pass a breeding soundness evaluation in the months of April, May, and June than at other times of the year. The

percentage of bulls with satisfactory semen quality was greatest from April through to June (81.9%, 86.7% and 87.5%) and lowest in September and February (57.1% and 57.7%). Similar results were obtained earlier for Holstein bulls by Everett *et al* (1978) who also reported the highest levels of semen volume, sperm concentration and total number of sperm per ejaculate during the months of April, May, and June.

It is unclear whether the observations made by Barth *et al* (2002) are due primarily to season, genetic selection, or a combination of the two. On the Canadian prairies it is standard practice for bulls to enter the breeding herd near the end of May or beginning of June, a period that coincides with high rates of satisfactory breeding soundness evaluations. It could be argued that the changes seen in beef bulls during the year may be the result of many decades of heavy selection for high levels of bull fertility in April, May and June. Barth *et al* (2002) suggest that these changes are the result of a combination of photoperiod, cold stress and body condition of the animal. More research is needed in this area to determine the actual effects of photoperiod on reproductive parameters domestic bovine bulls.

Rams exhibit more pronounced seasonal changes in reproductive parameters than cattle. Seasonal variations are seen in sexual interest, testicular size and sperm morphology for Suffolk, Lincoln, Columbia and Polypay rams (Mickelsen, Paisley *et al.* 1982). Mickelsen (1982) showed that between August and October libido, scrotal circumference, sperm motility and normal sperm morphology were at their maximum (8.4±0.7, 38.1±0.7 cm, 72.5±3.1% and 82.3±2.9%, respectively) for rams of 4 different breeds. Lowest levels for these parameters (4.4±0.7, 32.6±0.6 cm, 56.6±4.4% and 57.9±3.5%, respectively) were observed between February and April. This demonstrates that reproductive parameters of short day breeders improve after the summer solstice as a decline in day-length takes place. Short day breeders are animals that are reproductively active after the summer solstice during times of decreasing day length.

European red deer (Lincoln 1971) and North American wapiti (Haigh, Cates *et al.* 1984) provide two of the most extreme examples of photoperiodic effects on male fertility. During the months of May and June, red deer (*Cervus elaphus scoticus*) in Scotland experience complete testicular quiescence (Lincoln 1971). Testosterone levels in testicular tissue are extremely low (<0.298 and <0.231 ug/100g of tissue) in May and June, respectively. The epididymis is devoid of any sperm, and no spermatids or sperm are found in the seminiferous tubules and primary

spermatocytes are present only in small numbers. Seminiferous tubules are also at their smallest diameter (~190 μm) at this time. Lincoln (1971) reported slight increases in testicular testosterone concentrations (2.85 μg/100g) after the summer solstice (June 21) as day length started to decline. Spermatogenesis was reinitiated at this time as evidenced by seminiferous tubules containing spermatids and an increase in the number of spermatogonia. Seminiferous tubule diameter, testosterone concentrations and spermatogenesis continued to increase throughout July. By August there was complete resumption of spermatogenesis and the tubules reached their maximum diameter (~350 μm). Maximal concentrations of testicular testosterone (492 μg/100g) and measurements for tubule diameter (~360 μm) were attained at the beginning of the rut (September) and remained so until the end (October). By November testicular testosterone concentrations decreased (156 μg/100g) and tubule diameter began to decline (~320 μm). Spermatogenesis was greatly reduced at this time, as demonstrated by a corresponding decline in the diameter of seminiferous tubules (Lincoln, 1971). Testicular regression continued until April when there was minimal testosterone production, incomplete spermatogenesis and decreased testicular size.

Results obtained by Haigh *et al* (1984) using male North American wapiti (*Cervus elaphus canadensis*) agreed with the findings for the Scottish red deer. However, there were differences in the timing of peak testosterone levels. Lincoln (1971) did not record peak testosterone levels until mid September, which is already into the rut, whereas Haigh *et al* (1984) recorded peak serum testosterone levels (20 ng/ml) in August, prior to rutting activity.

2.3.2 Physiology of seasonal reproduction in temperate climates

The primary evolutionary goal of the male is to pass on his genetic characters to as many offspring as possible. It can be argued that for this reason semen quantity and quality is optimal when females are in estrus. In relatively constant seasonal environments where females are able to breed successfully at any time of the year, it would be advantageous for males to experience little to no seasonal reduction in their reproductive potential (Bronson 1985). As the breeding season becomes shorter, as it does in cold and temperate climates, males need to be at their maximal reproductive potential for a brief but specific time each year. These fluctuations in reproductive capability become more profound for males living in more northern latitudes where breeding activity is restricted to only a few weeks, or even days.

Many species of mammals breed during a restricted period at a specific time of the year. This ensures that offspring arrive when they are most likely to survive; at times when environmental conditions are optimal for both lactating females and growing offspring. Baker (1938) described the evolution of breeding seasons as organisms evolving the capacity to respond to certain stimuli by breeding. Baker further defined ultimate and proximal factors as the stimuli that regulate seasonal reproduction. Ultimate environmental factors, such as food availability and temperature, directly influence reproductive success by affecting the survival of mother and offspring. Proximal environmental factors, such as photoperiod and rainfall, are cues used by seasonal mammals to synchronize breeding seasons with their environment (ultimate factors) based on gestation periods.

The degree to which males of different species react to changes in season varies. Domestic cattle show little seasonal variation in reproductive parameters while rams show obvious reactions to changing seasons. Deer of temperate and cold climates illustrate an extreme case in which spermatogenesis ceases completely in the non-breeding season (Haigh, Cates *et al.* 1984).

Past studies demonstrate very clearly that the duration of daylight controls reproductive cycles in mammals of cold and temperate climates when ultimate factors such as nutrition are adequate (D'Occhio, Suttie 1992). Males and females of the same species react differently to changes in photoperiod. In deer, sexual recrudescence in males begins 4 to 6 weeks before the summer solstice whereas females of most deer ovulate for the first time after the summer soltice (Asher, Fisher *et al.* 1994). Early studies with sheep and deer demonstrate how some breeds or species experience a complete shift in reproductive cycles after a transfer across the equator from the northern hemisphere to the southern hemisphere (Marshall 1937). More recently, manipulation of the local photoperiod has allowed researchers to change the reproductive cycles of both rams (Lincoln, Short 1980; Simpson, Suttie *et al.* 1983/84) and red deer stags (Simpson, Suttie *et al.* 1983/84).

By alternating Soay rams between 16 weeks of short days (8L/16D; L=light, D=dark) and 16 weeks of long days (16L/8D) with an abrupt changeover in daylight length Lincoln *et al* (1980) were able to compress their annual reproductive cycle to 8 months rather than 12. Simpson *et al* (1983/84) were able to reduce the reproductive cycle of Suffolk cross rams to 6 months from the normal 12 through the manipulation of lighting regimes. Frequency of an

annual daylight cycle was doubled so that a complete daylight cycle was achieved in 6 months. Within this same study researchers induced red deer stags to grow two sets of antlers and experience two peaks of testicular activity within a 12-month period under the same lighting regime. By manipulating photoperiod Goss (1969) caused sika deer (*Cervus nippon)* bucks to grow two and sometimes three sets of antlers in a period of 12 months. In this experiment the author increased the frequency of an annual daylight cycle to 2 or 3 cycles in a 12-month period.

The use of controlled photoperiods shows that male ruminants, such as rams and sika deer bucks, have an endogenous reproductive rhythm (circannual cycle) that is entrained to daylight length cycles (Arendt 1986; D'Occhio, Suttie 1992). This is demonstrated by several species of male deer in the tropics that exhibit an annual antler cycle, although they are not synchronized with each other. Photo-refractoriness in photosensitive animals involves the loss of daylight length response after chronic exposure. After being kept on constant 16L/8D, 8L/16D, or 24L/0D sika bucks revert back to expressing cycles of antler growth, which is closely linked to testicular function, but in irregular intervals (Goss 1969). A review by D'Occhio and Suttie (1992) indicates that rams show spontaneous reversals in reproductive function if controlled photoperiods are greater than 12 weeks in duration such that sexual cycles in those rams become asynchronous with the enforced lighting regime.

It is well understood that responsiveness to day length occurs at the level of the pineal gland that receives photo-neural information via the suprachiasmatic nucleus and superior cervical ganglion. Although Lincoln *et al* (1989) demonstrated that cyclic changes in the pituitary and testicular activity still occur in rams having undergone a ganglionectomy (removal of the superior cervical ganglion) they also showed that these rams were unable to adjust their long-term rhythms to experimental lighting regimes. Similar work with white-tailed deer (*Odocoileus virginianus)* bucks and red deer stags demonstrated that these species still exhibit seasonal cycles in reproduction although synchronization with day length is lost (Lincoln 1998).

Research by Lincoln *et al* in 1981 and 1982 with Soay rams re-affirmed that the pineal gland is the main source of melatonin production and it is the duration of the melatonin signal within a 24 hour period that relays photoperiodic information (Lincoln 1998). Melatonin is secreted during hours of darkness resulting in longer elevations of serum melatonin in periods of short daylight than in periods of long daylight. Melatonin supplementation in spring and summer has been shown to induce a short day response in white-tailed bucks, red deer stags, and

fallow deer (*Dama dama)* bucks (D'Occhio, Suttie 1992). Premature testicular activity, cleaning of antlers, and rutting behaviour were observed when melatonin was administered either orally 6 hours before sunset or subcutaneously with an implanted slow-release capsule to mimic short days during ambient long day photoperiods. Soay rams, on 16L/8D and given melatonin orally 8 hours before lights were turned off, showed reproductive stimulation similar to that of rams transferred to 8L/16D (see D'Occhio, Suttie 1992).

Melatonin regulates gonadotropin secretion by altering the pulsatile secretion of gonadotropin releasing hormone (GnRH) (Bronson, Heideman 1994). More specifically, melatonin modifies the sensitivity of the hypothalamus and pituitary to steroid negative feedback (Lincoln, Short 1980). The switch from long days to short days causes an increase in the duration of the nocturnal peak in melatonin secretion. This induces an increase in pulsatile GnRH secretion that then activates the reproductive axis by increasing LH pulse frequency and amplitude and FSH secretion. The increase in LH secretion acts on interstitial cells of the testes that respond by producing increased amounts of testosterone. In a study that monitored gonadotropin and testosterone production in a group of white-tailed bucks over a period of a year, there was a significant increase in LH production in the month of July when daylight hours become shorter (43º latitude north) (Bubenik, Morris 1982).

2.3.3 Seasonal reproductive cycle in bison

Until now no attempt has been made to investigate seasonal fluctuations in the fertility of male bison. Much of what is known to date about male reproduction comes from observational accounts of social organization (McHugh 1958) and reproductive behaviour (Berger, Cunningham 1994; Lott 1981). One of the earliest descriptions of bison behaviour was given by William Temple Hornaday (1881), superintendent of the National Zoological Park. He compiled a comprehensive historical account of the bison that included observations on reproductive behaviour. McHugh (1958) published an article on the social behaviour of the American bison in Yellowstone National Park and, more recently, Berger and Cunningham (1994) completed a case study of the behavioural and ecological aspects of reproduction at Badlands National Park.

The premise that bison are seasonal breeders arises from observations that bison have their offspring during a well defined period in the spring and that breeding activity is confined to a few months in late summer (Berger, Cunningham 1994; Hornaday 1889). How restricted bison

are to a seasonally confined reproductive period remains unanswered as it is unclear what regulates this apparent seasonal reproductive cycle in bison. To what degree each sex influences this seasonal reproductive cycle, beyond the environmental factors, is unknown and any physiological information for the male bison would help to understand reproduction in bison. Unfortunately there are no accounts of seasonal variations in male reproductive parameters and current beliefs rely on information available from studies of female bison.

The North American breeding season, or "rut", for bison generally occurs from July through to the end of October. Hornaday (1889) wrote that the "breeding season of buffalo is from July 1st to October 1st. Lott (1981) observed that 46% of observed breedings on the National Bison Range in 1972 occurred in the last two days of July and first two days of August. A three year observational study (1951 to 1953) on free-ranging bison in Yellowstone National Park indicated that breeding activity was most active between the 15th of June and September 30th (McHugh 1958). At Badlands National Park in South Dakota, bison were observed to be sexually active from early July to the end of August (Berger, Cunningham 1994).

There is no published information on seasonal fertility for bison bulls despite the fact that semen tests are common practice in today's commercial bison industry. The majority of testing takes place prior to the breeding season (June), however some bulls are tested during late fall and early winter in preparation for auction sales. To our knowledge no data has been compiled on the outcomes of sequential semen tests and no inferences have been made from changes in semen quality throughout the year. The extent to which bison bulls are seasonal and where they belong in the continuum of seasonal change discussed above is a matter of speculation. Bison bulls might show very little fluctuation in semen quality, much like the bovine bulls (Barth, Waldner 2002; Everett, Bean *et al.* 1978).

Since bison have evolved in the more northern latitudes of the northern hemisphere it could be expected that male bison have a fertility pattern similar to that of wapiti and red deer (Haigh, Cates *et al.* 1984; Lincoln 1971). Spring anestrous would be biologically advantageous for survival in bison, as it would ensure greater calf survival. Calf survival would be optimal when arrival of offspring occurs during favorable climatic conditions and when lactation coincides with periods of high quality forage. More work is necessary to completely understand estrous activity in bison. The other possibility may be that bison bulls are more similar to the ram and exhibit less dramatic changes somewhere in between domestic cattle and cervids. An

understanding of the estrous activity in female bison from available information helps to form a hypothesis for male bison.

Kirkpatrick (1991) attempted to characterize the estrous cycle in bison during the presumed breeding season, July through to the end of August. The author noted that a majority of the conceptions were happening in the first or second estrous (Kirkpatrick, Kincy *et al.* 1991). By measuring urinary pregnanediol-3-glucuronide, Kirkpatrick *et al* (1991) demonstrated that 18 of 29 females sampled during the month of August showed a continual increase in progesterone production (>200 ng/mg Creatine (Cr)). This persistent increase in levels of pregnanediol-3-glucuronide was recognized as elevated progesterone production due to pregnancy. Eight of the 29 cows in Kirkpatrick's study demonstrated cyclic pregnanediol-3-glucuronide levels indicating an estrous cycle ranging in length from 19 to 26 days. What occurred physiologically outside of the presumed breeding season was not revealed by these data.

Matsuda *et al* (1996) presented some of the first reliable endocrine evidence that female bison may be seasonally polyestrous, with estrous cycles beginning in late July or early August. Although data was collected only from April to December in year 1 and June to November in year 2, the data indicated a period of anestrous activity up to the expected time of sexual activity. Progesterone production was measured by urinary pregnanediol-3-glucuronide and fecal progesterone from April 1st to November 30th. Low levels of pregnanediol-3-glucuronide (<200 ng/mg Cr) and progesterone (<200 ng/g feces) were observed from April to the beginning of August indicating that these animals were not experiencing any estrous activity during that time. Estrous patterns of progesterone production, or luteal activity (>200 ng/mg Cr and P_4), were observed in early August and recognized by rising and subsequent decreases in pregnanediol-3-glucuronide and progesterone concentrations. These cyclic patterns of progesterone production lasted for 16 to 32 days and occurred several times during the remaining months of the study. These data concur with the 19 to 26 day cycle lengths reported by Kirkpatrick *et al* (1991).

Results from Matsuda *et al* (1996) support earlier conclusions made by Rutley in 1995 who measured estrous activity a group of female bison using fecal progestin concentrations. Non-pregnant females exhibited low levels of ovarian activity (fecal progestin concentrations ranged from 4.2 ng/ml to 13.22 ng/ml) during the months of April, May, and June. Lactational anestrous (fecal progestin concentrations less than 10 ng/ml were considered to represent non-luteal activity) lasted an average of 100 days and was characterized by mean fecal progestin

levels of 7.67 ± 2.12 ng/ml. Resumption of luteal activity was indicated in both lactating and non-lactating animals in August by increases in fecal progestin levels ranging from 4.8 to 42.3 ng/ml. Rutley concluded from these data that bison cows go through a lactational and/or a spring (seasonal) anestrous starting in April. This could explain observations of the rarity of bison calves born in January, February and March.

It is difficult to draw firm conclusions from the data presented by Rutley (1995) due to the small number of animals. Only 6 plains and 5 wood bison cows were studied, and many of the cows seemed to be predisposed to reproductive problems such as low conception rates, dystocia and abortions. Bison from 6 different farms arrived at the research centre a few weeks before parturition and a year later 3 more cows were added to the project. Of the original 6 plains cows only one successfully reared a calf (born in August). After 3 other cows were added to the group 3 cows were open, 2 cows aborted and 2 cows died while calving. Rutley (1995) concluded that handling close to parturition was detrimental and may have been the cause for these reproductive failures. There was also great variation between animals in fecal progestagen levels for all physiological states throughout the study and an accurate interpretation of these results is difficult at best. The authors showed evidence of possible trends but a more controlled study using greater numbers of animals would be needed to make any definitive conclusions on seasonal reproductive cyclicity of female bison.

3. ONSET OF PUBERTY IN BISON BULLS

3.1 Introduction

The lack of physiological data on the onset of puberty in bison bulls is troubling when one considers the growth and current state of the commercial bison industry which, for the past 30 years, has been growing at 25% a year. The present estimated Canadian bison population 175,000 animals with about 100,000 in Alberta and 55,000 in Saskatchewan. Numbers have now reached levels where the supply of animals is exceeding demand and market value is depressed. This has forced producers to improve management and reduce production costs, but with so little known about the biology and physiology of the animal this is a difficult task. Improved reproductive management could make bison operations more profitable. However, there are no published reports regarding the effect of reproduction management changes on efficiency or profitability of commercial operations raising bison for meat or breeding stock.

In the past, breeding bulls were selected on attributes that had no direct connection with reproductive capacity and performing semen evaluations was not common practice. The industry now uses younger, unproven bulls for breeding and today very few potential breeding bulls are sold without a semen evaluation. This presents a problem as little is known, with the breeding soundness evaluation. There is no information about when young bison reach certain milestones in the usual sexual development of bison bulls under 2 years old such as puberty and bulls less than 24 months of age are often tested and evaluated without established test criteria. No clear guidelines exist to aid veterinarians in making a decision about the reproductive potential of bison bulls less than 24 months of age, or for that matter, breeding soundness of older animals.

