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Investigation of the mode of ovulation and ovarian cycle in bears

By

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Abstract

Literature focusing on the reproductive mechanisms in bears is often limited and only has a narrow scope. However, with six out of eight bear species being considered threatened or endangered, it seems critical that more information about reproduction be obtained through empirical studies. One of the reasons that little is known about reproduction in bears is due to a variety of reproductive mechanisms that bears have evolved that make studying reproduction difficult. This in addition to their solitary nature, makes long-term studies of wild bears challenging. These reproductive mechanisms include: induced ovulation, pseudopregnancy, delayed implantation (embryonic diapause), seasonal breeding, multiple paternity, and polyestrus (with subsequent ovulation events). The current thesis focuses on two separate, but related, projects that I was involved in, focusing on reproduction in American black bears (*Ursus americanus*) and the polar bear (*Ursus maritimus*). In the first portion of my thesis, I re-examine the ovulation mechanism in American black bears, and in the second portion of my thesis, I work to determine whether a captive female polar bear had ovarian activity.

In bear biology, it has been accepted that American black bears are coitus-induced ovulators, although there is little empirical evidence that supports this. Induced ovulation is a process in which ovulation is caused by an external stimulus. In Chapter 2, we work to determine whether coitus is the only stimulus that induces ovulation in American black bears. We did this using physiological measures of estrus (vulva score), steroid analyses on urine and serum samples during embryonic diapause, and histology of ovarian structures. All of these measures serve as proxies for or direct evidence of ovulation. Females were categorized into three groups: mated, not observed mating, and isolated. These groups were compared to detect differences in estrus length, length of time it takes to exit estrus after mating, progesterone (P4) concentrations during embryonic diapause and estrogen (E2) patterns during estrus. In the current study, we found that the length of estrus and the time it takes to exit estrus were not statistically different between the female groups. Likewise, we found that the

concentrations of P4 during both early and late embryonic diapause were equally elevated. We also found that isolated females exhibited E2 patterns indicative of ovulation. Finally, we confirmed that two of our females that were not observed mating had CL present on their ovaries via histological analysis. From this, we were able to conclude that American black bears are ovulating due to a stimulus other than coitus. We were unable to determine what stimulus, if any, induces ovulation in or if American black bears are spontaneous ovulators due to limitations in study design and the field site.

Similar to American black bears, polar bears are also considered coitus-induced ovulators although there is little empirical evidence supporting this. Because polar bears are considered coitus-induced ovulators, it would be expected that a mature female, housed singly, without any male stimuli would exhibit no signs of pseudopregnancy or ovulation due to no possibility for coital or other stimulation. Although this prediction agrees with the current literature, zoo keepers at the Milwaukee County (MKE) Zoo reported that their 30 year old female was exhibiting the behavioral signs of pregnancy. As result, the MKE Zoo asked Dr. Spady to look into ways to determine whether their female was still cycling. Because P4 is associated with pregnancy and pseudopregnancy, and is known to increase after fertilization and again at implantation, we decided to use this as our measure for ovarian activity. Keepers at the MKE Zoo collected fecal samples whenever possible for four years and then shipped them to CSUSM. In the lab at CSUSM, we used liquid phase extraction to separate the steroid metabolites from the fecal samples. We then measured the concentrations of progestins (P) in the samples via an EIA. We found that in 2010 and 2014, the female from the MKE Zoo exhibited P patterns that match what would be expected in a pregnant/pseudopregnant bear. In 2011, we determined that the female was not cycling and likely exhibited a luteal cyst which would have caused elevated P concentrations at times that would otherwise not be expected. In 2015, we also suspected that this female exhibited a luteal cyst due to elevated P concentrations early in the year, but because we do not have samples from later in the year, we were unable to determine if this female exhibited an active luteal phase and was therefore cycling. Because we were able to observe an active luteal phase in this female, we can infer that this female also likely ovulated. Because this female was isolated throughout the study, then it is likely that, like in American black bears, polar bears are not only coitus-induced ovulators.

Knowledge obtained through the work of this thesis can be used to better inform both captive and wild management. The fact that ovulation may not require coitus to occur in both American black bears and polar bears means that ovulation and pseudopregnancy are occurring at higher rates than currently expected. If this is true, this knowledge can be used to better inform methods for pregnancy determination. Furthermore, this knowledge can better inform management of captive populations, for example, the reduction of invasive ovulation induction techniques during artificial insemination procedures. There is still much to learn in regards to reproduction in bears, but this thesis reopens issues that may have been misleading researchers in previous years.

Acknowledgements

There are a large number of people who aided in my success during my graduate career at California State University San Marcos (CSUSM). I would like to thank the entire Department of Biological Sciences for providing a challenging and rewarding work environment to do my studies in. Each faculty member that I interacted with was truly interested in my success and goals, not only throughout the master's program but throughout life as well.

I would like to acknowledge and thank my advisor, Dr. Thomas Spady, for his support, encouragement, and push for excellence throughout my graduate program. Dr. Spady constantly reminded me that to be successful in science, you must look past a problem to find a solution, and that someone who can solve problems would be the most successful scientist. I am also appreciative of the learning environment that Dr. Spady's lab provided me both in the field and in the lab at CSUSM. I plan to apply the knowledge and experiences that I gained from Dr. Spady into my own classroom and life in the future.

Alexia Gee and Stephanie Baumann were two of the undergraduate students that I was lucky enough to work with and manage in Dr. Spady's lab during my time at CSUSM. Their work in processing our polar bear fecal samples, performing enzyme immunoassays, and performing data analysis were critical contributions to the third chapter of this thesis.

I would also like to extend many thanks to my committee members, Dr. Deborah Kristan and Dr. Thomas Jensen. Despite their already busy schedules, both committee members took time to provide consult about classes, my thesis, and/or my paper that was recently submitted to a peer-reviewed journal for publication.

I would also like to extend many thanks to my parents, Carl and Debra Stewart. I am the first person in my family to attend college, and more importantly, pursue a graduate-level degree. My parents ensured that I was given the chance to pursue opportunities that they themselves were not given. I could not be more thankful to have parents that see the importance in obtaining a higher education and supporting me while I work to reach my

vocational goals. Finally, thanks also needs to be extended to my significant other, Devin Emery. Devin and my parents were very understanding in the time demands of my Master's program. I am forever grateful for their patience and support, and their willingness to care for my dog during my time in the field.

I would like to conclude with a quote by Bob Goff. This quote is in reference to the learning environment that I was lucky enough to experience while at CSUSM:

"We all want to have a place where we can dream and escape anything that wraps steel bands around our imagination and creativity."