As herds expand and efficiency of production becomes a priority for the industry the demand for greater knowledge about bison physiology is increasing. It has long been known that relatively young bison bulls are able to impregnate females. Observations made on bison living under natural conditions indicate that bison bulls will breed successfully when they enter their third breeding season at 26 months of age (Berger, Cunningham 1994; McHugh 1958). It is for this reason that the bison industry chooses 2-year-old bulls for breeding.

Keen *et al* (1999) has performed the only scientific study to date dealing with semen

quality for bison. His focus was on the relationship between scrotal circumference measurements and semen quality for bulls aged 28 to 30 months. At this age samples were successfully obtained from 92% of the bulls sampled (n=234 over 5 years) and of those 65.3% had greater than 70% sperm with normal morphology. They also found that scrotal circumference was a significant predictor of percentage of normal and motile sperm as well as percentage of primary sperm defects. However, this study does not address puberty and reproductive development and they discuss that not all bulls may have been sexually mature. In their minds the 8% of bulls for which sampling was unsuccessful may have represented prepubertal bulls.

The purpose of the present study was to investigate reproductive development in bison bulls. As breeding bulls are selected based on semen evaluations it is essential for veterinarians and producers to understand their reproductive development. To our knowledge this would be the first scientific data to document the pubertal development in bison bulls as it pertains to semen quality. This information will enable veterinarians and producers to make better choices by understanding where bison bulls are in reproductive development at a given age. Results may show at what age it is worthwhile to start subjecting bison bulls to semen evaluations, and what results might be expected based on age.

3.2 Methods and materials

3.2.1 Animal selection

Twelve pre-pubertal bison bulls, 13 months of age (born May 2000), were randomly selected from a bison herd close to Saskatoon, Saskatchewan (52º 10´ N, 106º 4´ W). Bulls of this age were chosen because it was believed that they had not started functional spermatogenesis, whereby sperm cells are present in the ejaculate and are of normal morphology. As a majority of bison calves are born in May it was thought that bulls born in that month would be the best representation of animals on commercial operations. Data were collected for a period of 12 months, from June 2001 to May 2002. The bulls were maintained on a feeder ration comprised of alfalfa/grass hay and screening pellets fed ad-libitum throughout the study period. They also had free access to water, and salt and minerals by use of trace mineralized salt blocks.

The bulls selected for this study were representative of bison bulls currently under commercial production. However, some bias was inevitable because bull selection was not

random. The owner of these bulls deliberately did not include the lightest bulls from the age group, and the selected group also did not contain any bulls with potentially high breeding stock value because the owner was not willing to risk injury to these bulls through frequent handling. Bulls in the study had three different sires, but the number of bulls from each sire was not high enough to investigate a heritable genetic influence on the onset of puberty. Therefore, potential genetic influence on the onset of puberty in bison was not investigated.

3.2.2 Examination of internal and external reproductive tract

Samples were collected from each bull once a month from 13 through 24 months of age and all collections were done out of doors. Between collections bison bulls were kept as one group in a pen (approximately 100'X200') and had no visual or physical contact with the mature herd residing on the same farm. On the day of collection they were run out of the pen into a holding alley designed to hold bison as a group for greater lengths of time. They were individually taken from the holding alley and run into a narrow box-stall with a scale. From there they were put in a hydraulic squeeze especially designed for bison and restrained with the head gate and movable side panels which put pressure on the side of the animal. The back end of the animal was accessible through a side door about 7 inches wide and a bottom door about 12 inches high running the length of the squeeze was used to access the animal for semen collection. Prior to semen collection the rectum was cleared of feces and the accessory sex glands, including the prostate, vesicular glands, and ampullae, were examined by rectal palpation. The scrotum and testes were palpated to confirm the presence of both testes. Any abnormalities of the accessory sex glands and testes were recorded. Due to the facility design and the agitation of bulls it was deemed too dangerous to measure scrotal circumference even though it is well known that scrotal circumference is a reliable predictor of semen quality in young beef bulls (Arteaga, Baracaldo *et al.* 2001; Coe 1999).

3.2.3 Semen collection and evaluation

The rectum was cleared of any fecal material and the accessory genital glands were massaged for a minimum of one minute immediately prior to stimulation. Samples were collected by electroejaculation using a Pro-Jac machine (Reprotronics, Medicine Hat, Alberta) with a segmented probe (60mm) and a Lane Pulsator IV machine (Lane Manufacturing Co.,

Denver, Colorado) with a non-segmented (60mm) probe. The Pulsator IV with the non-segmented probe was initially chosen for sample collections. However, on the first collection (18/06/01) the bulls reacted very negatively to the stimulation. Negative reaction to stimulation was recognized by excessive kicking and thrashing as well as jumping up and down in the squeeze. Bison normally take a few moments to settle down after their head has been caught. If they are receiving no adverse treatment they normally take to handling with little movement. We observed reaction past the expected reaction at being restrained. On subsequent occasions the Pro-Jac system was used as it was thought to be less adversely stimulating to the bulls. A subjective assessment indicated that by using only the rear segment of this probe agitation was minimized. The Pulsator IV remained as a back up machine and was used when the segmented probe was damaged on 07/11/01. As bulls got older, and the internal reproductive tracts increased in size, the Pulsator IV was used as the need for a segmented probe was not as great.

The probe was inserted completely and held in place by positioning the animal's tail between the connector arms. Stimulus was delivered in a pulsatile rhythm with increasing intensity from initiation through to ejaculation as described for bovine bulls (Barth 2000). Pulse stimulation was applied for 2 to 3 seconds with an interval of 1 to 2 seconds between each pulse. Success or failure of penile protrusion, erection and ejaculation were recorded on each occasion and any abnormalities of the penis and prepuce were noted. If the first attempt at semen collection was unsuccessful the bull was allowed to rest for a minimum of 5 minutes before a 2nd attempt. A bull was subjected to a maximum of 2 stimulation sequences. If no sample was obtained after two collection attempts it was recorded as an unsuccessful collection. All procedures were approved by the University Committee on Animal Care and Supply (UCACS) of the University of Saskatchewan.

Semen was collected into a test tube with a plastic cone attached and mounted on a handle fitted with a water canister and thermometer. Water within the canister was maintained at 37ºC. Between collections the apparatus was placed in a water bath maintained at 37ºC. For collections done in cold weather the apparatus was placed in a felt sleeve made from winter boot liners. Immediately after collection the tube containing the sample was transferred to a warm water bath.

Evaluations of fresh semen were done immediately after collection in a heated building adjacent to the squeeze. A warm stage was used to keep microscope slides and stain warm.

Seven criteria were used to judge the quality of the ejaculates: density, volume, gross motility, individual progressive motility, morphology, percent live staining, and concentration. Volume was measured to the nearest 0.50 ml using the 1 ml calibrations on the collection tubes. An estimate of density was made using a 4 point scale color and appearance (Barth 2000). Translucent or watery samples were classified as 1 (poor). Samples with a slightly skim-milk or opaque milk-like appearance were classified as 2 (fair) and 3 (good), respectively. Samples in category 4 (very good) were those with a thick grainy appearance and a cream colour.

Gross motility was estimated by microscopy using undiluted semen under bright field microscopy at 40X magnification. Categories for gross motility ranged from 1 to 4. Samples with no swirling activity and very little individual sperm motion were judged as a 1 (Poor). Samples with no swirling activity but evident individual sperm motion were classed as a 2 (fair). When slow swirling activity was seen the sample was classified as a 3 (good) and a sample with vigorous swirling activity containing vanishing dark eddies was judged as a 4 (very good).

Individual progressive motility (IPM) was determined by duplicate counts using fresh semen diluted in phosphate buffered saline (PBS) solution under 40X magnification without phase contrast. Sperm concentrations were determined with a hemacytometer by counting sperm in 4 square millimeters on each side. Concentrations were reported as the number of sperm per ml of ejaculate (10^6/ml). The product of concentration (10^6/ml) and volume (ml) of ejaculate gave the total number of sperm per ejaculate (10^6/ej).

Two smears of each sample were prepared for evaluation of sperm morphology. One smear was stained on site with Eosin-nigrosin stain and a duplicate smear was prepared on site to be stained later in the laboratory with Feulgen's stain. Eosin-nigrosin is a live-dead stain, which stains only dead sperm, and was used to determine percentage of living by counting 200 sperm per smear. Sperm morphology was evaluated using oil emersion microscopy for both smears of each sample. Two differential counts of 100 sperm under 1000X magnification were performed. When the count for normal sperm differed by more than 5%, a 3rd count of 100 sperm was performed. For data analysis, counts were averaged for each slide. Smears stained with Feulgen's stain were used to verify the frequency of nuclear vacuoles if these were detected with Eosin-nigrosin stain as they are sometimes difficult to detect with the latter.

Abnormal sperm were classified into 5 different categories according to descriptions used for bovine bull evaluations (Barth 2000). Head defects included knobbed acrosomes, nuclear

vacuoles, and misshapen heads. Distal midpiece reflexes (DMR), proximal cytoplasmic droplets and mitochondrial abnormalities were classified as midpiece defects. A sperm was arbitrarily determined to have a DMR when the angle in the distal midpiece was less than 90º from the straight line a sperm cell normally exhibits. With an angle greater than 90 º and in the absence of any other abnormalities the sperm was classified as normal. Kinked or coiled tails and multiple tails were classified as tail defects. Remaining categories were other defects and normal sperm. Distal cytoplasmic droplets and abaxial tails were not classified as defects as they are not known to affect fertility (Barth 2000).

All fresh semen and stained smears were evaluated by myself throughout the study. Guidelines for bovine bulls as stated by the Western Canadian Association of Bovine Practitioners (Barth 2000) were used for evaluating semen samples for satisfactory levels of semen quality for breeding. Minimum requirements of 60% progressive motility and 70% normal sperm were used to classify a sample as satisfactory.

3.2.4 Testosterone assay

3.2.4.1 Sample preparation

Fecal samples for all bulls were collected prior to electro-ejaculation and kept cool in transit to the laboratory. Samples were transferred to 20 ml scintillation vials and kept frozen at -20°C until they were lyophilized. Dried fecal samples were manually ground using a metal weighing spatula. Approximately 0.2 g of each sample was placed into 13x100 mm polypropylene tubes. For testosterone extraction, 5 ml of AnalaR Methanol (MeOH) was added to each tube and the contents vortexed for 8 periods of 1 minute each over a 2 hour period. Tubes were refrigerated overnight, vortexed again the following morning, and then centrifuged for 20 minutes at 1500g. One ml of supernatant was aliquotted into polypropylene tubes and air dried. Aliquots were reconstituted with 50:l absolute ethanol (EtOH) followed by 1.0 ml phosphate buffered saline (PBS). These samples were capped, vortexed and kept overnight in a fridge and assayed the next day.

3.2.4.2 Methanol extraction efficiency

The efficiency of testosterone extraction from bison feces using methanol was assessed. Testosterone (150:l) was added to each of four, 0.2g samples of dried feces and these were

subjected to the extraction procedure described above. However, when using methanol for extraction of testosterone from bison feces, the extract assumes a green colour that interferes with the scintillation counter. To correct for colour interference, the fist 4 samples were compared to 4 additional samples (0.2g each) to which 150:1 of ^{3}H testosterone was added. An additional fecal sample was extracted (no ^{3}H testosterone added) for use as a colour control for the total counts. One millilitre of each sample extract was aliquotted into scintillation vials and air dried. Aliquots were reconstituted with 50 :l of absolute EtOH and 0.5 ml PBS. Scintillation fluid (BetaMax, 3 ml, MP Biomedicals) was added to all scintillation tubes, and the radioactivity measured. The average recovery of testosterone (n=8) was 95.6%, indicating that this method of methanol extraction was reliable when extracting available testosterone from bison feces.

3.2.4.3 Radioimmunoassay of testosterone

Fecal testosterone was measured using a competitive binding radioimmunoassay (RIA) where the test (or standard) antigen competes against a constant amount of tracer-labelled antigen for a limited, constant number of antibody sites. If a standard or test sample contains a low concentration of the antigen, after the competitive binding reaction reaches steady-state, there will be a high concentration of tracer bound to the antibody and the radioactive counts will be high. Conversely, if there is a high concentration of antigen in the sample, after the competitive binding reaction reaches steady-state, there will be a low concentration of tracer bound to the antibody and radioactive counts will be low. These counts can be used to calculate a standard curve, and then to calculate the content of the antigen in a sample.

Fecal testosterone in this study was measured using a solid phase iodinated radioimmunoassay (Coat-A-Count® Total Testosterone assay kit; Diagnostic Products Corporation; Los Angeles, CA). The minimum detectable limit of the assay, as provided by the manufacturer, is 0.04 ng/mL, which would convert to approximately 1 ng/g feces (0.04 ng/mL * 5mL/ 0.2 g feces). A standard curve for testosterone was prepared by adding known amounts (0.05, 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 ng/ml) of testosterone to PBS. Two methods were used to verify that the assay used and the standard curve were reading and calculating testosterone in a reliable fashion. Firstly, known amounts (0, 0.5, 1.0, 2.5 and 5 ng/ml) of testosterone were added to an extract containing 2.5 ng/ml of testosterone. Secondly, a known sample of high concentration (7.5 ng/ml) was serially diluted (1/2, 1/5 and 1/10) in PBS, as was a known sample

of low concentration (2.5 ng.ml). All samples were analyzed in duplicate and all resulting curves had similar slopes to the standard curve created using PBS. Intra-assay coefficients of variation were 9.8%, 7.9% and 4.5% for extracts with concentrations of 0.50, 1.91 and 7.33 ng/ml, respectively. Inter-assay coefficients of variation were 12.1% and 11.0% for extracts with concentrations of 0.52 and 1.72 ng/ml, respectively. This assay was therefore shown to reliably measure bison fecal testosterone. Final results are expressed as nanograms of testosterone per gram of feces (ng/g).

3.2.5 Data analysis

Descriptive statistics (Statistix7, Analytical Software, Tallahassee, FL.) were performed on the data to determine normality. Relationships between age, weight and the onset of puberty were determined by logistical regression using the generalized estimating equations (GEE) model (SAS 8.2 for Windows, SAS Institute, Inc.; Cary, NC). Relationships for the continuous variables of body weight, fecal testosterone production, sperm morphology, volume and individual motility to age were analyzed by linear regression using the GEE model assuming a normal distribution.

Generalized estimating equation models extend generalized linear models (GLM's) for the situation of correlated data (Ghisletta, Spini 2004; Ballinger 2004). Having clustered data (1 bull across time) violates the statistical assumption about independent observations of traditional regression methods. In this project data are collected for bison bulls across time, and these repeated observations are correlated over time. By taking into account this intra-cluster correlation there is less chance of estimation bias occurring in parameters' standard errors. In this analysis GEE was also appropriate to handle the small data set and its randomly missing data. An autoregressive correlation structure was specified for analysis.

Spearman's Rank Correlations (Statistix7, Analytical Software, Tallahassee, FL.) were used for determining simple non-parametric correlations between various sperm morphology classes and age, gross motility and total number of sperm and testosterone and sperm defects.

Onset of puberty was defined as the time in development where an ejaculate contained a minimum of $50x10^6$ sperm showing at least 10% IPM. Due to poor field conditions, the reliability of using 10% IPM as part of the equation to define onset of puberty was tested against using 15% live sperm on slides stained with eosin-nigrosin. A 5% adjustment factor was added

when using live sperm as it is assumed that not all live sperm are progressively moving forward at time of staining. Therefore, 10% IPM was equivalent to 15% live sperm. A kappa value was calculated to measure the agreement between these two measures of sperm motility. If agreement was found between these 2 measures, the counts of IPM was be used in the analysis. If there was no agreement the value of 15% live sperm was used to determine the onset of puberty.

3.3 Results

3.3.1 Animals

All bulls (n=12) remained in the study for the entire period. On 2 occasions no data were recorded for 1 bull. Bull R21 was not subjected to the sampling procedure on the 3rd collection (14/08/01) due to trauma experienced during handling. On a separate occasion (04/11/01) bull R18 escaped from the pen 2 days prior to collection and joined another group of animals. It was not possible to catch him, but he was present for subsequent collections. Between the sampling dates of 04/10/01 and 04/11/01 the research bulls were unavoidably mixed with other bison bulls of similar age and from the same farm for management purposes and they remained together for the remainder of the study period. Bulls not included in the study were occasionally removed for slaughter. Feeding strategy did not change for the duration of the study period and feed intake was not monitored. Bulls did not have access to pasture for grazing as they were maintained under a confined feeding management system.

Changes in mean (±SD) body weights (kg) over the entire study period are shown in Figure 3.1. At 13 months of age the mean weight was 263 ± 18.56 kg. A linear increase (Y = 19.26, 95% CI = 17.29-21.22, $P<0.0001$) in bull weight was found, with bulls weighing an average of 475 ± 52.21 kg at 24 months of age. Although feed intake was not monitored, mean (±SD) average daily gains (ADG) were calculated for each month between semen collection periods using data from monthly weighing and are shown in Figure 3.2. Mean (±SD) average daily gain for the entire 12 month period was 0.68 ± 0.12 kg/d. ADG increased for the periods June to July, July to August and August to September (0.98 ± 0.39 kg/d, 1.17 ± 0.63 kg/d and 1.23 ± 0.43 kg/d, respectively) after which they declined to 0.10 ± 0.23 kg/d for January to February. Average daily gains increased again for the last 3 months (March, April and May) of the study period to 0.80 ± 0.41 kg/d at 24 months of age. There was a slight decrease in ADG

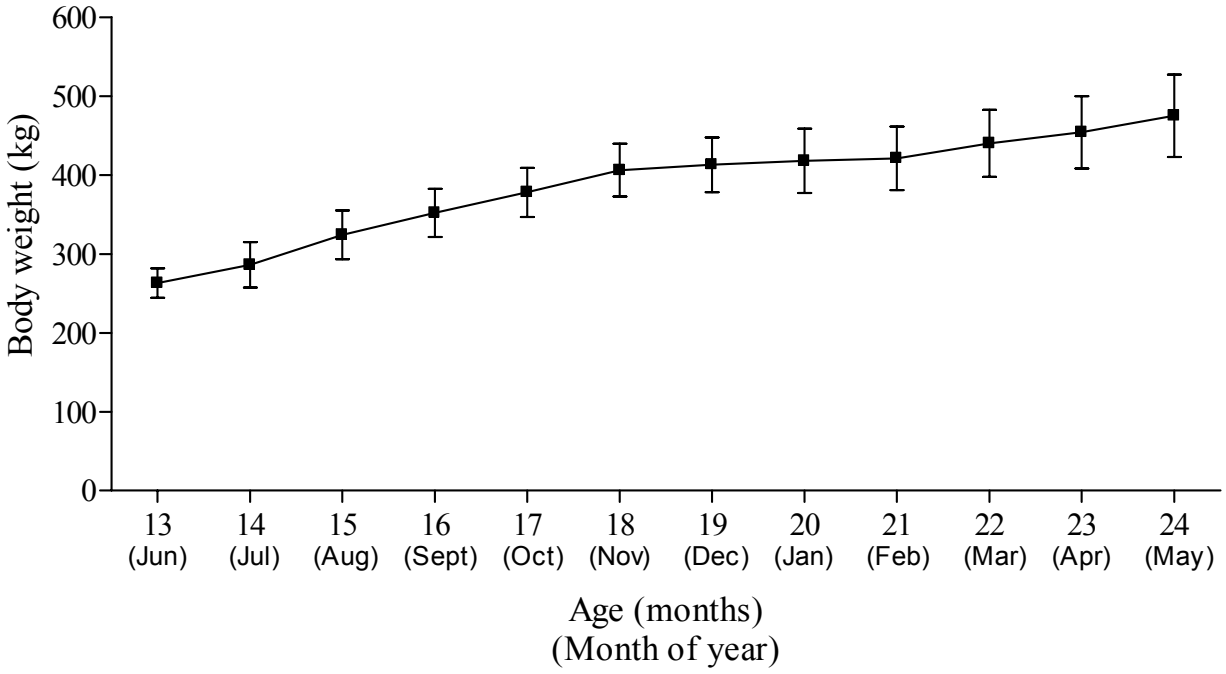

Figure 3.1 Mean (±SD) body weights for bison bulls at monthly semen collections over a 12-month study period (n=12).

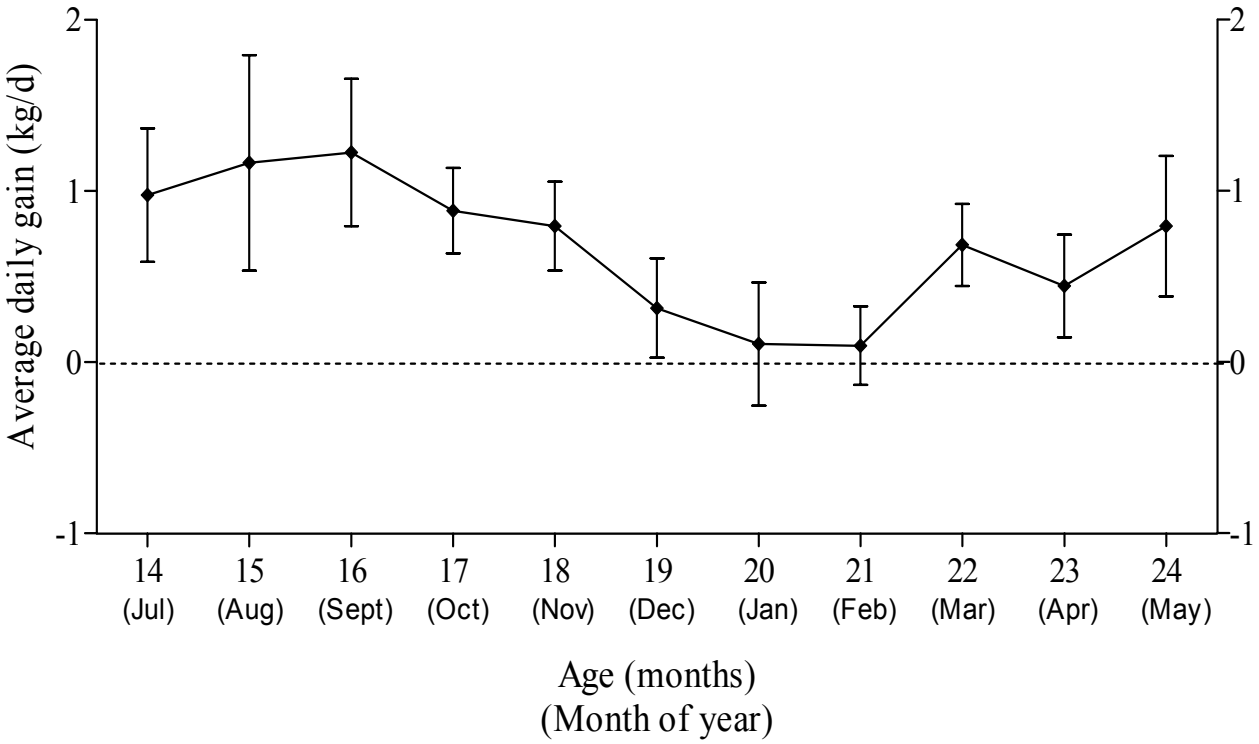

Figure 3.2 Mean (±SD) average daily weight gains for bison bulls measured monthly over a 12-month study period (n=12). 0 = No net gain in mean body weights.

from the March to April collections. No extreme changes in the weather were recorded and no other obvious stressors could be identified.

3.3.2 Examination of internal and external reproductive tract

Rectal palpation on each sampling date showed no abnormalities of the internal reproductive tract for any of the bulls. Ampullae were difficult to recognize. Palpation of the scrotum revealed that the left testis of bull B6 had not completely descended at the first semen collection (18/06/01). Both testes were fully descended at the next sampling period (16/07/01) but were noticeably smaller than those of all other bulls for the entire study period based on palpation. Visual examination of the penis and prepuce showed no abnormalities throughout the study.

3.3.3 Semen collection and evaluation

A bull was classified as immature (not producing sperm) until the age at which sperm were first visible in the ejaculate under light microscopy at 40X magnification. Any subsequent collections not yielding sperm after two attempts were recorded as unsuccessful collections. A total of 7 collections from 4 bulls were unsuccessful over 12 months, due to the intractability of the bison bulls. It was deemed unsafe to collect samples on these occasions for both personnel and the animals.

Bull B15 yielded unsuccessful results on 4 occasions (04/11/01, 09/12/01, 14/01/02, and 12/04/02) due to agitation caused by handling. This particular bull was the last one through the chute on most occasions.

3.3.4 Testosterone assay

The assay recovered 95.6% of the fecal testosterone from test samples and was therefore considered to be accurate. Mean (±SD) fecal testosterone concentrations (ng/g) did not show a significant trend during the study period (Figure 3.3). Rising levels were observed from 13 to 16 months of age peaking at 86.32 ± 25.88 ng/g. From 16 months of age levels decreased to a low of 32.04 ± 39.01 ng/g at 19 months. A 2nd increase (81.14 ± 28.95 ng/g) occurred at 21 months of age with levels decreasing again to 42.02 ± 16.75 ng/g two months later.

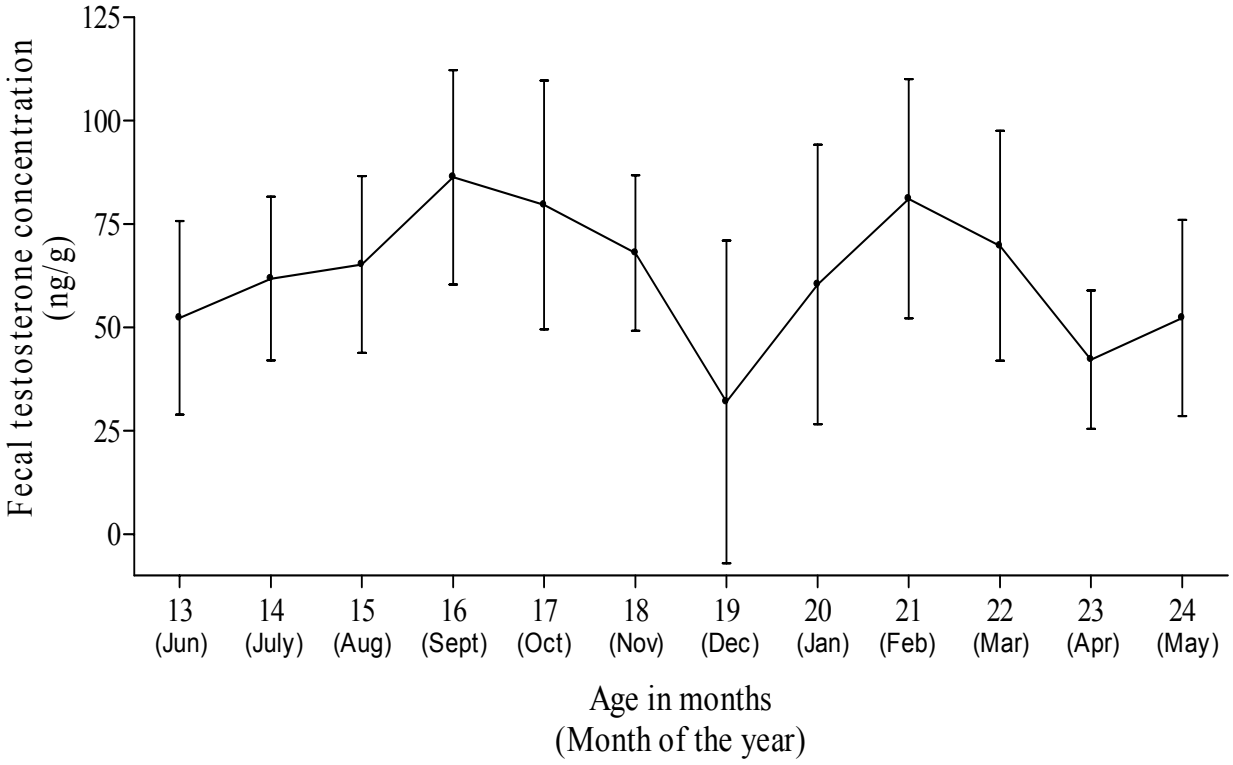

Figure 3.3 Mean (±SD) bison fecal testosterone concentrations at monthly intervals over a 12-month study period (n=12).

3.3.5 Onset of puberty

Agreement between the two methods for assessing the sperm viability was very high (Kappa = 0.81, P<0.0001), therefore the definition using $50X10^6$ sperm with at least 10% IPM was used to determine the onset of puberty in this study.

Characteristics of semen at the first appearance of sperm, at onset of puberty, and at 13 and 24 months of age are given in Table 3.1 and 3.2, respectively. Ejaculates from 4 bulls contained sperm on the first collection and by 17 months of age all bulls' ejaculates contained sperm. Age was the most significant factor (OR = 1.87, 95% CI = 1.05 – 3.33) in determining the onset of puberty. On average bulls in this study reached onset of puberty at 16.5 months of age. Three bulls reached the onset of puberty at 14 months of age with 10 out of the 12 bulls attaining it by 18 months of age. The last 2 bulls to reach puberty were 21 months of age (Figure 3.4).

Mean (±SD) body weight at puberty was 353 ± 52.8 kg (Table 3.1). When analyzed without regard to age, weight had a significant effect on the onset of puberty (OR = 1.04, 95% CI = 1.02 – 1.05, P<0.0001). Eight of the 12 bulls reached the onset of puberty by 400 kg; however, when considered together with age, weight was no longer significant in determining the onset of puberty. Weight at puberty was missing for one bull (B19) for an unaccountable reason.

Changes in semen morphology from 13 to 24 months of age are shown in Figure 3.5 and Table 3.2. There was a linear increase in the number of normal sperm with increasing age (Y = 6.39, 95% CI = 5.55 - 7.22) as well as decreases in the number of proximal cytoplasmic droplets (Y= -2.92, 95% CI = (-3.90) – (-1.93), P<0.05) and head defects (Y= -4.51, 95% CI = -5.50 - -3.53, P<0.05). There were no significant changes in midpiece and principal piece defects from 13 to 24 months of age. There were significant increases in IPM (Y= 4.54), gross motility (Y = 0.21), concentration (Y= 32.13), density (Y= 0.17) and volume (Y= 0.07) as bulls increased in age. There were no significant changes in the percentage of live sperm with age. The characteristics of semen at onset of puberty are listed in Table 3.1. Data were adjusted between bulls for onset of puberty to be time zero. At onset of puberty (n=12) sperm showed low mean (±SD) proportions of normal cells (21.5 ± 15.9 %) with head defects (51.7 ± 17.8 %) and proximal cytoplasmic droplets (26.6 ± 22.7%) being the main defects observed (Figure 3.6). The main head defects noted were nuclear vacuoles and malformed heads. Head defects and proximal cytoplasmic droplets were present in greatest numbers before onset of puberty and

Table 3.1. Mean (±SD) age, weight, fecal testosterone concentration and semen characteristics of bison bulls at first successful collection of sperm and at the onset of puberty.

Characteristic	First sperm	Onset of Puberty*
Age (months)	14.3±1.3	16.5±2.5
Body weight (kg)	296.5±42.1	353.4±52.8
Testosterone (ng/g)	52.9±15.6	76.9±29.8
Sperm morphology (%)		
Normal	4.0±4.8	21.5±15.9
Head defects	62.5±25.1	51.8±17.8
Midpiece defects	16.0±9.7	14.6±9.4
Tail defects	4.1±2.9	1.3±1.0
Proximal droplets	26.4±23.0	26.6±22.7
Ejaculate		
Live sperm (%)	31.1±26.7	51.0±22.0
IPM (%)	6.1±10.0	34.9±23.5
Concentration (10^6/ml)	44.0±57.0	167.1±153.4
Total sperm number (10^6)	58.2±98.1	340.5±254.3
Volume (ml)	1.9±1.0	2.6±1.6

*****Onset of Puberty is defined as the point in development where the ejaculate first contains a minimum of $50X10^6$ sperm having at least 10% individual progressive motility.
IPM- individual progressive motility

Table 3.2. Mean (±SD) body weight, fecal testosterone concentration and semen characteristics of bison bulls at 13 and 24 months of age.

Characteristic	Age (months)	
	13	24
Body weight (kg)	263.6±18.6	475.4±52.2
Testosterone (ng/g)	52.3±23.4	52.2±23.7
Sperm morphology (%)		
Normal	6.3±6.7	67.9±15.2
Head defects	51.1±32.3	15.1±6.1
Midpiece defects	10.3±8.2	8.3±7.9
Tail defects	6.1±3.9	1.2±0.8
Proximal droplets	23.5±22.9	2.7±4.4
Ejaculate		
Live sperm (%)	48.3±42.1	67.7±20.3
IPM (%)	0	49.2±20.0
Concentration (10^6/ml)	0	467.9±487.9
Total sperm number (10^6)	0	912.8±988.8
Volume (ml)	0.9±0.2	2.0±0.6

IPM- individual progressive motility

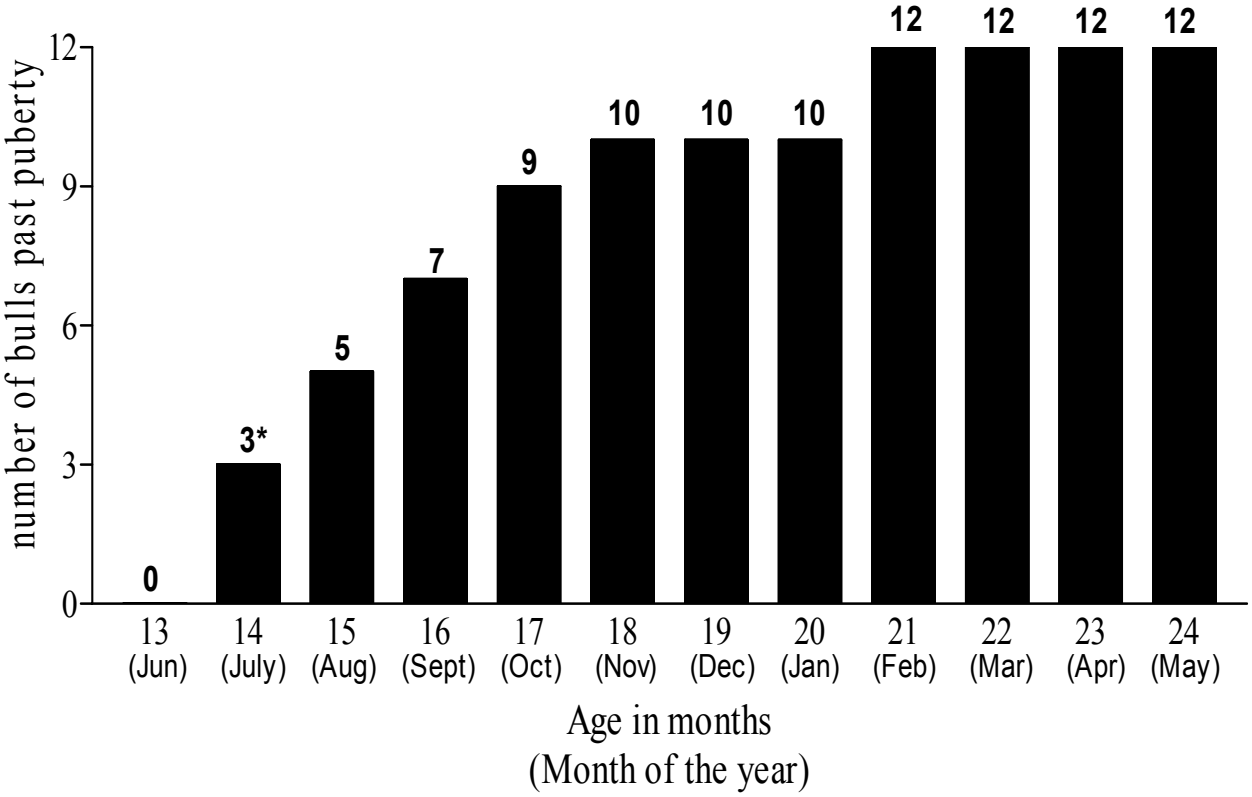

Figure 3.4 The numbers of bison bulls that had reached onset of puberty at the ages shown (n=12). *Each bar represents the number of bulls out of a group of 12 that have passed the onset of puberty.