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Terms and abbreviations

Term	Abbreviation	Meaning
Coitus	-	Sexual intercourse between two conspecifics.
Corpus albicans; corpora albicans (pl)	CA	Reproductive structure that forms subsequently from the corpus luteum. The CA is no longer produces progesterone and occurs when regression of the luteal cells occurs.
Corpus hemorragicum; corpora hemorragiuca (pl)	CH	Reproductive structure that forms immediately from the ruptured follicle. This structure will then become the corpus luteum.
Corpus luteum; corpora lutea	CL	Reproductive structure that forms from the CH. This structure actively producing progesterone to maintain pregnancy, unless the animal has a delay in implantation. If delayed implantation occurs, the CL will remain relatively inactive until just after CL reactivation and implantation.
Delayed implantation (embryonic diapause)	DI	Process in which the blastocyst remains “free floating” inside the uterus of the female, with delayed development. Delayed implantation occurs from the end of the mating season until just after CL reactivation.
Estradiol	E2	In sexually-mature females, E2 aids in the development of the follicle. E2 is highest just before the follicle ruptures, releasing the egg.
Estrogen	E	A class of sex steroids that serves as ligand for the estrogen receptor and is produced primarily by the ovaries.
Estrus (behavioral)	-	Behavioral estrus is the time in which a female is sexually receptive to a male. This can include remaining in the presence of a male for a prolonged period of time, soliciting a male, and standing for a male to mount the female.
Estrus (physiological)	-	Physiological estrus is characterized by marked vulvar swelling and inflammation. For the purpose of this thesis, a female in physiological estrus is a female that has a vulva score of 2.5, or greater. Behavioral and physiological estrus were shown to be correlated in Himelright et al. (2014).
Follicular phase	-	Initial stage of the ovarian cycle where the follicles develop and mature. The follicular phase ends with ovulation. Estrogen is highest during the follicular phase, just prior to ovulation.
Induced ovulation	-	Ovulation mode that requires an exogenous stimulus. The most common stimulus that induces ovulation is vaginal stimulation from coitus. Other male stimuli may induce ovulation.
Luteal phase	-	Portion of the ovarian cycle, just following ovulation, where preparation for pregnancy occurs. Progesterone is highest during the luteal phase and pregnancy.

Term	Abbreviation	Meaning
Polyestrus	-	A trait observed in females where more than one estrus can occur in a single mating season.
Progesterone	P4	A steroid associated with the luteal phase and pregnancy or pseudopregnancy. P4 is primarily associated with an active CL and helps maintain pregnancy. P4 also affects denning behavior and metabolism.
Progestins	P	A class of sex steroids that serves as ligand for the progesterone receptor and is primarily produced by CL on the ovaries.
Pseudopregnancy	-	Also known as a “false pregnancy”. Reproductive state where a female exhibits the behavioral, metabolic, and endocrine signs of a true pregnancy without actually being pregnant.
Seasonal breeders	-	Animals who can successfully mate and give birth only during a specific time of the year.
Sequential ovulation	-	Ovulation events that occur subsequently in a single mating season.
Vulva score	-	Ranking system used to determine physiological estrus. The scale is from 0-3, with 0.5 increments. A vulva score of 2.5 or greater is indicative of physiological estrus.

Chapter 1: Introduction

Little is known about the physiological mechanisms involved in the reproduction of bears. American black bears (*Ursus americanus*) and polar bears (*Ursus maritimus*) are two species that were examined in the following thesis. Six of the eight bear species are considered threatened or endangered (IUCN Red List, 2015). American black bears are one of the species that is currently not considered threatened, in contrast, polar bears are considered vulnerable (IUCN Red List, 2015). By learning more about the reproductive mechanisms in these species and developing ways to monitor or determine ovarian activity, changes can be made to management techniques for the remaining captive and wild populations of bear species.

Theory for the presence of induced ovulation

Induced ovulation is a trait that is unique to seasonally-reproducing mammals. There are many possible explanations for why having induced ovulation increases fitness in these species (Lariviere and Ferguson, 2003). Common characteristics that species with induced ovulation have are: 1) solitary nature for most of the season, 2) presence of baculum or other penis structures that would increase stimulation, 3) reduced energy expenditure by ovulating only when a mate is present, and 4) exhibit long periods of intromission (Lariviere and Ferguson 2002; 2003).

Bears, including American black bears, superficially fit into this theoretical paradigm for exhibiting induced ovulation (Boone et al., 2004). American black bears are solitary in nature and often only encounter a mate or conspecific few times throughout the year (Powell, Zimmerman, and Seaman, 1997; Lariviere and Ferguson, 2003). Due to their solitary nature, it is also assumed that in the wild, it is difficult for bears to find mates. Because of this, the idea that females are expected to only ovulate when in the presence of a male is reasonable, because this would increase the likelihood for fertilization and would decrease the energetic costs associated with producing eggs that will not be fertilized (Lariviere and Ferguson, 2003). Male American black bears have baculum, which is expected to increase stimulation of the female's reproductive tract during intromission (Marks and Erickson, 1966; Lariviere and Ferguson, 2002). It is also known that American black bears have prolonged mating durations (Gonzales et al., 2013), which is believed to ensure proper stimulation of the female's reproductive tract (Dixson, 1995). It is true that American black bears exhibit many of the behaviors and physiological mechanisms that many known induced ovulators have, yet little empirical data exists to support or refute this paradigm.

Although American black bears have many characteristics that other induced ovulators have, this does not mean that American black bears should be assumed to be induced ovulators. In the current literature, American black bears are documented having characteristics that would question the need for having induced ovulation. For example, American black bears are polyestrus with sequential ovulations (Himelright et al., 2014). Because American black bears are not limited to one estrus and one ovulation during the mating season, it seems less beneficial to have induced ovulation. It is currently proposed that induced ovulators have less energetic costs by ovulating only when in the presence of a male, because that egg will likely be fertilized (Lariviere and Ferguson, 2003). Maternal parental investment in most mammals includes egg production, ovulation, gestation, parturition and lactation (Schatten and Constantinescu, 2008). Gestation and lactation are the two most energy-taxing stages (Key and Ross, 1999), suggesting that the energy lost due to egg production is minimal and is not likely to be reason for induced ovulation to occur. Assuming that American black bears are induced ovulators due to the presence of a baculum is also not appropriate (Lariviere and Ferguson, 2002). American black bears, and most other bears, have long, slender baculum which would not likely aid in stimulating the female's reproductive tract (Abella et al., 2013). Instead, a long, slender baculum may be used for better semen transfer, by allowing the baculum to enter and hold open the female's otherwise convoluted cervix (Boone et al., 1999;

Abella et al., 2013). Because there is little empirical evidence and little evidence in the current literature supporting that American black bears are induced ovulators, we believe that it is important to re-examine this issue.