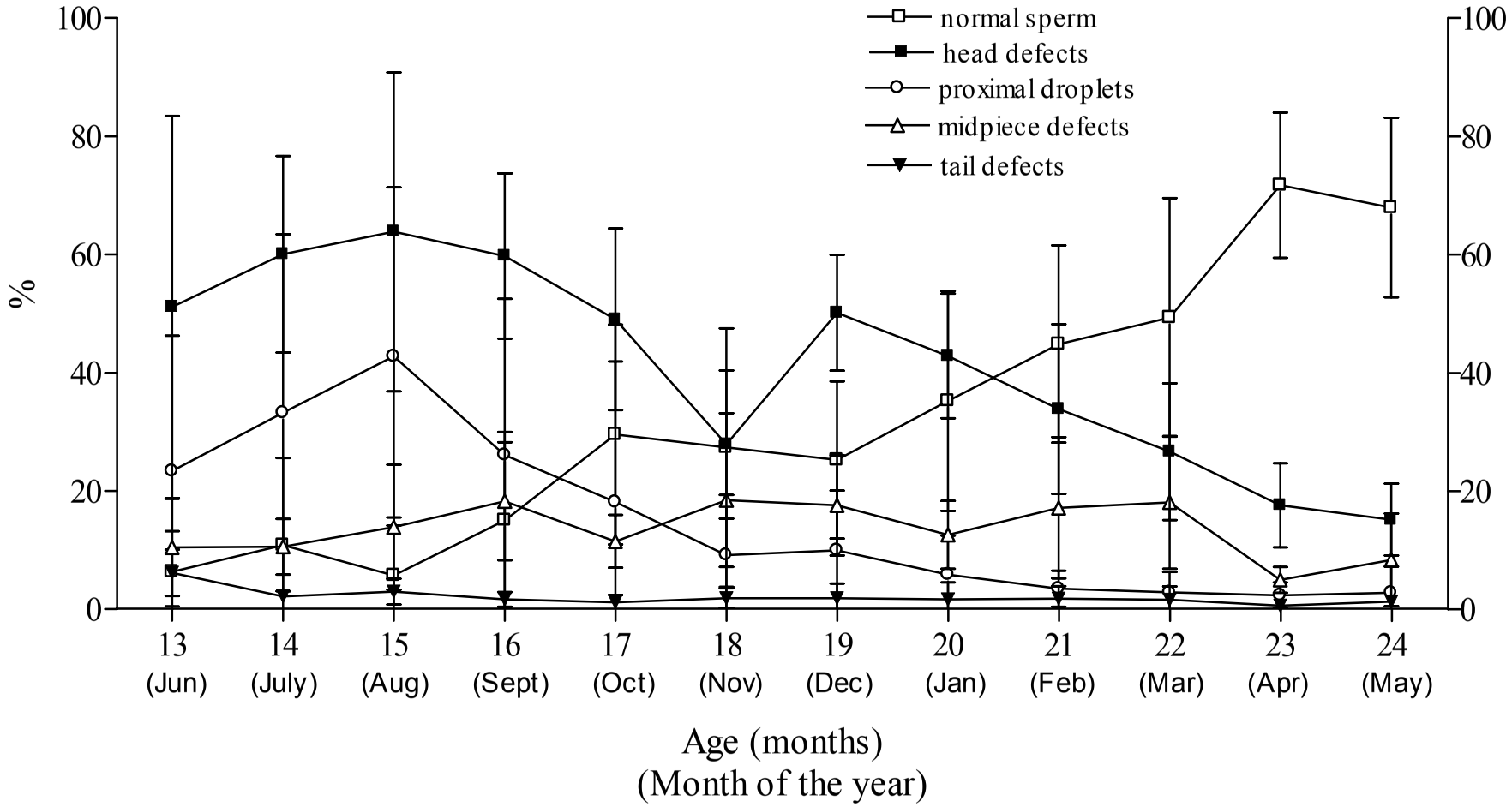

Figure 3.5 Percentage (mean ± SD) of bison sperm having the characteristics shown in ejaculates collected from 13 to 24 months of age (n=12).

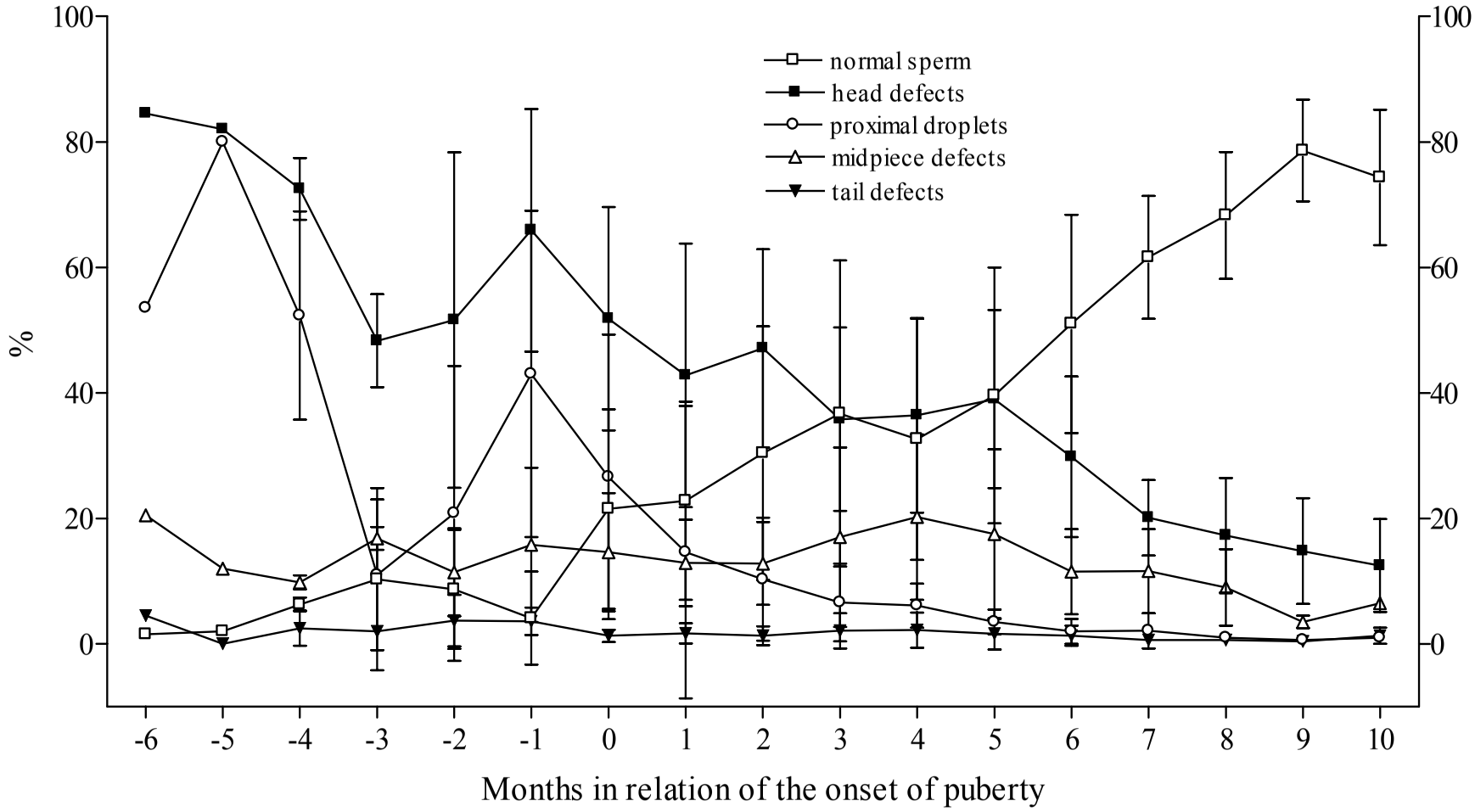

Figure 3.6 Percentage (mean ±dSD) of bison sperm having the characteristics shown in ejaculates before and after the onset of puberty. 0 = Onset of puberty.

decreased to 12.5% and 1%, respectively, 10 months after onset of puberty (n=3). The proportion of normal sperm stayed low before the onset of puberty but increased significantly from onset of puberty (21.5 ± 15.9%, n=12) until the end of the study (74.3 ± 10.8%, n=3).

Changes in IPM, concentration and total number of sperm before and after the onset of puberty are shown in Figures 3.7, 3.8, and 3.9. Individual progressive motility steadily increased significantly from 4 months prior (1.8 ± 2.5%, n = 5) to onset of puberty to 10 months after (54.3 ± 22.5%, n=3).

Concentration (10^6/ml) and total number of sperm (10^6/ej) increased significantly at onset of puberty (Figures 3.8 and 3.9). One month prior to onset of puberty mean concentration of sperm (25.5 ± 25.5 X 10^6/ml, n=7) and total number of sperm (31.9 ± 46.2 X 10^6, n=11) were low. Concentration and total number of sperm increased to 167.1 ± 153.4 X 10^6/ml and 340.5 ± 254.3 X 10^6, respectively. In the months following onset of puberty sperm numbers increased to 746.8 ± 811.0 X 10^6 sperm/ml of ejaculate (n=3) and 1,511.7 ± 1,599.0 X 10^6 total sperm (n=3).

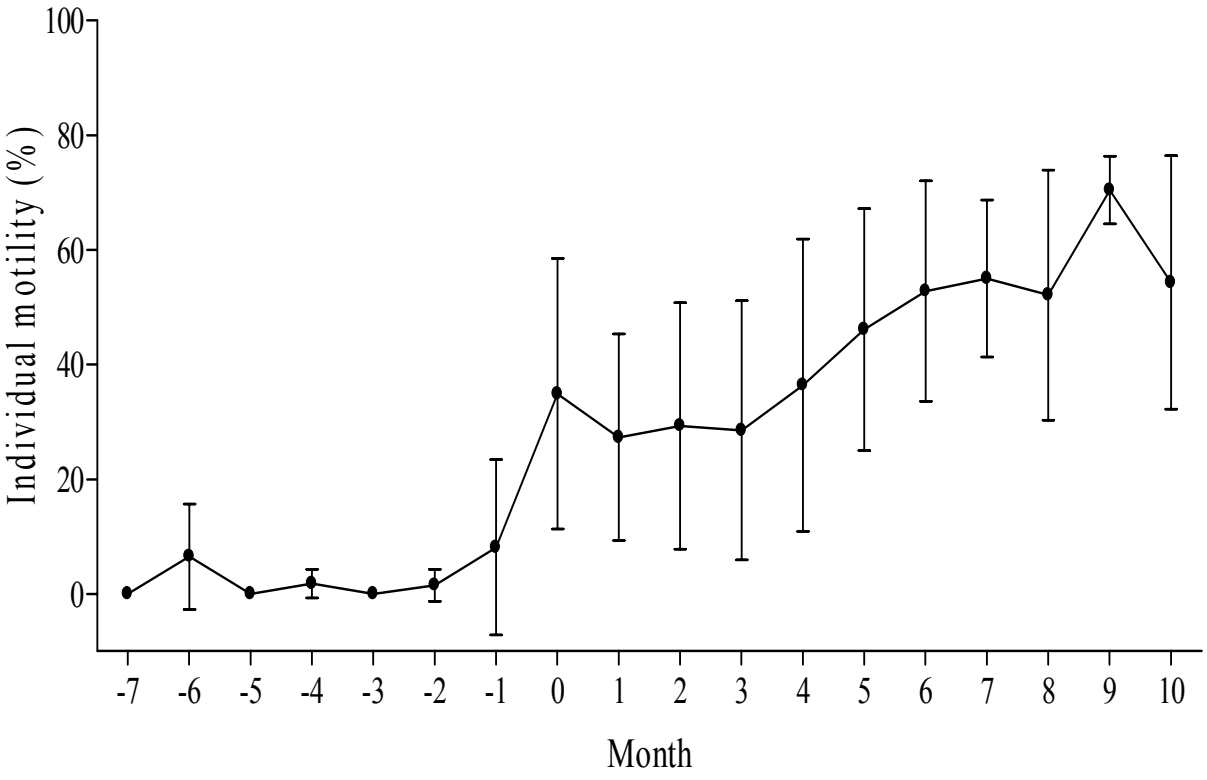

Figure 3.7 Percentage (mean ± SD) of individual progressive motility of bison sperm in ejaculates before and after the onset of puberty. 0 = Onset of puberty (n=2 at -4 mos, n=12 at 0 mos, n=9 at +9 mos, n=7 at + 7 mos).

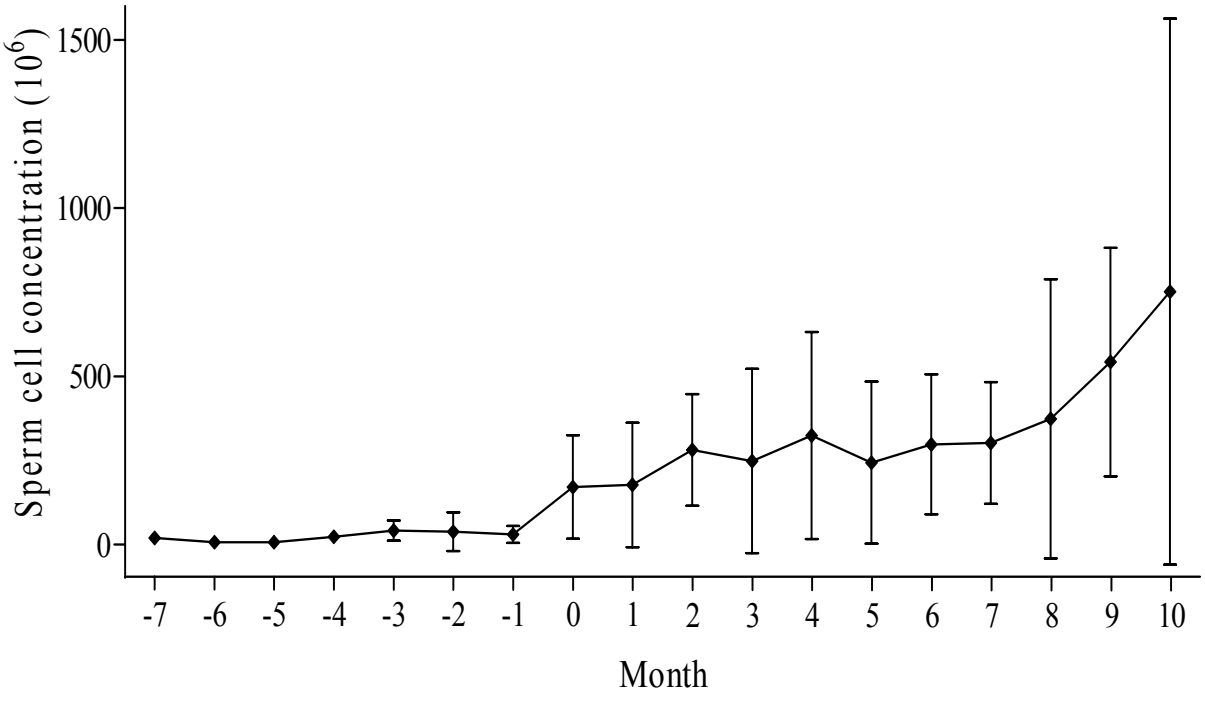

Figure 3.8 Mean (±SD) bison sperm concentration in ejaculates before and after the onset of puberty. 0 = Onset of puberty (n=2 at - 4 mos, n=12 at 0 mos, n=9 at +9 mos, n=7 at + 7 mos).

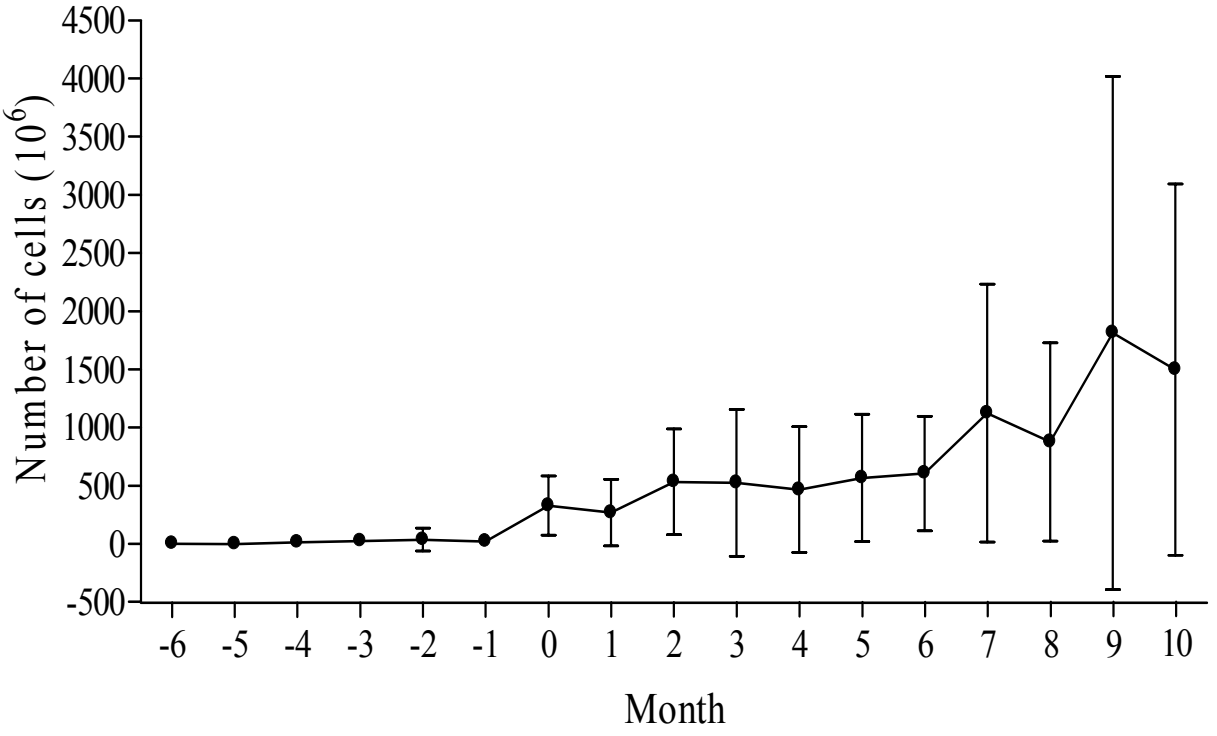

Figure 3.9 Mean (±SD) number of bison sperm per ejaculate before and after the onset of puberty. 0 = Onset of puberty (n=2 at - 4 mos, n=12 at 0 mos, n=9 at +9 mos, n=7 at + 7 mos).

3.4 Discussion

Until this study there have been no published scientific reports on the onset of puberty in bison bulls, probably because commercial bison farming is relatively new. Knowledge about physical and physiological pubertal development is important to efficiency of commercial bison farming, but less important under natural free ranging situations where the most information about reproduction has been generated. Another reason for a lack of observation on puberty in bison is the fact that they are not easily handled, making research difficult for both bison and researchers. Once frightened or upset, few bison will stand and cooperate during semen sample collection and, in the present study, samples were unobtainable from some bulls on various collection attempts.

Successful collection of a semen sample from a bison by electroejaculation takes practice. On occasions where samples were unobtainable for a particular animal after the previous collection date provided a sample, operator inexperience may have been the prime reason. My technique improved and modifications to the procedure described for beef bulls by Barth (2000) were made as the study progressed. Stimulation for the bison bulls was started at lower current and were not taken as high as one would for some beef bulls. Stimulation and adjustments in stimulation were also done more slowly than that described for beef bulls. For collections at the beginning of the study that resulted in no samples it is probable that bulls entered the research project at a time in development where spermatogenesis was just beginning. The bulls' agitation and behaviour may also have resulted in unsuccessful collections.

The point at which puberty arrives is more difficult to define for males than for females. In its broadest sense puberty is a developmental physical process that is initiated by the maturation of the reproductive tract and is completed when the male can produce sufficient viable sperm to impregnate the female. Coincidentally, males must develop appropriate reproductive behaviour and the desire to mount and copulate with a female in estrus. The attempt to identify puberty as a specific point in physiological development is difficult at best and this has led scientists to identify the onset of puberty. The bull's ability to produce an ejaculate containing a minimum of 50 million sperm having at least 10% IPM (Wolf, Almquist *et al.* 1965) is the most widely accepted definition for the onset of puberty in domestic cattle. This definition was used in this study.

Free ranging bison have been studied extensively but this has not provided much insight

into the age at onset of puberty and development in semen production. From his behavioural observations, Lott (1974) concluded that bison bulls in the National Bison Range in Montana are sexually mature at 3 years of age but competition with larger bulls prevents them from breeding before the age of 5. Others have noted that the majority of tending behaviour and aggressive encounters are associated with bulls over the age of 6 years (Berger, Cunningham 1994; McHugh 1958; Wolff 1998). It has been established that bulls between the ages of 8 and 11 years are the most aggressive in attaining estrus females (Maher, Byers 1987; McHugh 1958). Berger *et al* (1994) reported that the prime breeding bulls of Badlands National Park in South Dakota were even older at 11 to 12 years. The author noted that young bulls are not entirely excluded from breeding as some of the copulations in the Badlands were performed by bulls 4 to 5 years of age. Yearlings and 2-year-old bulls have also been seen to exhibit tending behaviour when given the chance but these bonds are mostly with heifers which were probably not in estrus (McHugh 1958). None of the above studies addressed the physical capability of animals younger than 2 or 3 years to impregnate females.

Commercial bison producers use 2-year-old bulls as breeding sires with high levels of success. This suggests that bison bulls have adequate libido, both in terms of libido and semen quality before the age of 26 months; however, there is little information on the timing of key stages in sexual development.

Keen *et al* (1999) have performed the most comprehensive study to date on semen collection and semen quality in bison, but the study was performed on bulls aged 28 to 30 months. These results demonstrate that, in terms of semen quality (guidelines used by the Society for Theriogenologists), many bison (65.3%) mature before 28 months of age. Haigh *et al.* (2001) suggest that bison semen quality improves from an unsatisfactory immature state to a satisfactory (WCABP guidelines) quality around 24 months.

This study showed that bison bulls attained onset of puberty at all ages from 14 to 18 months with two bulls reaching onset of puberty at 22 months. Variation in the age of onset of puberty was expected as variation among breeds of cattle is common and, unlike cattle, bison have not undergone extensive selection. Bison included in this study showed greater variation in the age of onset of puberty than cattle (Arteaga, Baracaldo *et al.* 2001; Lunstra, Echternkamp 1982; Lunstra, Ford *et al.* 1978) even if the two later maturing bulls are ignored. One could hypothesize that the greater variation seen in bison is a result of the lack of selection.

The last 2 bulls to attain onset of puberty were consistently the last to develop by all other criteria including body weight and semen traits. These 2 bulls have skewed the results and when they are removed from the analysis, the mean age at onset of puberty decreased by 1 month to 15.5 months. However, bulls such as these may be common in bison populations, indicating that approximately 20% of bison bulls may develop at a much slower rate than the species average.

These results support the hypothesis that bison attain onset of puberty and mature later than the common *Bos taurus* bulls of North America (Lunstra, Echternkamp 1982; Lunstra, Ford *et al.* 1978). Interestingly, the age for onset of puberty reported here for bison bulls is similar to that of *Bos indicus* breeds of cattle (Fields, Hentges *et al.* 1982; Silva-mena 1997). While bison included in this study attained onset of puberty at 16 months of age, more than 60% of beef bulls in western Canada are classified as mature at this age, based on satisfactory semen characteristics (Arteaga, Baracaldo *et al.* 2001). This difference in age at onset of puberty may be a reflection of the selection pressures for reproductive efficiency in beef and dairy bulls. However, Arteaga *et al.* (2001) concluded that even though young beef bulls are selected for greater scrotal circumference, which is correlated with good semen quality and earlier onset of puberty, the proportion of yearling bulls with mature semen traits is no higher today than it was 25 years ago.

In this study, age was the only reliable predictor for onset of puberty even though body weight was also investigated. However, when age was controlled in the analysis, weight became a significant predictor for onset of puberty. As expected the bulls increased in weight continuously during the study. The apparent interaction between age and weight in determining the onset of puberty makes it difficult to separate the true independent effect of age and weight. A larger and more controlled study monitoring feed intake would have to be performed to investigate the interaction between and/or independent effects of body weight and age on the onset of puberty in bison.

In studies of male cattle, serum testosterone increased in a linear fashion as spermatogenesis progressed and the testes developed (McCarthy, Hafs *et al.* 1979; Schams, Schallenberger *et al.* 1981). In this study, owing to the difficulty of collecting blood and the stress of handling and collecting semen, testosterone was analyzed in feces. Because serum testosterone concentration is very sensitive to any immediate changes in secretion caused by increased stress it was determined that any measures of serum testosterone would not have been an accurate measure of actual testosterone production at the time of semen collection. It is

believed that due to the nature of bison behaviour fecal concentrations would be more stable and give more accurate measures of actual testosterone production over time. This technique has been used successfully in the past with female bison in determining progesterone production patterns (Matsuda, Bellem *et al.* 1996; Rutley 1995).

This study is the first attempt to demonstrate production patterns of testosterone for developing bison bulls. The bison in this study did not show a clear trend in fecal testosterone production and levels varied greatly between bulls at all ages and between sample collections. Although variation in testosterone production has also been observed in domestic bulls, there was still a general trend of increasing levels during sexual development (Evans, Davis *et al.* 1995; Schams, Schallenberger *et al.* 1981).

Bulls included in this study were mixed with other bulls of the same age between the November and December semen and fecal collections. This entire group of bulls was confined in a holding pen measuring 100' X 200'. Although behaviour between collections was not observed the fall in the average fecal testosterone concentrations at the November and December collections may have been due to the stress of readjustment in social status, with subordinate bulls lowering their testosterone production. This explanation would also account for the large variation in testosterone levels for the December collection as some bulls in this study may have been more aggressive in attaining a dominant social position while others were subordinate to dominant behaviour. The overall increase in testosterone levels by the January collection may indicate that social ranking among the bulls was re-established at a new equilibrium. The relatively long time period of decreased testosterone levels can not be attributed entirely to stress, therefore there may be another unaccounted variable attributing to it.

Sperm first appeared in the ejaculate between 13 and 15 months but few, if any, were normal. A month after sperm were first seen, morphology began to improve. Although there is the possibility of investigator inexperience with the collection procedure at the beginning of the study, the extremely low numbers of normal sperm suggests that sperm first appear in ejaculates around the age of 14 months in bison. Lunstra *et al.* (1982) reported the appearance of first sperm in beef bulls to be earlier at 8 to 12 months of age.

This study also showed that as bison bulls increased in age from 13 to 24 months the percentage of morphologically normal sperm increased. This has been shown frequently in beef bulls (Arteaga, Baracaldo *et al.* 2001; Lunstra, Echternkamp 1982). In bison, from 13 to 15

months of age the mean number of normal sperm remained very low. The greatest improvements happened from 19 to 23 months of age. At the onset of puberty a high percentage of sperm abnormalities were present. Of these the most common were proximal cytoplasmic droplets, nuclear vacuoles, pyriform heads, and bent tails. As bulls progressed through development these abnormalities decreased. This pattern is very similar to that of beef bulls (Evans, Davis *et al.* 1995; Lunstra, Echternkamp 1982). As in this study on bison bulls, a study conducted on a large number of beef bulls in western Canada it was observed that proximal cytoplasmic droplets were the main sperm defect seen in semen samples of immature bulls (Arteaga, Baracaldo *et al.* 2001). Proximal cytoplasmic droplets are a sign of abnormal spermatogenesis and a large percentage of them in semen from young bulls can be a sign of immaturity. Sperm continue maturation in the epididymis and the movement of the cytoplasmic droplet from a proximal to distal position happens as the sperm moves from the caput to the cauda epididymis. This movement of the droplet appears to be associated with the onset of sperm motility (Barth, Oko 1989).

Concentration of sperm in ejaculates increased markedly from the onset of puberty to the end of the observation period. This follows the same pattern that has been described for beef bulls (Evans, Davis *et al.* 1995; Lunstra, Echternkamp 1982). Even though these are the first data to describe the concentration of sperm in semen of developing bison bulls they may not be a true representation of actual concentrations and should be interpreted with caution. It has been well established that semen from beef bulls collected by electroejaculation is often more dilute than that collected by an artificial vagina (Arteaga, Baracaldo *et al.* 2001; Evans, Davis *et al.* 1995). Use on an artificial vagina in bison would be extremely dangerous and at present electroejaculation is the only feasible means of collecting semen from bison.

By allowing seminal plasma to drip off before placing the collection handle to catch sample every attempt was made to collect only the sperm rich fraction of the ejaculate. For these reasons data presented here would be an estimate for sperm cell concentration of developing bison bulls rather than an accurate assessment.

Individual progressive motility showed the same pattern as sperm concentration. There was a significant increase at the onset of puberty and a continued increase to the end of the study. Under the conditions of collection accurate counts for IPM were difficult to obtain, and these data should be seen as estimates; none the less they showed a similar trend to that seen in beef

bulls (Evans, Davis *et al.* 1995).

To our knowledge the onset of puberty in bison bulls has not been studied before. Results from this study give the first data to create baseline parameters for reproductive development. Not surprisingly, bison bulls attained onset of puberty at a later age than most of the bovine bulls in North America, but the developmental process was very similar. Male bison show similar changes in sperm morphology during puberty to bovine bulls, although they to occurred over a longer period of time. Average age at puberty was not attained until after bulls had gone through their 2nd breeding season. Rothstein *et al.* (1991) who observed yearling bison bulls preferred to form social partnerships with other males, indicate that yearling bulls going through their 2nd breeding season are reproductively immature both behaviourally and physiologically. By the time bulls reach 24 months of age, just prior to their 3rd breeding season, they should have a mature ejaculate and show mature male behaviour.

Six of the 12 bulls (50%) in this study reached the standard used to determine adequate semen quality WCABP by 24 months and 3 other bulls came very close. Inadequate sperm motility was the determining factor in failing those 3 bulls but they were not far off from exhibiting adequate motility. If, as seems likely, this small difference is accounted for by the adverse conditions of collection, then 9 of the 12 bulls (75%) had reached the standard by 24 months. Unfortunately data collection in this study did not continue until the start of the bulls' third breeding season in August therefore it is not possible to speculate on how many bulls in this study would have had adequate semen quality by that time.

Based on our results it should be recommended that to be chosen as potential breeding sire young bulls 24 months of age should successfully fulfill requirements for a breeding soundness evaluation. However, due to the nature of the bison industry, many young bulls are tested at 19 to 21 months of age when, according to our results, the mean individual motility and the mean normal sperm morphology were 29-54% and 25-45%, respectively. Based on the results of this study, bulls that do not meet the minimum criteria between 19 and 21 months of age may be developing normally and should not be hastily discarded. If all other attributes were favourable it would be worth testing such bulls again at 24 months of age. Producers that purchase young bulls with less than adequate semen quality due to immaturity should be cautioned to buy them only with a guarantee that they perform a successful breeding soundness evaluation at 24 months.

4. SEASONAL FERTILITY OF BISON BULLS

4.1 Introduction

Bison (*Bison bison*) have evolved for almost 40,000 years in North America but remarkably little is known about them (Steelquist 1998). Bison behaviour and their interaction with their environment have been studied in some detail through observational studies (Berger, Cunningham 1994; Lott 2002; McHugh 1958; Roe 1970). However, there is a lack of information on their basic physiology. Regarding reproduction, some effort has been made to investigate seasonal ovarian function in female bison (Kirkpatrick, Kincy *et al.* 1991; Rutley 1995), but definitive information on seasonal testicular function does not exist.

Observations of wild bison in North America leave little doubt that their reproduction is seasonal. Seasonality in reproduction of bison has been documented as early as the European expansion into the western frontier (Hornaday, 1882), and more recently by Berger *et al* (1994). Breeding activity begins with the congregation of smaller herds in late July to early August with solitary males entering the larger herd from surrounding areas. These small herds are made up mostly of females, but also contain yearling bulls that are too immature to be on their own as bachelors. Mature bulls (6 years and older) in their prime do the majority of the breeding at the beginning of the breeding season, with males 3 to 5 years of age doing more breeding once the mature bulls have left the herd to resume solitary life (Berger, Cunningham 1994). When the breeding season ends in October the remaining bulls leave the larger herd to seek a more solitary life, or to form small bachelor herds containing 3 to 5 bulls, depending on their ages. At this time females and younger males (1 to 2 years of age) once again split into smaller herds until the next breeding season. Consequently, 50% of the births observed in Badlands National Park, South Dakota occurred within a period of approximately 25 days and 80% within 50 days (Berger, Cunningham 1994).

As the commercial bison industry continues to grow and administrators of government owned herds are required to make difficult management decisions there is a greater need to understand the reproductive biology of bison. Information on the male's role in the seasonal regulation of reproduction would be useful for both conservation and commercial production efforts.

Bison bulls selected for breeding in private herds are subjected to breeding soundness evaluations on a regular basis. Semen evaluation is an entry requirement in many of the select breeding stock sales. These usually occur between October and April. Presently semen evaluations are based on criteria developed for domestic cattle in the absence of better information. These criteria do not take into account possible seasonal fluctuations in semen quality.

Many ruminants around the world exhibit seasonal reproduction, with members of the Cervidae being among the most seasonal. North American wapiti bulls show dramatic increases in circulating androgen levels in late July to early August that are associated with significant increases in scrotal circumference and semen quality towards the end of August, just before the first hinds show estrus (Haigh, Cates *et al.* 1984). Scrotal circumference peaks in early September, decreasing significantly by December, and reaching its lowest value in early April. Semen quality follows much the same pattern, resulting in a physiological cycle in which wapiti stags are infertile for 3 to 4 months of the year (Haigh, Cates *et al.* 1984).

Marked seasonal changes in scrotal circumference and semen quality were also noted in Suffolk, Lincoln, Columbia and Polypay sheep breeds, and, in a few rams, periods of complete azoospermia were observed (Mickelsen, Paisley *et al.* 1981; Mickelsen, Paisley *et al.* 1982). Significant seasonal changes in semen quality have also been observed in many bovine bulls of Western Canada (Barth, Waldner 2002).

An objective of the present study was to determine the presence and magnitude of any seasonal fluctuations in semen quality of bison. The results of this study are expected to give insights into seasonal changes in male fertility. Results may serve as an aid to veterinarians performing semen evaluations during various months of the year, and to biologists interested in conserving genetic material by cryopreserving semen from older bulls.

4.2 Methods and materials

4.2.1 Animal selection

4.2.1.1 Abattoir samples

A total of 288 bison bulls were selected from North West Foods, Ltd. (formerly Alsask Cutting Edge Foods, Ltd) in Edmonton, Alberta to study sperm quality and testicular tissue changes over a period of 12 months. The bulls ranged in age from 18 to 33 months (Grinde

2003). They came from farms in Alberta, Saskatchewan and Manitoba. Samples were collected from the first bulls processed by the abattoir on the chosen day, to a maximum of 30. Both testes, encapsulated in the vaginal tunic, were removed from each bull by severing the spermatic cord proximal to the vascular core. Each pair was placed in separate, individually identified, plastic bags and immediately put on ice to slow decomposition. Samples were transported from the abattoir for 20 minutes to the Veterinary Pathology Lab (VPL) in Edmonton, Alberta for processing. Live weights were unavailable for most bulls and, therefore, a warm carcass weight was recorded for analysis.

4.2.1.2 Mature live bull samples

Semen was collected from mature bison bulls in November, February, April and June. There were 21 bulls from 3 farms in Alberta (Farm 1, n = 9, 56º 39' N, 111º 13' W; Farm 2, n = 7, 54º 34' N, 113º 31' W, and Farm 3, n = 5). The farms were selected because the owners were willing to allow the use of their breeding bulls. The age of the bulls ranged from 2.5 to 8 years. No collections were performed during the rut (July through to the end of September) since bison bulls cannot be safely handled at this time.

Bulls on Farm 1 received a 25% alfalfa, 75% fescue hay fed ad-libitum, and free choice minerals (Breeder mix - #15-7455, Unifeed). Bulls at Farm 2 were maintained on tame pasture supplemented with first cut alfalfa hay (~8% CP) and free choice winter mineral and salt. The bulls on Farm 3 had free access to grass hay, minerals and water. Feeding strategies for all groups did not change during the study. All bulls were kept separate from females in other pastures but maintained fence line contact.