Reproductive mechanisms in bears

Bears have evolved a large variety of reproductive physiology mechanisms. In American black bears, these traits include seasonal polyestrus (Gonzales et al., 2013), polyestrus with sequential ovulations (Himelright et al., 2014), multipaternity (Schenk and Kovacs, 1995), induced ovulation (Boone et al., 2004), delayed implantation (Wimsatt, 1963), and pseudopregnancy (Hellgren et al., 1990).

Seasonally polyestrous females are females that have more than one estrous cycle during the mating season, thus providing multiple mating opportunities throughout the mating season. Gonzales et al. (2013) also hypothesized that males used chemosensory cues to determine a female's fertility status. Under certain social conditions, females were capable of synchronizing their estruses, which further supported the conclusions of Gonzales et al. (2013) that chemosensory cues played a role during the mating in American black bears. During the mating season, when resources are most available, it is likely that solitary species encounter other individuals more often in areas rich in resources, such as a water source or an area that would promote a high-fat diet. If females synchronize their estrous cycles, then males would be able to monopolize on these females. This would increase mating successes in both males and females.

In American black bears, each estrus in a polyestrous female have sequential ovulation events (Himelright et al., 2014). In this study, 13 out of 16 females were determined to by polyestrus via vulva score analysis. In a subset of these females, three out of four polyestrous females were found to have sequential ovulation events, as determined by morphological analysis of ovaries. Sequential ovulation events were determined by the number of ovulation-related structures and their stage of development. Ovulation structures include the corpus hemorragicum (CH), corpus luteum (CL) and corpus albicans (CA) (Schatten and Constantinescu, 2007; Figure 1). A CH forms immediately after the follicle has ovulated, and is identified by hemorrhaged tissue; CH indicates a fresh ovulation event (Schatten and Constantinescu, 2007; Figure 1). A CL forms from the CH and will produce progesterone (P4) to support pregnancy; CL indicates a recent (but not new) ovulation event (Douglas et al., 1994; Morales et al., 2000; Figure 1). Finally, a CA is a CL that is regressing and undergoing atresia; CA indicates an old ovulation event, likely from a previous mating season (Douglas et al., 1994; Morales et al., 2000; Figure 1). An example of how sequential ovulation was determined in Himelright et al. (2014) is through morphological assessment of the ovary, where a female found with a CL and a CH would be considered to have sequential ovulation; whereas a female with two CL of similar developmental age would not be considered to have sequentially ovulated. The key conclusions made by Himelright et al. (2014) were that American black bears exhibit estrous cycles that are independent from pregnancy, meaning that each subsequent estrus in a single female is independent from the previous estrus.

Multiple paternity was documented in a single litter in American black bears by Schenk and Kovacs (1995). Schenk and Kovacs (1995) suggested that there are likely no monogamous bonds in bears and that through multiple copulations, females increase the genetic diversity and fitness of their litter. The observation that males and females mate with multiple individuals of the opposite sex was a key conclusion made by Schenk and Kovacs (1995). The results from Schenk and Kovacs (1995) laid the groundwork for what we know about large carnivore reproduction because little information was known at the time and the field is still further developing today.

Induced ovulation is a process by which ovulation occurs as a result of an external stimulus (usually coitus) (Milligan, 1974). Boone et al. (2004) concluded that the American black bear is an induced ovulator. The authors studied seven nonmated and eight mated female American black bears. The authors found that one out of the seven nonmated females had CL, and seven out of the eight mated females had CL. In this study, serum P4 was also analyzed as a measure of ovulation. Boone et al. (2004) stated that the mated females had significantly higher concentrations of P4 (0.70-2.70 ng/mL) than did the nonmated females (0.32-1.50 ng/mL). Due to a higher percentage of mated females having CL and these females having high serum P4 concentration, the conclusion of induced ovulation was reached. Boone et al. (2004) further concluded that coitus is the stimulus inducing ovulation in all bears.

Delayed Implantation (DI) occurs when a blastocyst (an early structure in embryonic development that later becomes the embryo) remains "free" inside the uterus for some time before implanting to the uterine wall, leading to gestation (Mead, 1981). Wimsatt (1963) found that American black bears in the New York area ovulated in June. When looking for blastocysts, Wimsatt (1963) found that unimplanted blastocysts were found from late-June through as late as November. Furthermore, pregnant females that were examined in December were found to have embryos that had implanted within a week to two weeks prior to being harvested. From this evidence, Wimsatt (1963) was able to conclude that American black bears exhibit long delay in implantation of conceptuses from the time of mating.