4.2.2 Sample collection and evaluation

4.2.2.1 Epididymal sperm

The vaginal tunic was removed from each testicle and it was weight to the nearest gram. Testicular abnormalities were noted and monorchid bulls were removed from the study. The distal one third of the cauda epididymis, with remaining deferent duct attached, was dissected from the testis with a scalpel. Sperm were flushed from the cauda-epididymis into a Petri dish by inserting a blunted 21 gauge needle into the deferent duct and flushing the tubules with 3 ml of PBS. Two smears were made from each flush and labeled with a sample number and date.

Slides were prepared with Eosin-nigrosin and Feulgen stains as described by Barth *et al* (1989). The remainder of the sample was placed into a labelled conical tube with 1 ml 10% buffered formalin for transport and storage at the spermatology laboratory at the Western College of Veterinary Medicine (WCVM).

4.2.2.2 Fresh Semen

Samples were collected by electroejaculation using a Pulsator IV (Lane Manufacturing Co., Denver, Colorado) and a non-segmental (60mm) probe. All collections were done outdoors on bulls restrained in a hydraulic squeeze designed specifically for bison. Bison were handled in the same manner as described in the previous chapter. The rectum was cleared of feces and the accessory genital glands were massaged for a minimum of 1 minute to stimulate the ampullae and vesicular glands. The ejaculator probe was inserted and held in place by positioning the animal's tail between the connector arms. Stimulation was delivered in a pulsatile rhythm with increasing intensity until the bull ejaculated as described for bovine bulls (Barth 2000). Pulse stimulation was applied for 2 to 3 seconds with an interval of 1 to 2 seconds between each pulse. Success or failure of penile protrusion, erection, and ejaculation was recorded on each occasion and any abnormalities of the penis and prepuce were noted. If the 1st was unsuccessful the bull was allowed to rest for a minimum of 5 minutes before a 2nd collection was attempted. If no sample was obtained after 2 attempts it was recorded as an unsuccessful collection and no further attempts were made on that day. All procedures were approved by the University Committee on Animal Care and Supply of the University of Saskatchewan.

Semen was collected in a test tube with a plastic cone attached was mounted on a handle fitted with a water canister and thermometer. Water within the canister was maintained at 37ºC. Between collections the apparatus was placed in a water bath maintained at 37ºC. For collections done in cold weather the apparatus was placed a in a felt sleeve made from winter boot liners. Immediately after collection the tube containing the sample was transferred to a warm water bath.

4.2.2.3 Semen and sperm evaluation

Evaluation of fresh semen was done immediately after collection in a heated building adjacent to the squeeze. A water bath and warm stage, each set to 37ºC, were used to keep

materials warm and to reduce cold shock. All fresh semen and stained smears were evaluated by myself throughout the study. Guidelines for bovine bulls given by the Western Canadian Association for Bovine Practitioners (Barth 2000) were used for evaluating all samples.

Ejaculate quality was judged on the basis of density, volume, gross motility, individual progressive motility, morphology, live/dead ratio, and concentration. Volume was measured to the nearest 0.50 ml using the 1 ml calibrations on the collection tubes. An estimate of density was made using a 4-point scale based on color and appearance (Barth 2000). Translucent or watery samples were classified as 1 (poor). Samples with a slightly skim-milk or opaque milk-like appearance were classified as 2 (fair) and 3 (good), respectively. Samples in category 4 (very good) appeared thick, grainy and cream in colour.

Gross motility was estimated by microscopy using undiluted semen under bright field microscopy at 40X magnification. Categories for gross motility ranged from 1 to 4. Samples with no swirling activity and very little progressive motility were judged as class 1 (Poor). Samples with no swirling activity but evident progressive motility were classed as 2 (fair). When slow swirling activity was seen the sample was classified as 3 (good) and a sample with vigorous swirling activity containing vanishing dark eddies was judged as 4 (very good).

Individual progressive motility (IPM) was determined by duplicate counts of motile sperm using fresh semen diluted in phosphate buffered saline (PBS) solution under 400X magnification without phase contrast. Sperm concentrations were determined by counting the number of sperm in 4 square mm on each side of a hemacytometer. Concentrations were recorded as the number of sperm per ml of ejaculate (10^6/ml). The product of the concentration (10^6/ml) and volume (ml) of ejaculate gave the total number of sperm per ejaculate (10^6/ej).

Oil emersion microscopy on Eosin-nigrosin stained smears was used to evaluate sperm morphology (Barth 2000). Two differential counts of 100 sperm at 1000X magnification were performed. A 3rd count of 100 sperm was performed when the total of normal sperm for the first 2 counts differed by more than 5%. Percentage of live sperm was determined by performing 2 counts of 100 sperm per slide. Morphology counts were averaged for each slide, with the average being used in the final analysis.

Sperm abnormalities were classified into head, midpiece and principle piece defects, other, and normal sperm according to descriptions used for bovine bull evaluations (Barth 2000). Head defects included knobbed acrosomes, nuclear vacuoles, and misshaped heads. Distal

midpiece reflexes (DMR), proximal cytoplasmic droplets and mitochondrial abnormalities were classified as midpiece defects. A sperm was arbitrarily determined to have a DMR when the angle of the bend in the distal midpiece was less than 90º. With an angle greater than 90º, and in the absence of any other abnormalities the sperm was classified as normal. Kinked or coiled tails and multiple tails were classified as principal piece defects. The remaining 3 categories were detached heads, other, and normal cells. Distal cytoplasmic droplets and abaxial tails were not classified as defects as they are not known to affect fertility in bovine bulls (Barth 2000). These same procedures were used to evaluate stained smears of samples collected from bulls at the abattoir.

4.2.2.4 Histology

At the same time that semen smears were prepared, 10 pairs of testes were chosen randomly for histological analysis by pulling sample numbers from a hat. A 1 cm x 1 cm x 0.5 cm section of parenchyma was cut from each of the upper, middle and lower thirds of one testis of each pair. Tissue sections were fixed in marked containers containing Bouin's solution and shipped to the WCVM. Samples were removed from the Bouin's solution approximately 20 hours later and placed in jars containing 70% alcohol.

Tissue sections were embedded in paraffin and 5 μm thick sections were stained with Harris haematoxylin and eosin, and periodic acid-Schiff. For every cross section mounted and stained, 10 round seminiferous tubules were arbitrarily chosen for measurements. Both lumen and tubule diameter were measured with an ocular micrometer scale that was calibrated with a stage micrometer to the nearest tenth of a micron. The thickness of the epithelium was estimated by subtracting lumen diameter from the tubule diameter and dividing by two. Spermatogonia, spermatocytes, elongated and round spermatids were counted and the stage of spermatogenesis was indentified for each tubule measured, as described by Curtis and Amann (1981).

4.2.3 Testosterone assay

4.2.3.1 Sample preparation

Fecal samples for all bulls were collected prior to electro-ejaculation and kept cool in transit to the laboratory. Samples were transferred to 20 ml scintillation vials and kept frozen at -20°C until they were lyophilized. Dried fecal samples were manually ground using a metal

weighing spatula. Approximately 0.2 g of each sample was placed into 13x100 mm polypropylene tubes. For testosterone extraction, 5 ml of AnalaR Methanol (MeOH) was added to each tube and the contents vortexed for 8 periods of 1 minute each over a 2 hour period. Tubes were refrigerated overnight, vortexed again the following morning, and then centrifuged for 20 minutes at 1500g. One ml of supernatant was aliquotted into polypropylene tubes and air dried. Aliquots were reconstituted with 50:l absolute ethanol (EtOH) followed by 1.0 ml phosphate buffered saline (PBS). These samples were capped, vortexed and kept overnight in a fridge and assayed the next day.

4.2.3.2 Methanol extraction efficiency

The efficiency of testosterone extraction from bison feces using methanol was assessed. Testosterone (150:l) was added to each of four, 0.2g samples of dried feces and these were subjected to the extraction procedure described above. However, when using methanol for extraction of testosterone from bison feces, the extract assumes a green colour that interferes with the scintillation counter. To correct for colour interference, the fist 4 samples were compared to 4 additional samples (0.2g each) to which 150:1 of ^{3}H testosterone was added. An additional fecal sample was extracted (no ^{3}H testosterone added) for use as a colour control for the total counts. One millilitre of each sample extract was aliquotted into scintillation vials and air dried. Aliquots were reconstituted with 50 :l of absolute EtOH and 0.5 ml PBS. Scintillation fluid (BetaMax, 3 ml, MP Biomedicals) was added to all scintillation tubes, and the radioactivity measured. The average recovery of testosterone (n=8) was 95.6%, indicating that this method of methanol extraction was reliable when extracting available testosterone from bison feces.

4.2.3.3 Radioimmunoassay of testosterone

Fecal testosterone was measured using a competitive binding radioimmunoassay (RIA) where the test (or standard) antigen competes against a constant amount of tracer-labelled antigen for a limited, constant number of antibody sites. If a standard or test sample contains a low concentration of the antigen, after the competitive binding reaction reaches steady-state, there will be a high concentration of tracer bound to the antibody and the radioactive counts will be high. Conversely, if there is a high concentration of antigen in the sample, after the competitive binding reaction reaches steady-state, there will be a low concentration of tracer

bound to the antibody and radioactive counts will be low. These counts can be used to calculate a standard curve, and then to calculate the content of the antigen in a sample.

Fecal testosterone in this study was measured using a solid phase iodinated radioimmunoassay (Coat-A-Count® Total Testosterone assay kit; Diagnostic Products Corporation; Los Angeles, CA). The minimum detectable limit of the assay, as provided by the manufacturer, is 0.04 ng/mL, which would convert to approximately 1 ng/g feces (0.04 ng/mL * 5mL/ 0.2 g feces). A standard curve for testosterone was prepared by adding known amounts (0.05, 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 ng/ml) of testosterone to PBS. Two methods were used to verify that the assay used and the standard curve were reading and calculating testosterone in a reliable fashion. Firstly, known amounts (0, 0.5, 1.0, 2.5 and 5 ng/ml) of testosterone were added to an extract containing 2.5 ng/ml of testosterone. Secondly, a known sample of high concentration (7.5 ng/ml) was serially diluted (1/2, 1/5 and 1/10) in PBS, as was a known sample of low concentration (2.5 ng.ml). All samples were analyzed in duplicate and all resulting curves had similar slopes to the standard curve created using PBS. Intra-assay coefficients of variation were 9.8%, 7.9% and 4.5% for extracts with concentrations of 0.50, 1.91 and 7.33 ng/ml, respectively. Inter-assay coefficients of variation were 12.1% and 11.0% for extracts with concentrations of 0.52 and 1.72 ng/ml, respectively. This assay was therefore shown to reliably measure bison fecal testosterone. Final results are expressed as nanograms of testosterone per gram of feces (ng/g).

4.2.4 Data analysis

4.2.4.1 Abattoir samples

Data was grouped into 4 seasons (periods of 3 months) in relation to the summer solstice (June 21) for the purpose of statistical analysis. Data from January 1st through March 31st were grouped as Season 1 (S1, n=57). April 1st through June 30th data made up Season 2 (S2, n=71) with data from July 1st through September 30th being Season 3 (S3/BS, n=56). Season 4 (S4, n=45) included data from October 1st through December 31st. Season 3 (July to September) corresponds to the late July to mid-September breeding season of bison observed under natural conditions in North America (Berger, Cunningham 1994).

The precise age of the slaughtered bulls was unknown but it was estimated from their carcass weight and the time of year. As the majority of bison under commercial bison

production are born in May; ages of bulls in the abattoir at any given month can be reasonably estimated. Using age estimates, an attempt was made to correct for age by eliminating possible outliers based on carcass weight in relation to the month of the year. Industry practice is to bring long yearlings (animals 16 to 17 months of age) off grass and move them into a feedlot or onto a feeder program. Bulls are fed over the winter and a majority of them go to slaughter at 23 to 24 months of age. However, bulls are shipped anytime from 20 to 31 months of age and it is possible for bulls to arrive for slaughter as young as 17 months of age and as old as 36 months of age. These differences in age at slaughter are due to the relatively low levels of genetic selection for weight gain and different feeding strategies used by the industry to achieve slaughter weight. To be more certain of including bulls between 20 (at January collection) and 31 months of age (at December collection) the outlying heavy bulls in January, February, March, April and May were excluded from the data set as they were assumed to be over 31 months of age. For the months of August, September, October, November and December the outlying lighter bulls were excluded as possibly being under 20 months of age. This was most effectively accomplished by only including bulls within 50 kg of the mean hot carcass weight in the analysis (n=229, range of 11 to 26 bulls). An attempt to correct for age was necessary to improve confidence in conclusions about the effect of season on semen quality.

Differences in sperm morphology, testicular weight, testicular histology and carcass weight for seasons 1, 2 and 4 from season 3 (breeding season) were analyzed using generalized linear model (GLM; PROC GENMOD, SAS 8.2 for Windows, SAS Institute, Inc.; Cary, NC). Season 3 (S3/BS) was used as the reference season. To test the hypothesis that bison from the abattoir were still undergoing sexual maturation, linear regression was used to illustrate the relationship between carcass weight and month. Pearson's correlation coefficients were calculated for carcass weight and month, carcass weight and testicular weight, as well as testicular weight and month. The level of significance was set at 0.05.

4.2.4.2 Mature live bull samples

Semen quality parameters, body weight and fecal testosterone from mature live bulls were analyzed using generalized estimating equations model (GEE; PROC GENMOD; SAS 8.2 for Windows, SAS Institute, Inc.; Cary, NC). The reference collection was set for June (pre-breeding season) as it was the closest collection prior to the breeding season. Semen quality

traits analyzed were sperm morphology, individual progressive motility, gross motility, sperm concentration, density and volume. The rational for using GEE was discussed in the previous chapter and the same applies in the analysis of these data. The level of significance was set at 0.05.

4.3 Results

4.3.1 Abattoir samples

Fifty-nine of the 288 bulls sampled at the abattoir were excluded from the final analysis: sperm could not be flushed from the epididymis of 8 bulls, 1 was monorchid and 50 had carcass weights that differed by more than 50 kg range around the mean. Differences in carcass weights over time (n = 229) are shown in Figures 4.1 and 4.2. There was a linear increase ($Y = 261.9 + 5.70X$, $r = 0.84$, $P<0.05$) in carcass weights from January to December indicating that the bulls were still growing at the time of slaughter.

Changes in testis weight are shown in Figure 4.3. Mean testis weights were significantly lighter in S1 (358.0±52.5 g), S2 (434.9±63.6 g) and S4 (442.5±76.4 g) than in S3/BS (523.6±67.2 g). Mean testis weights were lightest throughout late autumn and early spring (S4 and S1) and heaviest throughout the breeding season of July to the end of September (S3/BS). Testis weights were only moderately correlated (r=0.44) with hot carcass weights for the 12 month collection period.

4.3.2 Mature live bull samples

Due to aggressiveness and handling difficulties one mature bull from Farm 2 was not collected in the June semen collection. Semen collections on Farm 3 were performed by the local veterinarian who did not collect data on body weights, IPM, or sperm concentration, or obtain feces for fecal testosterone assay. Fecal samples were not collected from Farm 2 for November.

Mean (±SD) body weights for mature bulls for the four collections are shown in Table 4.1. Four body weights from the spring collection and 7 weights from the June collection are missing for bulls from Farm 1 due to malfunction of the scale. Body weights for bulls from Farm 3 are missing for the January collection (25/01/02) as the scale seized due to extreme weather conditions (-31ºC). Body weights for bulls from Farm 3 for the June collection are not

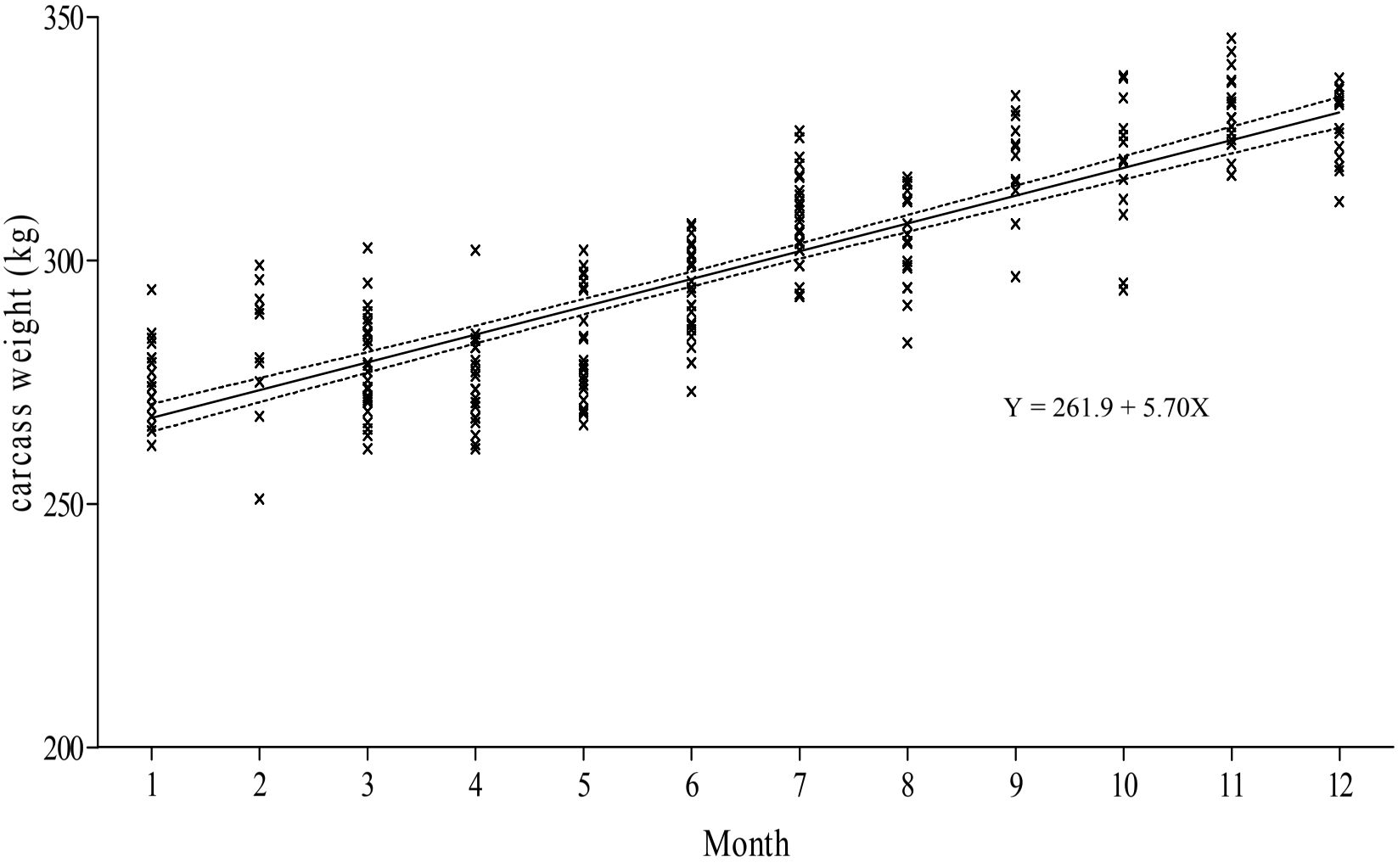

Figure 4.1 Distribution of warm carcass weights for bison over a period of 12 months (n=229). 1=January to 12=December.

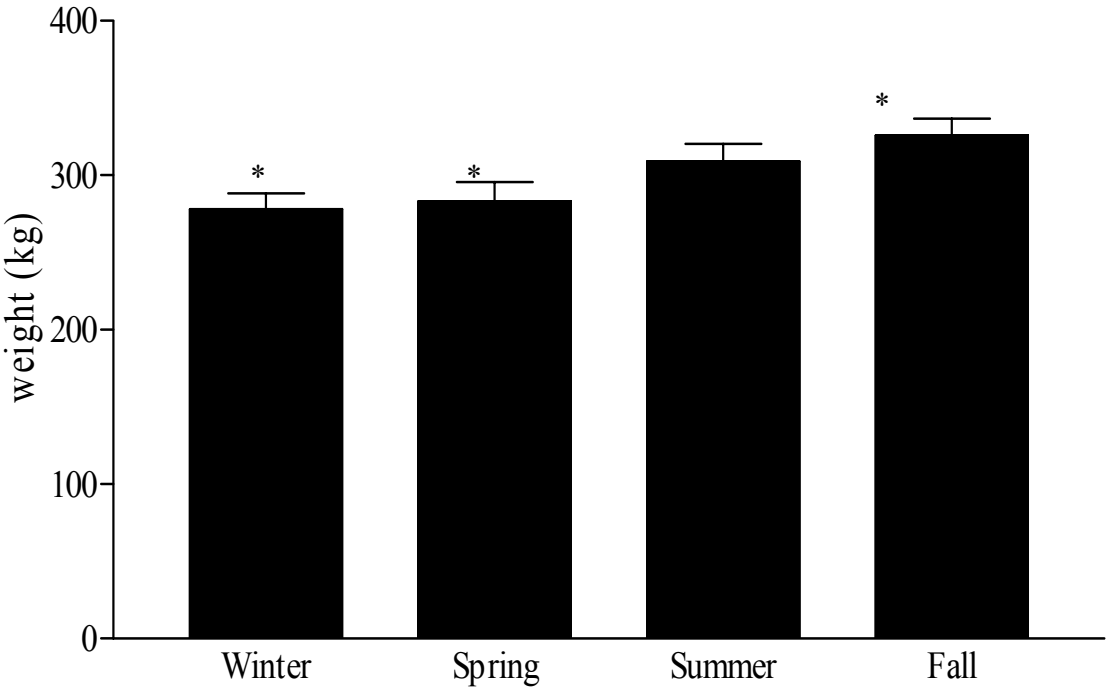

Figure 4.2 Mean (+SD) seasonal warm carcass weights for bison for a 12 month period. Winter - S1 (Jan-Mar), n=57; Spring - S2 (Apr-Jun), n=71; Summer - S3/BS (Jul-Sept), n=56; Fall - S4 (Oct-Dec), n=45). *Are different from S3/BS; $P<0.05$.

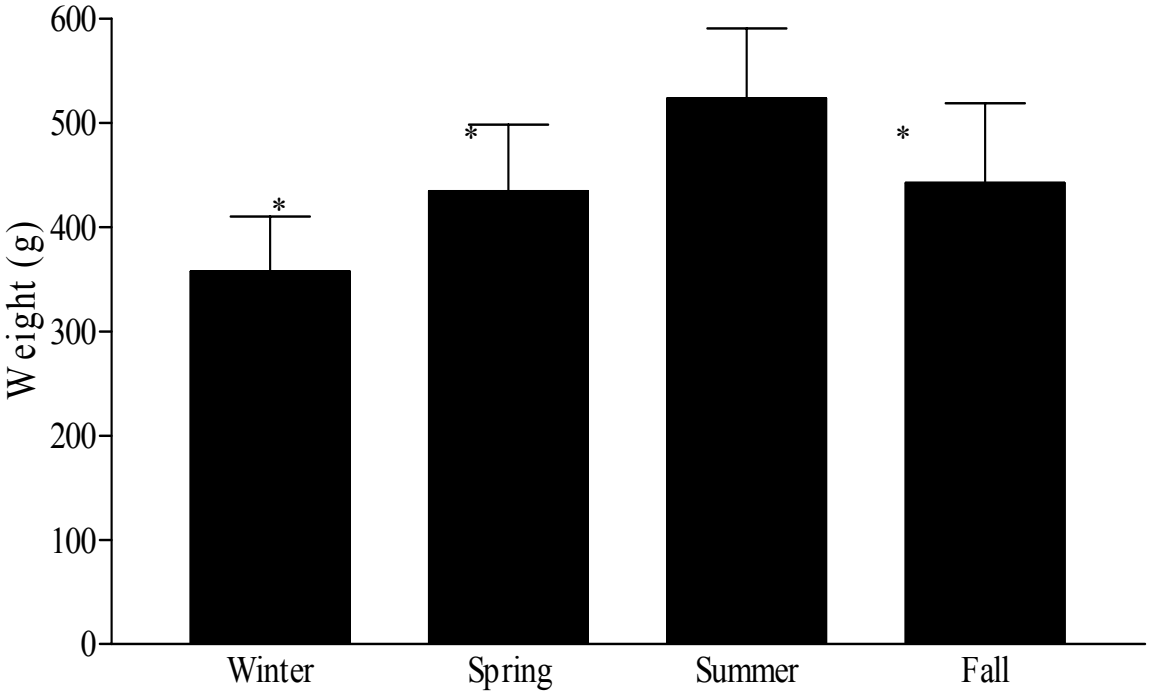

Figure 4.3 Mean (+SD) seasonal combined bison testes weights for a 12 month period. Winter - S1 (Jan-Mar), n=57; Spring - S2 (Apr-Jun), n=71; Summer - S3/BS (Jul-Sept), n=56; Fall - S4 (Oct-Dec), n=45. *Are different from S3/BS;$P<0.05$.

Table 4.1. Mean (±SD) body weight, fecal testosterone concentration, and semen characteristics of mature bison bulls at four times during the year.

Characteristic	Pre-Breeding* (June)	Post-Breeding (November)	Winter (January)	Spring (April)
Body weight (kg)	731.4±137.9	776.7±142.6	723.6±121.7	723.9±178.7[1]
Testosterone (ng/g)	128.6±67.4	63.1±31.7[1]	53.8±27.8[1]	48.5±33.3[1]
Sperm Morphology (%)				
Normal	73.8±9.1	44.1±24.3[1]	50.5±28.5[1]	60.0±27.3
Head defects	19.2±8.5	29.7±17.0[1]	34.1±23.6[1]	14.1±10.2[1]
Midpiece defects	3.8±3.6	15.3±17.8[1]	11.4±9.6[1]	11.6±13.3[1]
Tail defects	0.3±0.3	0.7±1.3	2.5±4.1	2.9±4.5
Proximal droplets	0.7±0.7	2.7±3.9	4.1±6.1	5.1±10.1[1]
IPM (%)	68.8±14.2	55.6±20.5[1]	44.1±24.9[1]	50.3±25.4[1]
Sperm concentration (10^6/ml)	711.3±616.5	824.5±529.6	510.9±481.7	520.4±701.7

* reference season. All other seasons were compared to the pre-breeding sampling period.
[1] numbers in the same row are significantly different ($P<0.05$) from the value for the reference season.
IPM – individual progressive motility

available as no collections were performed on these bulls during this period.

Analysis of body weights showed an interaction between the collection period and farm. The results are reported as differences in body weight between collections, adjusted for farm of origin. Even though differences between collection periods were not statistically significant, the bulls gained weight between June and November (Y = -4.8, 95%CI = -32.7 – 23.2, P>0.05; see Figure 4.1). They continued to lose weight over the winter (Y = -89.7, 95%CI = -145.5 – -33.9, P<0.05) and but started to regain weight by April (Figure 4.1). However, mean weights were less in April than June (Y = -19.9, 95%CI = -32.1 – -7.8, P<0.05). Mean bull weights on Farm 1 were heavier (Y = 211.3, 95%CI = 332.0 – 90.5, P>0.05) than from Farm 3 and only slightly heavier (Y = 106.7, 95%CI = -239 – 25.8, P=0.11) than bulls from Farm 2.

4.3.3 Semen evaluation

4.3.3.1 Epididymal sperm

Spermiograms for eosin-nigrosin and Feulgen stained slides from abattoir samples are shown in Figure 4.4. There were no significant differences in the proportion of normal sperm between S1, S2 and S3/BS for slides stained with eosin-nigrosin (Table 4.2). Slides from S4 had a mean of 9.4% more normal sperm (mean ± SD) compared to S3/BS (70.8±19.4% and 61.3±18.5% (P<0.05), respectively). Fewer (P<0.05) head defects were observed in S4 compared to S3/BS, which would account for the increased number of normal sperm for that season. More (P<0.05) midpiece and "other" defects were observed in S1 than any other season, including the breeding season (S3/BS). Levels of these defects were not enough to significantly reduce the proportion of normal sperm in a single sample. There were no significant differences from the breeding season (S3/BS) in the number of principle piece defects, proximal cytoplasmic droplets, and detached heads.

Differential counts for Feulgen stained slides gave similar results (Table 4.2). Head defects are generally easier to detect with Feulgen's stain than with eosin-nirgrosin stain and this was reflected in the results. S1 samples showed significantly more abnormal sperm than in S3/BS (-9.6%, 95%CI -18.3 – -1.0%, P<0.05). Although there were no differences in the proportion of normal to abnormal sperm from S2 to S3/BS, this proportion increased significantly from S3/BS to S4 (10.5%, 95% CI 1.5 – 19.6%, P<0.05). Of the head defects observed, the diadem defect had the greatest impact with a mean of 9.4% fewer (95% CI 17.5 –

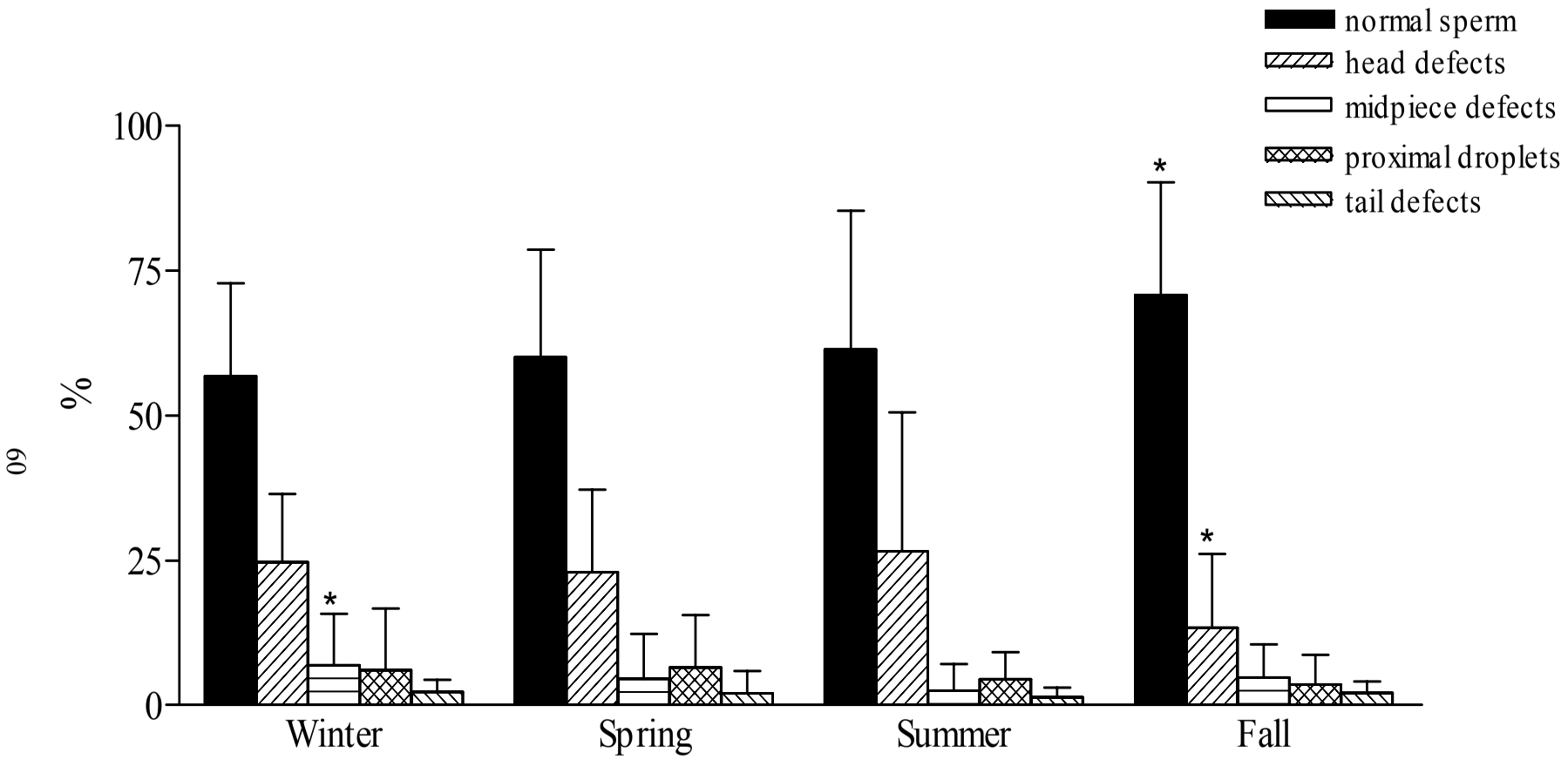

Figure 4.4 Mean (±SD) percentage of bison sperm cell characteristics for epididymal sperm during four seasons using Eosin-nigrosin stain. Winter – S1 (Jan-Mar), n=57; Spring – S2 (Apr-Jun), n=71; Summer – S3/BS (Jul-Sept), n=56; Fall – S4 (Oct-Dec), n=45. *Are different from S3/BS:P<0.05.

Table 4.2. Mean (±SD) carcass weight, testes weight, epididymal sperm characteristics, and histology measurements of bison bulls collected at abattoir for four seasons during a 12 month period (n=229).

Characteristic	Season			
	S3-BS* (Jul – Sept)	S1 (Jan – Mar)	S2 (Apr – Jun)	S4 (Oct – Dec)
Carcass weight (kg)	309.4±11.4	278.4±10.4[t]	283.6±12.5[t]	326.2±10.9[t]
Testes weight (g)	523.6±67.2	358.0±52.5[t]	434.9±63.6[t]	442.5±76.5[t]
Sperm Morphology				
Eosin-Nigrosin (%)				
Normal sperm	61.3±23.9	56.8±16.0	60.1±18.5	70.7±19.4[t]
Head defects	26.6±24.0	24.7±11.8	23.0±14.2	13.4±12.7[t]
Midpiece defects	2.5±4.6	6.9±8.9[t]	4.6±7.7	4.8±5.7
Tail defects	2.1±2.0	2.3±2.1	2.1±3.8	1.4±1.7
Proximal droplets	4.5±4.7	6.1±10.6	6.5±9.1	3.4±5.1
Feulgen's stain (%)				
Normal sperm	58.2±26.3	48.5±20.5[t]	55.0±22.8	68.7±19.9[t]
Diadem defect	23.8±25.0	23.8±15.7	20.9±21.8	14.4±16.2[t]
Single apical vacuole	7.2±11.8	6.8±10.2	6.1±6.9	4.7±9.6
Pyriform defects	5.3±6.0	7.2±6.6[t]	6.2±5.6	3.5±3.0
Midpiece defects	6.1±4.8	16.7±18.1[t]	10.7±10.4[t]	5.5±4.0
Histology (μm)				
Tubule diameter	192.1±21.2	168.6±18.9[t]	173.4±20.4[t]	178.3±17.7[t]
Lumen diameter	84.3±20.5	74.4±18.2[t]	73.2±16.7[t]	76.4±15.8[t]
Epithelial thickness	54.0±8.5	47.1±7.18[t]	50.1±8.0[t]	51.0±8.7[t]

*Breeding season. Used as the reference season for statistical analysis.
[t] Numbers in the row are significantly different ($P<0.05$) from the breeding season (S3-BS)

1.1%, P<0.05) diadem vacuoles appearing during S4 than S3/BS. The occurrence of diadem vacuoles did not change significantly from S1 and S2 to S3/BS. The number of pyriform shaped heads in any season did not differ significantly from the breeding season except in S1 when this defect was seen more often. Midpiece defects were significantly greater in S1 and S2 than in S3/BS but not S4.

4.3.3.2 Fresh semen

Sperm morphology data from mature bulls are shown in Table 4.1. There were significant between-farm differences in the proportion of normal sperm to sperm with head defects. Therefore, calculations for differences between collections were adjusted for between-farm differences. The proportion of normal sperm for samples collected in June were higher than those for samples collected in November, January or April, but the differences from April were not statistically significant. When compared with June, head defects were significantly higher in November and January and lower in April. Semen samples from bulls on Farm 1 had consistently higher levels of normal sperm than those from Farm 3; but neither farm differed significantly from Farm 2.

Changes in midpiece defects, proximal cytoplasmic droplets and tail defects were observed, but levels of these defects occurred at relatively low levels (Table 4.1). On average, midpiece defects decreased in frequency of appearance from the November to June collections. Midpiece defects were at their highest levels in November and significantly decreased in frequency by the June collection. Levels were still significantly higher in January and April than in June.