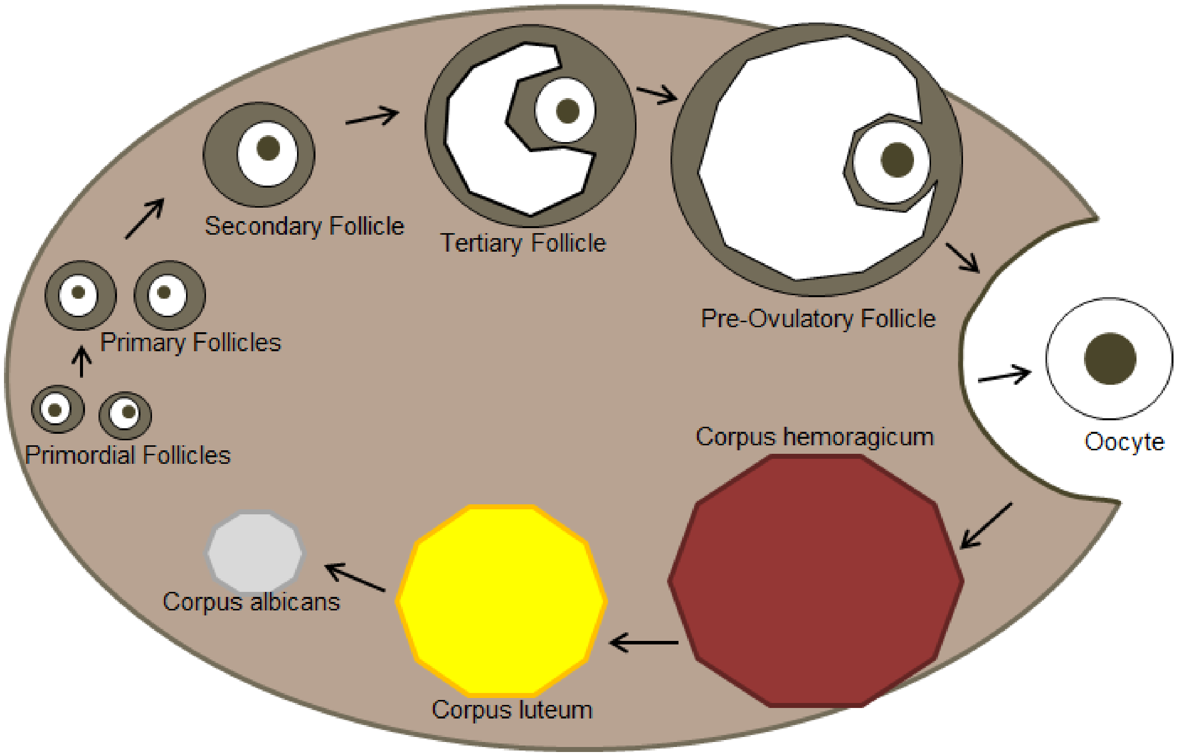

Figure 1: Depiction of the follicular and luteal phases of the ovarian cycle. The formation of primordial follicles is stimulated by follicle stimulating hormone (FSH). The concentration of estrogen (E2) produced by the follicle(s) increase as the primordial follicles transition into primary, then secondary, then tertiary, and finally, pre-ovulatory follicles. When ovulation occurs, the oocyte is released along with a bolus of E2. The freshly exploded follicle remnant is also known as the corpus hemoragicum (CH). The CH then becomes luteinized by luteinizing hormone (LH), becoming a corpus luteum (CL), which produces progesterone (P4) to support implantation and maintain pregnancy; CL activity and P4 production decreases after parturition. Following pregnancy, while lactating, the CL is still present and is producing low quantities of P4. After the mother is no longer lactating, the CL regresses and becomes a corpus albicans (CA). CA cells continue to regress and are absorbed into the body, CA do not produce P4.

Induced ovulation

Induced ovulation is defined as a process where eggs are released due to an external stimulus (Milligan, 1974). The stimulus triggering ovulation can be hormonal (Foster and Hisaw, 1935), physical stimulation (Boone et al., 2004), olfaction of pheromones (More, 2006), and visual (Pearce and Oldham, 1988). Physical stimulation, typically coitus, is a common stimulus for inducing ovulation. Following the stimulus, an increase in luteinizing hormone occurs, which then triggers ovulation (Kumar and Farouk-Sait, 2011). In species where females are induced ovulators, males have evolved ways to increase stimulation during coitus. As a result of selective pressures, males developed penises with spines (e.g. domestic cats (*Felis catus)*; Schatten and Constantinescu, 2007) and baculum (e.g. striped skunks (*Mephitis mephitis*); Wade-Smith and Richmond, 1980; Dixson, 1995) to induce ovulation. The purpose of these structures as a means to better stimulate ovulation is only circumstantial and has not yet been supported (Lariviere and Ferguson, 2002). It is important to note that not all species with baculum are coitus-induced ovulators. For example, the raccoon (*Procyon lotor*) has a baculum, but is induced to ovulate due the presence of a male without coitus or other physical contact (Morris, 1975). It is also important to note that not all species that exhibit induced ovulation have baculum, as seen in guinea pigs (*Cavia porcellus*) (Blaustein and Feder, 1979).

Induced ovulation is a trait that is observed in carnivores, especially those that are seasonal breeders that also have delayed implantation (DI) (Lariviere and Ferguson, 2002). For example, Wade-Smith and Richmond (1978) determined that the striped skunk (a mustelid) is an induced ovulator which also exhibits DI, and Enders (1952) similarly reported that DI and induced ovulation also occurs in the American mink (*Mustela vison*). Induced ovulation was documented by Wade-Smith and Richmond (1978), the authors studied mated (n=19) and nonmated (n=6) females of the striped skunk. After 36 hours, the time in which it takes to ovulate (Wade-Smith and Richmond, 1978), the females were killed and their ovaries were observed. Of the mated females, 17 had ovulated, determined by the presence of CL, and two had not yet ovulated but had enlarged follicles. Of the nonmated females, all six had not ovulated. Wade-Smith and Richmond (1978) presented clear evidence to support that the striped skunk is an induced ovulator.

It has been widely accepted that ursids, like the American black bear (Boone et al., 2004), are induced ovulators. This also includes the Asiatic black bear (*Ursus thibetanus*) (Okano et al., 2006; Chang et al., 2011), brown bears (*Ursus arctos*) (Craighead et al., 1995), and polar bears (Rosing-Asvid et al., 2002).

Delayed implantation

DI is often observed in mammals that have limited resources during various times of the year. In seasonally reproducing species, delayed implantation provides a means to prolong gestation so that offspring are born at the most favorable time of year (Sandell et al., 1990). In the American black bear, mating occurs during early summer, bears begin to den during the fall, then in mid-winter the bears give birth to their young in the den (Lariviere and Ferguson, 2002). Delayed implantation allows for this seasonal pattern to occur (Wimsatt, 1963). Just after fertilization, blastocysts enter a diapaused state inside the uterus, in the late fall the blastocysts implant into the endometrium and begin gestation, until birth in January or early February (Wimsatt, 1963). Understanding the reproductive behavior of animals is critical in explaining the adaptive benefits of delayed implantation and induced ovulation, and how these two traits coevolved.

The period of delayed implantation can be observed through hormone concentrations in the blood, urine and feces. P4 concentrations are relatively low before mating, increase slightly during the period of delayed implantation, and then sharply increase just prior to or approximately the same time as implantation occurs (Tsubota et al. 1987, 1998). The rise in P4 is due to the development of CL, a reproductive structure that develops as a result of ovulation and helps to establish and maintain pregnancy in mammals (Jarrell and Dziuk, 1991). During DI and immediately following implantation, the CL nearly doubles in size, increasing its capacity to produce elevated levels of P4 (Wimsatt, 1963). Because P4 is linked to ovulation and implantation in seasonally reproducing species, P4 levels can be monitored and compared to the trend described by Tsubota et al. (1987; 1998) to understand ovulation patterns in bears.

Identification of corpus luteum and its role in reproduction

The CL is believed to prevent follicular development to the tertiary follicular stage and supports the maintenance of pregnancy through the production of P4 (Wimsatt, 1963; Erickson, Nellor, and Petrides, 1964; Schatten and Constantinescu, 2007). One or multiple CL can be found on an ovary in bears (Tsubota and Kanagawa, 1993) and both ovaries can have CL present at the same time (Erickson, Nellor, and Petrides, 1964). The size of a given CL is primarily determined by the amount of luteal cell vacuolation (Dharmarajan et al., 1988). Large vacuoles in CL indicate high luteal activity, meaning high P4 production (Amelkina et al., 2015). Due to the unique degree of vacuolation, this characteristic can be used to determine whether a structure on the ovary is a CL or some other structure.