When the occurrence of proximal cytoplasmic droplets was averaged across the three farms they increased slightly from November to April. Levels of proximal cytoplasmic droplets decreased significantly from April to reach their lowest levels in June. No significant differences were noted between farms. There were no differences observed in the level of tail defects between seasons and between farms.

Data for semen quality parameters are recorded in Table 4.1. Although no differences were seen between farms for IPM there were differences over the 12 month collection period. Individual progressive motility was lowest in the January collections and highest in the June collections. Individual progressive motility was significantly improved for the June collections

when compared with the November, January, and April collections. Using the June collection as the reference collection there were no significant differences for sperm concentration, gross motility, volume and density. Data on the above mentioned semen parameters were not collected for bulls from Farm 3 as the local veterinarian did not record them. Samples from Farm 3 were collected by project personnel (25/01/02) on 1 occasion, but even though IPM was recorded weather conditions (-31ºC and wind) exposed the semen sample to cold shock and motility numbers were deemed invalid. Farm 3 was therefore omitted from the analysis of individual progressive motility.

4.3.4 Histology

Results of germ cell counts and tubular diameter measurements are reported in Table 4.2. Seminiferous tubular diameter, lumen diameter and epithelial thickness were all significantly greater in S3/BS than in any other season. The total number of spermatogonia in the breeding season (S3/BS) did not differ significantly from other seasons. However, the number of spermatocytes, round spermatids and elongated spermatids in the tubules were all significantly higher during the breeding season, in concordance with the larger tubule diameters and epithelial thicknesses observed during this season.

4.3.5 Testosterone assay

Fecal testosterone concentrations were analyzed for bulls from Farms 1 and 2 with the results listed in Table 4.1 and shown in Figure 4.5. Significant differences in fecal testosterone concentrations were observed between sample collection periods. Concentrations were highest for the June collection and decreased in each subsequent collection to reach their lowest levels in the April. No fecal samples were collected at Farm 3 and there were no significant differences between Farms 1 and 2.

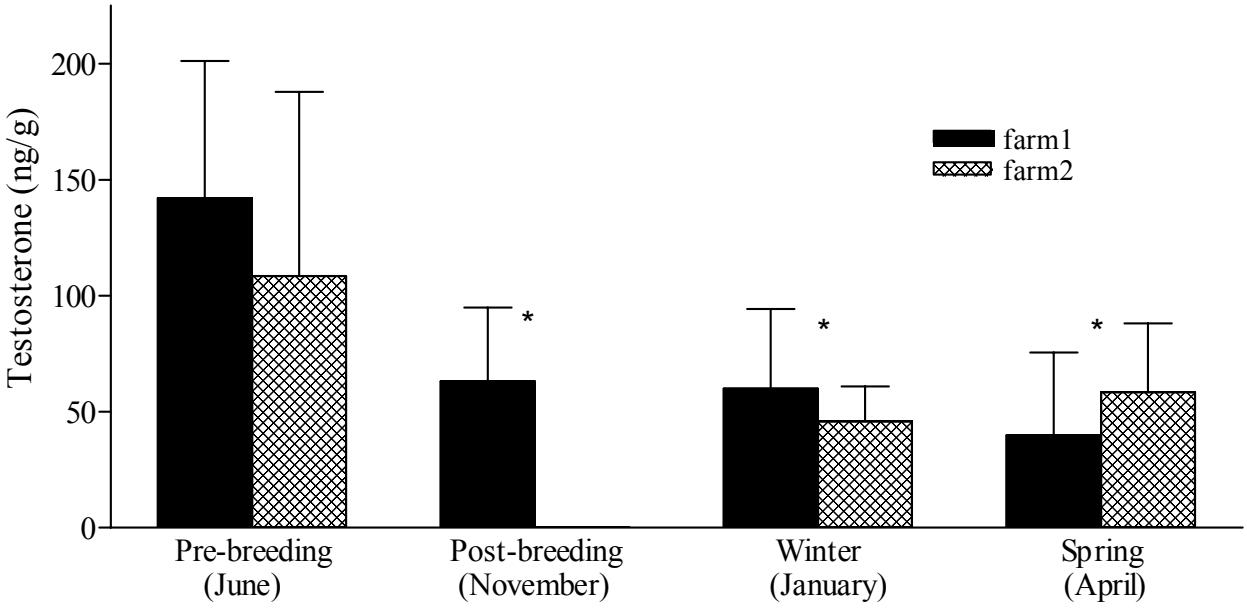

Figure 4.5 Mean (+SD) bison fecal testosterone concentration at 4 sample collections over 1 year (Farm1, n=9; Farm2, n=7). Data for fecal testosterone concentration was not collected for Farm1 in November.

4.4 Discussion

There are no previously published scientific reports on the changes in semen quality and physical characteristics of the testes in relation to time of year. Keen, Rupp *et al.* (1999) attempted to evaluate breeding soundness examination procedures in bison bulls by testing bulls in October every year for 5 years. However, the researchers made no attempt to determine whether testing at various times of the year would influence semen quality parameters measured. To my knowledge the present study is the first attempt to investigate semen quality in bison bulls over a period of 12 consecutive months.

4.4.1 Abattoir samples

The study of sperm morphology from the abattoir samples did not provide a distinctive seasonal pattern. Haigh *et al.* (1984) found that semen quality in elk stags improved quite rapidly shortly before the breeding season and remained elevated during the rut (September to end of November). Barth *et al*. (2002) found that the proportion of beef bulls with satisfactory semen quality increased in late spring and early summer.

However, epididymal samples from slaughtered bison bulls showed that the proportion of morphologically normal sperm was highest in the fall, not the summer. There was no apparent improvement in semen quality from winter and spring to summer. It was expected that if a seasonal pattern existed, the percentage of normal sperm would increase during the breeding season (S3/BS – July to end of September). In this study the percentage of normal sperm was increased in the fall, the season after breeding activity in bison has ceased.

It is the current practice to slaughter most bison between the ages of 18 to 36 months. It was concluded in Chapter 2 bison bulls between the ages of 13 and 24 months are in the early or intermediate stages of sexual maturation. The combination of these 2 factors may explain why the expected results in sperm morphology were not obtained in this study. Assuming that bulls used for the present analysis were first sampled at 20 months of age and the last sampling occurred at 32 months, many of the bulls in this part of the study were progressing through puberty. Therefore the changes observed in sperm morphology may have been a result of maturation rather than time of year.

I was unable to determine if an interaction of age and season were responsible for the changes seen because the precise age of the bulls was unknown. The time at which bison are

sent to slaughter is determined by body weight and not age. Even though most bison are born within two months in the spring we cannot assume that all bison appearing at the slaughterhouse in April or May are 24 months of age. Determining age at slaughter was attempted by taking a digital image of incisors on the kill floor. However, plant personnel felt this procedure slowed the processing line too much and did not allow us to continue with it.

An employee of Veterinary Pathology Lab in Edmonton, Alberta collected all samples from the abattoir. The epididymal samples gave spermiograms of poor quality for both the eosin-nigrosin and Feulgen stained slides. Improper handling and storage of samples and materials during and between collections, and inexperienced personnel preparing the slides most likely contributed to this. Sperm on these slides were often extremely difficult to evaluate because cells were too dense and there was no clear definition between stain and cell. As a result the proportion of abnormal sperm may have been over- or underestimated. Therefore, sperm traits reported for samples taken from the abattoir may not accurately reflect sperm morphology throughout the year.

Dramatic changes in scrotal circumference throughout a 1-year period are observed in mature wapiti stags (Haigh, Cates *et al.* 1984). There are rapid increases in mean scrotal circumference in early September, just as the animals are entering the rut. Shortly after the increase there is a definite, but gradual, decline in circumference measurements and by April they reach levels that were last observed in late August. These fluctuations in testicular size were attributed to the seasonal changes occurring in parenchymal tissue; which is made up of the seminiferous tubules interstitial tissue, of elk stags (Haigh, Cates *et al.* 1984).

Even though scrotal circumference could not be measured in this study, a combined testes weight was calculated for the bulls sampled at the abattoir to test the possibility of seasonal changes in parenchymal tissue mass. Testes were significantly heavier during the summer period (S3/BS) than at any other season with the testes being lightest in the fall. This may be an indication of seasonality, although it contradicts with the observations of semen quality. It was expected that variation in testicular weights would follow that of semen quality throughout the seasons.

It is well understood that in domestic beef bulls, a high correlation exists between scrotal circumference and paired testes weight (Coulter, Foote 1979) and parameters of semen quality (Barth 2000). Keen, Rupp *et al.* (1999) concluded that scrotal circumference was positively

correlated with body weight and the percentage of normal sperm in bison bulls between the ages of 28 and 30 months.

Results of this study in bison aged between 28 and 30 months differ from Keen's, as bulls had improved sperm morphology at a time when paired testes weights were low. In the present study, there was no correlation between sperm morphology and testes weight, or carcass weight. Because the correlation between scrotal circumference measurements and semen quality traits is high in beef bulls it would make sense to assume that such a relationship also exists in bison bulls. However, more work needs to be done to positively correlate scrotal circumference measurements for bison bulls with sperm cell and semen quality. Difficulty in obtaining accurate scrotal measurements for bison bulls may hinder immediate progress in this area.

There are no standard feeding practices for finishing bison and they are slaughtered at various ages depending on body weight (Grinde 2003). One could argue that bulls slaughtered in the fall were younger than those slaughtered in the summer since age at slaughter was unknown. A proportion of producers finishing bison aim to have animals finished to slaughter weight by the time bulls are 18 to 22 months of age, corresponding with the winter season. Other producers aim to have bulls finished by 24 to 28 months of age, corresponding to the spring season (Grinde 2003). It is most likely however, that bulls slaughtered in the fall were older than those slaughtered in summer as both the carcass weights and the percentage of normal sperm were higher in the fall.

Histological sections of the testes followed the same seasonal trend that was seen in testicular weights. Previous studies have shown that testicular weights provide an accurate estimate of the amount of sperm producing parenchyma and the total sperm production (Coulter, Foote 1979). Not surprisingly, the diameter of the seminiferous tubules was greatest in the summer season (S3/BS). Changes in both lumen diameter and epithelial thickness contributed to changes in tubular diameter. Spermatocyte, and round and elongated spermatid numbers were all increased during S3/BS from all other seasons. This agrees with the changes observed in paired testes weights.

It can not be determined whether the increases observed from S1 to S3/BS are due to the bulls increasing in age or to seasonal and environmental influences. The decrease observed in both tubular measurements (tubular diameter and epithelial thickness) and germ cell numbers during S4 from S3/BS may be an indication that bison undergo changes in reproductive potential

in relation to changing seasons. Moreover, that tubule diameter measurements observed in S4 (fall) are slightly greater than in S1 (winter) is further indication that bison in S4 were older than bison from S1.

As epithelial thickness and seminiferous tubular diameter decreased in S4 there were also significant decreases in the number of spermatocytes, and in round and elongated spermatids per tubular cross section. This agrees with observations made in Ile de France rams that undergo moderate seasonal changes in reproductive potential in Britain (Lincoln 1981). Lincoln (1981) reported that decreases in tubular diameter were the result of a reduction in the number of germ cells that successfully complete spermatogenesis, and also reported that there was a close relationship between seasonal changes in testicular weight and spermatogenesis. An out-of-breeding-season failure in the completion of spermatogenesis leads to a decreased germinal epithelium thickness and reduced tubule diamter. The degree to which spermatogenesis is not completed and testicular regression occurs varies between species (Lincoln 1981). In non-seasonal breeders such as the domestic bovine bull these changes are small and have no great effect on reproductive potential. On the other extreme, testicular regression can be severe with a failure of all germ cells to fully complete the spermatogenic cycle, thereby rendering males such as the North American wapiti sterile for parts of the year (Haigh, Cates *et al.* 1984; Lincoln 1981).

4.4.2 Mature live bull samples

It was planned that data from mature bulls be collected from a greater number of bulls from various farms in Alberta and Saskatchewan. Participation in this study guaranteed anonymity; but unfortunately several producers removed their bulls from the study, as they feared others in the industry would know any unfavourable results from their bulls. Therefore, data collected from mature bulls was limited to a small number of bulls and a data set with low statistical power.

Differences in average body weights between farms were expected as management practices and animal genetics might differ. Age was not a factor in between-farm differences, as all herds were comprised of bulls ranging from 2 to 8 years of age. One of the biggest genetic factors may have been the fact that bulls from the Farm 1 were wood bison (*Bison bison athabascae*), which are generally larger and heavier than plains bison (*Bison bison bison*) of

comparable age. However, any objective differences between the two types of bison have, to our knowledge, not been investigated, and definitely not for reproductive parameters. Moreover, bulls within herds differed for dam and sire and therefore genetic potentials of specific pedigrees were not available.

Bulls were maintained on different diets and in different environments between farms. Bulls at Farm 1 resided at more northern latitude and had to endure -40^{o}C weather for a period of 2 weeks during the study in January. Bulls in other locations experienced minimum temperatures of -30^{o}C. The effects of differences in latitude as they pertain to reproductive seasonality in bison are unknown. Reproduction in mammals is ultimately controlled by complex interactions between physical factors such as food, rainfall, humidity and temperature (Bronson, Heideman 1994). Photoperiod is known to time this seasonal cycle by working on the reproductive neuro-endocrine system via melatonin.

It was expected that the breeding bulls would lose weight during the breeding season due to a net expenditure of energy; however, they maintained their body weights through that time. The energetic costs of reproduction are mainly behavioural for males, as they need to maintain a physical dominance over other males and also attract females (Bronson 1985). Mating behaviour for bison bulls has been described extensively and it has been observed that bulls reduce the time used for foraging during the breeding season and increase their physical activity by partaking in aggressive encounters with other bulls (Berger, Cunningham 1994). However, under commercial production, bison have constant access to high quality feed and thus loses in body weight may have been minimal and not detected. In addition to this is that there may be a higher ratio of males to females in estrus that fighting in commercial herds may be reduced.

Semen samples from mature bison bulls at 4 times during the year did not show significant changes in sperm concentration, volume, density and gross motility. This contradicts what has been observed in mature dairy bulls where there were significant seasonal changes in concentration, volume and total number of sperm in the ejaculate (Everett, Bean *et al.* 1978). Highest yields for these semen traits were observed in the months of April, May and June. It might be expected that these traits of semen quality would be improved for bison bulls in June, prior to the start of breeding activity. However, it would be inaccurate to draw any firm conclusions because the power of this data set was low.

The fluctuations in the proportion of normal sperm and individual progressive motility

along with the rise in fecal testosterone before the beginning of the breeding season (June) are an indication that bison bulls have a degree of seasonality in their reproduction. It is also evident that any seasonal changes are more pronounced in bison than in domestic beef bulls (Barth, Waldner 2002) but they are not as pronounced as in North American wapiti (Haigh, Cates *et al.* 1984).

Bronson (1985) argued that in environments where females are able to reproduce during nutritionally challenging parts of the year, males should experience very little to no seasonal declines in reproductive potential. This theory could explain the apparent ability of bison bulls in this study to produce adequate quantities of semen throughout the year and show only moderate seasonal fluctuations in semen quality. The synchronous arrival of a majority of the calves in May and June indicates that bison exhibit seasonal reproductive behaviour. Although births in January, February and March are extremely rare, the birth of calves has been reported for all months of the year (Olson 2002).

In conclusion, even though this study was unable to provide definitive levels of quality for various semen traits during different seasons, results do indicate testicular change due to reproductive seasonality. There was some testicular regression because testicular weights and seminiferous tubular diameter were increased in the summer season. Results from bulls at the abattoir combined with observations on the mature bulls included in this study provide some evidence of seasonal fluctuations in semen quality. This study shows that reproductive seasonality in bison bulls warrants further investigation.

Results from this study are useful for producers of bison at a commercial level as well as conservation biologists. Genetic preservation is a concern in the free-ranging bison populations and the cryopreservation of semen has been considered as an option for accomplishing that goal. However, protocols for collecting and freezing semen and the time of year to collect semen have not been established. Veterinarians performing semen evaluations on bison bulls under commercial production now have some objective data to aid them in making decisions on the potential reproductive success of bulls they test. Semen evaluated in the winter will be done with the knowledge that quality may increase in June and July prior to the commencement of the breeding season. Therefore, bulls showing moderate to marginally satisfactory quality in the winter but possessing other desirable genetic traits may warrant another evaluation in late spring before making a definite decision on whether they should be culled from a breeding program.

5. CONCLUSION

Objectives of this project aimed to gather basic information on the reproductive development and physiology of male bison. Prior to this study one could reasonably assess when a bison bull had passed puberty and was able to be reproductively successful. Observational studies have shown, that bison bulls can successfully reproduce at approximately 27 months of age, or by the time they experience their 3rd breeding season. The point during physical development at which bison bulls produce sperm, and how long it takes from that point until they produce semen of adequate quality to pass standards for breeding soundness examinations, has been a guessing game until now. We found that sperm production, as evidenced by the presence of sperm in semen samples, begins at about 13 months of age and the majority of bulls achieve onset of puberty by 17 months. These results also indicate that the majority (75%) had attained maturity at 24 months of age. This type of information is useful in semen testing and in evaluating the future reproductive potential of young bison bulls.

There was also some indication that bison bulls exhibit seasonality in their semen quality but there was not enough information to determine if this influences seasonal reproduction in the species. To unravel the nature of the apparent seasonal reproduction in bison more work needs to be done on both male and female reproductive physiology. The presence of a spring anestrous suggests that female bison are seasonally reproductive by (Rutley 1995) however, there are still many questions to be answered about seasonal aspects of reproductive endocrinology and physiology. There is no information about the mechanisms that regulate the estrous cycle and whether domestication and improved nutrition would subsequently influence this cycle. There is some speculation among bison producers and scientists that with improved nutrition in commercial production bison will lose their seasonality in reproduction. From a biological standpoint, what is the need for seasonal reproduction when adequate feed is available year round?

More research on the basic development and physiology of bison is needed. However, the nature of bison behaviour makes it difficult to conduct research. Many reproduction research procedures are intensive and require the animals to be handled repeatedly, sometimes several times a day. Bison do not lend themselves well to repeated handling, especially on a daily basis.

Even weekly handling is problematic especially when stressful or painful procedures are required. Therefore, research on bison behaviour and methods of handling, as well as ingenuity in the design of handling facilities may be required before more intensive research on reproductive endocrinology and physiology can be undertaken.

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