Discussion of the cellular composition of CL is relevant to the purpose of this thesis. Erickson, Nellor, and Petrides (1964) describe the characteristics of CL in American black bears during the end of the breeding season and into pregnancy. Following ovulation, CL contain well-rounded nuclei and the cytoplasm contains secretory vacuoles (Erickson, Nellor, and Petrides, 1964). As pregnancy continues, the CL persists and becomes highly vascularized and its luteal activity increases (Erickson, Nellor, and Petrides, 1964). In early January, near parturition, Erickson, Nellor, and Petrides (1964) described that the CL becomes greatly reduced. Similar to the study by Erickson, Nellor, and Petrides (1964), Tsubota and Kanagawa (1993) described the characteristics of CL in Hokkaido brown bears (*Ursus arctos yesoensis*). Six bears were sampled between October to December and all were found to have CL present in their ovaries (Tsubota and Kanagawa (1993). Four bears were reported to have had unimplanted embryos, and one additional bear's pregnancy status was undetermined. The sixth bear did not have any embryos present in its uterus. The authors reported the CL were highly vascularized in five of the bears. The exception was the bear without an embryo, which had little vacuolization (Tsubota and Kanagawa, 1993). Because P4 concentrations indirectly indicate ovulation and P4 is produced by CL, it is important to understand the steroid patterns associated with reproduction in bears.

Steroid profiles during and post-mating season

Hellgren et al. (1990) published the progesterone profiles of pregnant and non-pregnant American black bears. The pattern that Hellgren et al. (1990) observed was a moderate rise in P4 during delayed implantation (3.3 ± 0.3 to 7.0 ± 0.4 ng/mL (mean ± SE)). Near or during implantation of the blastocyst, P4 then increased to 16.9 ± 1.4 ng/mL. After implantation, P4 concentrations decreased becoming undetectable several days after birth (< 0.1 ng/mL). Hellgren et al. (1990) reported that the serum P4 concentration patterns were similar in pregnant and non-pregnant females, suggesting that even non-pregnant females may have ovulated. Similar trends in serum P4 concentrations were observed in the Hokkaido brown bears observed by Tsubota et al. (1987). Figure 2 demonstrates the overall trend of P4 expected in a temperate zone bear. Tsubota et al. (1987) described that this pattern of serum P4 concentrations correlates with the presence and activity of CL. Wimsatt (1963) described that CL remain inactive during the period of DI, but become active and increase in size when implantation occurs. Tsubota et al. (1987) suggests that this increase in size likely correlates with the increase in P4 production that they observed at implantation.

Similar to P4 concentration patterns, estrogen concentrations also correlate processes occurring during the mating season (Figure 2). By monitoring estrogen concentrations, it can be determined when a female is approaching ovulation and estimate the time when ovulation has occurred. Concentration patterns of estradiol (E2), an estrogen hormone, were reported in American black bears by Tsubota et al. (1998). In a group of semi-free ranging American black bears, Tsubota et al. (1998) reported serum E2 concentrations from days 0-52 of estrus in 12 mated females. Elevated E2 concentrations were observed during early estrus and were followed by a ~50% decrease in E2 concentrations after estrus (Tsubota et al., 1998). The trend observed is likely due to follicular development, in which estrogen is being produced at high levels, causing maturation of the developing follicle. When ovulation occurs, estrogen will be at its highest concentrations (Figure 2). When the follicle ruptures at ovulation, estrogen concentrations decrease rapidly (Figure 2). These trends in estrogen concentration were described and observed by Johnston et al. (1985) in domestic dogs. Steroid concentrations (primarily P4) are often used to determine when a female is pregnant. The use of P4 to determine pregnancy is difficult in bears due to possible pseudopregnancy.

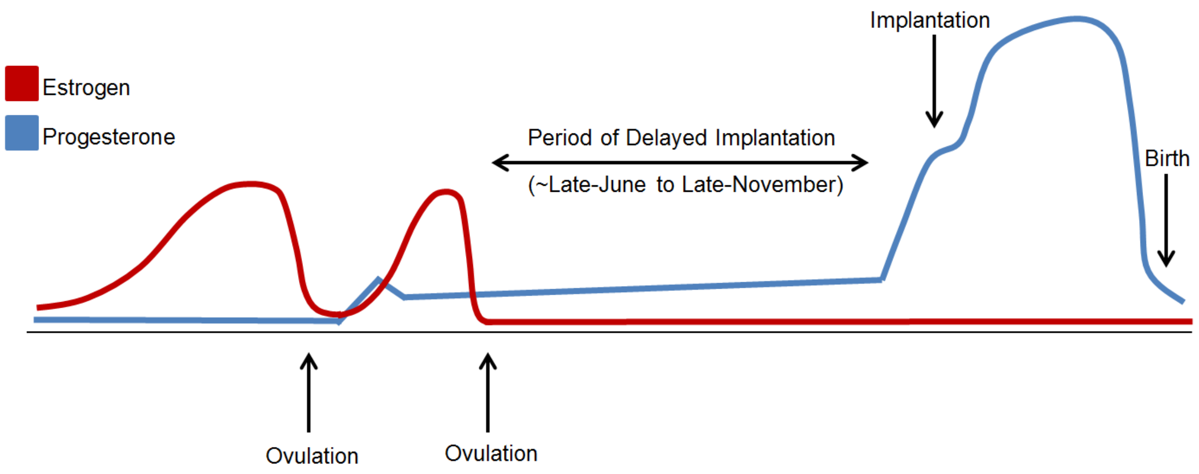

Figure 2: Figure demonstrates the expected concentration patterns of estrogen (E2) and progesterone (P4) in a temperate zone bear. At the beginning of the year, E2 is expected to be low and slowly increases until the mating season in late-spring. Peak E2 will be reached at the time of peak estrus, just before ovulation. At the time of ovulation, E2 will decline very rapidly, returning to baseline levels. American black bears are known to be polyestrus with sequential ovulations, so it is possible for E2 to peak an additional time and for a second ovulation event to occur. P4 will remain low until ovulation, where P4 will increase slightly, then remain at that slightly higher level during the period of delayed implantation. Implantation occurs near the fall equinox, causing P4 to rapidly increase. P4 remains high during pregnancy or pseudopregnancy and will decrease rapidly just prior to birth.

Pseudopregnancy

Pseudopregnancy, also known as false pregnancy, is a reproductive state in which a female that is not actually pregnant exhibits similar behaviors, hormone concentrations, and metabolism as pregnant females (Nelson, 2011). Pseudopregnancy can occur for the same duration as an actual pregnancy in carnivores (Mead et al., 1993; Nelson, 2011). American black bears were documented to exhibit pseudopregnancy by Hellgren et al. (1990), and this conclusion was further supported by Tsubota et al. (1998). Similar observations were also made in polar bears (Stoops et al., 2008 and 2012; Rosing-Asvid et al., 2012). Pseudopregnancy also occurs in other seasonal species of Order Carnivora, such as the wolverine (*Gulo gulo*) (Mead et al., 1993). The main piece of evidence for the occurrence of pseudopregnancy is elevated levels of P4 in females that were not observed mating (Hellgren et al., 1990; Mead et al., 1993; Tsubota et al., 1998). In each of these studies, P4 was measured from serum or fecal samples, however other fecal progesterone metabolites may be measured to determine pregnancy status (Stoops et al., 2008 and 2012). Due to physiological, behavioral, and endocrine similarities, pseudopregnancy is a phenomenon that makes studying pregnancy in bears difficult. With pseudopregnant bears having similar P4 concentrations to that of pregnant bears, it makes a simple blood, urine, or fecal test using P4 as a marker for pregnancy impractical. It is important to note that if pseudopregnancy occurs, ovulation must have also occurred, and if pseudopregnancy occurs in females that have not mated, it must be assumed that these nonmated females have ovulated. If pseudopregnancy is observed in nonmated females, then this provides evidence that that species is not a coitus-induced ovulator. The concept of pseudopregnancy may explain why Boone et al. (2004) observed elevated P4 concentrations or CL on the ovaries of females who did not mate.

Discrepancies in Boone et al. (2003 and 2004) studies

In 2004, a study performed by Boone et al. concluded that American black bears are coitus-induced ovulators. In this study, two groups of American black bears, held in separate study pens were observed. Group A had both males and females, which were allowed to mate. Group B had only females, which were not allowed to mate with males and were allowed only visual and olfactory contact with males. Following the mating season, the females from both groups were anesthetized and the ovaries were observed via laparoscopy. Laparoscopy is a routine surgical technique that uses a small incision to insert a fiberoptic camera probe inside the abdominal cavity to observe internal organs, such as ovaries and uteri (Schatten and Constantinescu, 2007). Boone et al. (2004) found that only the mated females (n = 8) had CL, whereas the nonmated females (n =7) had enlarged follicles and no corpus luteum (Boone et al., 2004). However, one nonmated female had two CL present on her ovaries. Furthermore, at least one nonmated female (different from the nonmated female with CL) exhibited P4 concentrations similar to concentrations found in mated females. Because a larger proportion of the mated females had CLs and elevated serum P4 levels, the researchers concluded that American black bears are coitus-induced ovulators (Boone et al., 2004). With the limited nature of analyzing ovaries via an *in vivo* laparoscopy technique, it is possible that Boone et al. (2004) may have missed identifying all of the morphological structures on the ovaries, as well as possibly misidentifying structures. In particular, superficial CL are much easier to observe via laparoscopy than are deep CL. It could be possible that Boone et al. (2004) identified structures of the ovary as CL when they were actually luteinized follicles. Luteinized follicles, also known as luteinized cysts, are cysts that form on the ovaries and encapsulate the follicle (Marik and Hulka, 1978). Luteinized follicles also produce P4 in similar levels to CL, which may cause observers to believe an animal has falsely ovulated (Marik and Hulka, 1978). Presumably, luteinized follicles look much like a CL but have an egg that can only be found when performing histology. Because of the state of the current literature and the limited supporting evidence of American black bears as coitus-induced ovulators, we thought that it was important to re-investigate the ovulation mechanism in American black bears. In addition to conclusions made by Boone et al. (2004), further discrepancies were found in a previous Boone et al. (2003) study.

Gonzales et al. (2013) found discrepancies between data collected in their study compared to data collected by Boone et al. (2003). Both Gonzales et al. (2013) and Boone et al. (2003) studied a semi-free ranging American black bear population at Bear Country, USA (BCUSA) in Rapid City, South Dakota. Both studies had a selected group of bears that were housed in smaller observation enclosures, separate from the semi-free ranging population. In these enclosures, the researchers were able to control which females had access to males, which females did not have access to males, and closely observe the mating in the bears (Gonzales et al., 2013; Boone et al., 2003). Gonzales et al. (2013) noted significant differences in their data compared to data reported in Boone et al. (2003). One notable difference was the length in which a male mounted a female to characterize successful mating. In Gonzales et al. (2013), the length of a successful mount (mount that led to intromission) was on average 33.8 ± 8.5 minutes (mean ± SD), however, Boone et al. (2003) characterized a successful mating as mount duration of 0.9 ± 0.6 minutes (mean ± SD). In contrast, Gonzales et al. (2003) reported that unsuccessful mounts had a duration of 1.2 ± 1.4 minutes (mean ± SD). Gonzales et al. (2013) reported that there was a 40-fold difference between mount duration between their study and Boone et al. (2003) (Gonzales et al, 2013). These comparisons lead me to believe that Boone et al. (2003) was not accurately distinguishing successful mounts from unsuccessful mounts. If true, then it is possible that some of the “mounted” females in the Boone et al. (2003) study were not actually mounted with intromission and/or ejaculation.

Interestingly, Boone et al. (2004) used the same bear population for their induced ovulation study. In this study, females were either isolated from males via a fence, or were kept in the pen with the main population and assumed to have mated. It is important to note that behavioral observations of mating interactions were not performed by Boone et al. (2004) and females that were classified as mated were assumed to have mated due to presence of males in the same study pen. If coitus is required for ovulation to occur, then successful mounts with intromission would be necessary to induce ovulation. From this, it is important to consider if the conclusions made by Boone et al. (2003 and 2004) were accurately informed. With such large discrepancies between Gonzales et al. (2013) and Boone et al. (2003), it is important that the classification of the American black bear as an induced ovulator be re-evaluated.

Bears may not be coitus-induced ovulators

In 2006, Okano et al. suggested that not all Asiatic black bears (*Ursus thibetanus*), a close relative of American black bears, require coitus as a stimulus for causing ovulation. In their study, the authors observed P4 levels of mated and nonmated female Asiatic black bears. Based on their P4 analysis, the authors determined that 2/2 of the mated females and 6/6 of the females with male contact without mating (e.g. separated via fence, but allowed visual, olfactory and some touch stimulation) were considered to have ovulated. The completely isolated (only male olfactory and auditory cues) females in this study were not considered to have ovulated based on P4 analyses. The authors then removed the ovaries of the isolated females to determine if any CL had developed. The authors found no evidence of CL, suggesting that the isolated females had not ovulated. Okano et al. concluded that the Asiatic black bear is indeed an induced ovulator, but are ovulating at high rates due to stimuli other than coitus (Okano et al., 2006). It seems reasonable to begin questioning whether other bear species are also not coitus-induced ovulators, as previously accepted.

With the findings of Okano et al. (2006), suggesting that the Asiatic black bear is not a coitus-induced ovulator, I questioned which other bear species are also not coitus-induced ovulators. It is currently believed that bears, including American black bears, are coitus-induced ovulators as presumed by data presented by Boone et al. (2004). However, this conclusion has not been empirically tested.

Objectives

There were two overall objectives for the following thesis: 1) determine if coitus is the only stimulus that induces ovulation in the American black bear, and 2) determine if a captive female polar bear exhibited signs of cyclicity through observation of P4 concentrations throughout multiple years. The first objective used a combination of techniques to provide evidence for whether ovulation occurred. A combination of vulva score methods, profiles of urinary estrogen concentration, concentration of serum progesterone during embryonic diapause, and morphological and histological observations of ovaries were used. The second objective was achieved by observing the concentration of progesterone metabolites from fecal samples throughout multiple years in a singly housed, captive, female polar bear. The information gained through the work of this thesis will aid in better informing bear biologists, reproductive physiologists, and managers of captive and wild populations of bears throughout the world.

Chapter 2: Are American black bears coitus-induced ovulators?

**Please note that this chapter was submitted for peer review publication on July 25, 2016 to Animal Reproduction Science.*

Abstract

It is widely accepted that American black bears are coitus-induced ovulators, although this has not been sufficiently tested empirically. The goal of the present study was to determine if coitus is the only stimulus that induces ovulation in American black bears. Behavioral observations, enzyme immunoassays and histology were performed on a population of semi-free ranging American black bears. Vulva score was used to measure physiological estrus. Urinary estrogen (E2) was measured in isolated females to document ovulation. Serum progesterone (P4) was measured in females to document corpus luteum (CL) activity. Ovary tissues were analyzed to confirm the presence of CL. We found that the overall duration of physiological estrus and how quickly females exit physiological estrus after mating were similar between females (DF = 29, t-stat = 1.3, P = 0.1 and DF = 23, t-stat = -0.7, P = 0.2, respectively). E2 in isolated females gradually increased, peaked at estrus and rapidly declined, indicative of ovulation. During early and late embryonic diapause, P4 was similarly elevated in mated females and females not observed mating (DF = 4, t-stat = -0.9, P = 0.2 and DF = 11, t-stat = -0.3, P = 0.4, respectively). Corpora lutea were confirmed via histology in two females that were not observed to have mated. Data in the present study indicates that coitus is not the only stimulus that induces ovulation in American black bears. Further research is needed to determine whether other stimuli induce ovulation or if spontaneous ovulation occasionally occurs in this species.

Highlights

- American black bears ovulate without coital stimuli at a high rate.
- Isolated females showed evidence of ovulation.
- It is not clear what other stimuli cause American black bear females to ovulate.
- The triggers of ovulation in American black bears need to be re-examined.

Keywords delayed implantation; embryonic diapause; progesterone; corpus luteum; estrus; pseudopregnancy

Introduction

Information on the reproductive mechanisms and behaviors in bears, including the American black bear (*Ursus americanus*), are few and not fully documented. American black bears are seasonally polyestrus (Gonzales et al., 2013), have sequential ovulation (Himelright et al., 2014), and are presumed to be coitus-induced ovulators (Boone et al., 2004).

Among Carnivora most closely related to bears, ovulation can be spontaneous, like in the domestic dog (Reynaud et al., 2012), or induced, like in striped skunks (Wade-Smith and Richmond, 1978). Induced ovulation is defined as a process where eggs are released (or ovulated) as a result of an exogenous stimulus (usually coitus) (Milligan, 1974). The stimuli that induce ovulation include: physical stimulation (coitus or tactile), olfaction of pheromones, and visual (Pearce and Oldham, 1988; More, 2006). Bears, including American black bears (Boone et al., 2004), Asiatic black bears (Okano et al., 2006; Chang et al., 2011), brown bears (Craighead et al., 1995), and polar bears (Rosing-Asvid et al., 2002) have been concluded to be induced ovulators. Induced ovulation has evolutionary advantages, especially in solitary seasonal species. In such species, there are low chances of encountering a mate near the time of ovulation. In these cases, induced ovulation would ensure that a female would ovulate around the time mating occurred. Species where males have a baculum (os penis) are also often coitus-induced ovulators (Lariviere and Ferguson, 2002). Bears, including American black bears, are solitary animals and have a baculum (Marks and Erickson, 1966; Boone et al., 2003). However, American black bears have also evolved strategies that would make coitus-induced ovulation unnecessary. American black bears are polyestrus (Gonzales et al., 2013), with each subsequent ovulation events (Himelright et al., 2014). This means that the solitary nature of American black bears need not increase evolutionary pressure to exhibit coitus-induced ovulation, as having more than one estrus with sequential ovulations, allows for multiple successful mating opportunities.

Since Boone et al. (2004) concluded that American black bears are coitus-induced ovulators, it has been widely cited that bears in general are coitus-induced ovulators (Okano et al., 2006; Czetwertynski et al., 2007; Reinwald and Burr, 2008; Swenson and Haroldson, 2008; Yamane et al., 2009; Kohira and Mori, 2010; Steyaert et al., 2012; Frederick et al., 2013; Stirling et al., 2016). This hypothesis has not been sufficiently tested empirically and data currently exists that does not support this hypothesis. In the Boone et al. (2004) study, one out of seven physically isolated females had CL present after the mating season, suggesting ovulation occurred. A study performed by Okano et al. (2006) concluded that in Asiatic black bears, females were ovulating at a high rate due to male stimuli other than coitus. Though Okano et al. (2006) did not provide suggestions of other male stimuli that may be inducing ovulation, Boone et al. (2004) suggested that other male stimuli may include olfaction, visual, or auditory cues. Because of these observations and currently limited supporting evidence that American black bears are coitus-induced ovulators, we thought that it was important to re-examine this dogma. The following study is retrospective, in that it is a culmination of data collected over six years for other research purposes, while analyzing these data we found evidence that was contradictory to the induced ovulation dogma. If coitus does induce ovulation in American black bears, we expected the following predictions to be supported by the data: 1) the duration of physiological estrus and how quickly females exit physiological estrus was expected to be shorter in mated females than in females not observed mating. The duration of physiological estrus was defined as the number of consecutive days that a females had a vulva score of 2.5 or greater. How quickly females exit physiological estrus was defined as the number of consecutive days that a female maintained a vulva score of 2.5 or greater after the first successful instance of mating. 2) Isolated females should not exhibit urinary estrogen (E2) concentration patterns that are indicative of ovulation (i.e. a gradual increase, followed by a rapid decline, once peak E2 levels have been reached). 3) Serum P4 concentrations should be more elevated in mated females during July and late-September to mid-October (representing the periods of early- and late-embryonic diapause, respectively) than in females not observed mating. 4) Females that were not observed mating should have no CL present in their ovaries.

Methods

Field site, animal disposition and study design

All research, animal handling and care were conducted in accordance with approved methodology by the Institutional Animal Care and Use Committee of California State University San Marcos (CSUSM), Office of Laboratory Animal Welfare assurance 4196. The subjects were sexually mature female and male American black bears, ranging from 4-8 years old and 5-15 years old, respectively. There were a total of 29 females (four physically-isolated females were repeat sampled in 2004 and 2005, five females with access to males in 2009, six females with access to males in 2011, six females with access to males in 2013, six females with access to males in 2015, and two isolated from males in 2015) and 20 males (four in 2009, five each in 2011 and 2013, and six in 2015). Subjects were selected from a semi-free ranging population at a privately owned wildlife reserve in South Dakota. The population was housed in one of two 60-hectare enclosures. Study females were housed in one of two 305 m x 488 m enclosures physically separated from the main population via a fence with hot wire. Females that were given access to males were housed in one study pen, while females isolated from males were held in the second study pen. Isolated females could still see, smell, and hear adjacent males in the present study, only physical stimulation of coitus was prevented in isolated females. Researchers collected behavioral observations from May 27 to July 6 2009, May 31 to July 7 2011, June 3 to July 23 2013, June 1 to July 14 2015. Only urinary samples were collected in 2004-2005, to analyze estrogen metabolites. During each year, females were housed in the study enclosures from May to February.

Vulva scores and behavioral recording

Vulvar swelling was semi-quantitatively scored, using methods described previously (Gonzales et al. 2013, Himelright et al. 2014), to determine when a female entered physiological estrus and its duration. Physiological estrus was defined at the time in which a female exhibits the physical signs of estrus, such as an engorged vulva. Vulva score is strongly correlated with behavioral estrus and therefore can be used as a physiological indicator of estrus in black bears (Gonzalez et al., 2013). A score of 2.5 or greater, on a scale of 0 to 3, is considered to be the period in which a female is in physiological estrus.

While males were housed in the pen with the females, observers recorded mating interactions. All observers recorded both successful and unsuccessful matings, as defined by Gonzales et al. (2013). Mating observations were made using focal sampling with all-occurrences recording (Martin and Bateson, 2007). In 2009, we recorded behaviors from 0900-1700 daily (624 cumulative hours), 0900-1730 daily (535.5 cumulative hours) in 2011, 0730-1930 daily (1152 cumulative hours) in 2013, and 0730-1730 daily (1320 cumulative hours) in 2015. During observation periods, the observers took breaks in shifts to minimize missed mating observations. We made no observations outside of these times. While it is possible that mating occurred outside of this time period, it is not likely. This assumption is based on 13 years of observing bears at this field site (TS personal observation), the bears exhibit little to no activity outside of daylight hours.

Tissue and serum sample collection, storage and processing

Blood samples were collected from the femoral artery or jugular vein of anesthetized females in 2011 in mid-July and in 2009 and 2015 in late-September to mid-October, corresponding to periods of early and late embryonic diapause/peri-implantation, respectively. Anesthesia was administered using a Telinject rifle (Telinject, Agua Dulce, CA) and 5-6 mg/kg Telazol (1:1 Tiletamine:Zolazepam) and 0.8 mg/kg Rompum (Xylazine) in 2009 and 2011 or BAM, a cocktail of butophanol tartrate (0.36 mg/kg), azaperone tartrate (0.12 mg/kg) and metatomidine (0.15 mg/kg) (ZooPharm, Windsor, CO, USA) in 2013 and 2015. BAM was reversed with 0.66 mg/kg Atipamezole and 0.2 mg/kg Naltrexone. Serum was separated by cooling the fresh blood at 0-4 ^{0}C for 15-30 min to enhance coagulation and centrifugation at 1000 X *g* for 30 minutes. Immediately after centrifugation, the serum was removed and stored at -20 ^{0}C until used for hormone analysis.

In 2011, ovaries were removed using laparoscopic assisted hysterectomy, as described in Himelright et al. (2014). The laparoscopic assisted hysterectomy allowed for *in vivo* examination of the ovaries using a laparoscope, a digital camera and a display monitor. Ovaries were excised from four females in July. Basic ovarian morphological analysis was performed by T. Spady and B. Himelright.

Ovaries were stored in 10% neutral buffered formalin, and cut into equal quarters prior to sectioning. Each quarter was divided into two layers by L. Stewart with a scalpel and placed into a tissue cassette in 70% ethanol. Processing, paraffin embedding and sectioning were conducted by the Sanford Consortium histology core facility at the University of California San Diego (UCSD).

Histology

Only the two females that were not observed mating during the 2011 season were analyzed via histology. Serial sections 10 μm thick were made by the Sanford Consortium lab, so that the entire ovary could be stained an analyzed. The serial sections were stained by L. Stewart in the lab at California State University San Marcos (CSUSM) with hematoxylin and eosin (H&E) following standard protocol. All of the H&E ovary sections were examined under a light microscope (Nikon, Melville, NY, USA) at 40X and 400X magnification to confirm identification of CL and verify absence of luteinized follicles with unovulated oocytes. The entire slide was observed for each section and the cell types and the presence of oocytes were recorded.

Urine sample collection

Operant conditioning techniques with positive food rewards were used by T.Spady to train four adult female American black bears to urinate in designated areas on command in 2004 and 2005. Twice daily, at 0900 and 1400 hours, all study bears were called to kennels adjacent to their enclosure. The kennels were positioned on a dry, clean concrete surface. The individuals were separated in the kennel with sliding, divider doors. Bears were offered water sweetened with honey (10% w/v) to hydrate bears if needed. Bears were then given the command to urinate. Wells made of silicon gel were placed in the areas of the kennel where the bears urinated, to pool the urine and facilitate collection. Using a divider door, the bear and urine sample were separated and the sample was collected using a clean plastic syringe (1-20 cc, as needed). This allowed for clean and fresh urine to be collected, with known sample identities.

Hormone analysis on urine and serum samples

E2 metabolites were measured directly from urine samples collected in June 2004-2005 (leading up to estrus, i.e. proestrus), without extraction, using commercially available E2 enzyme immunoassay (EIA) kits (Assay Designs Inc., Ann Arbor, MI). Serum P4 was measured, after ether extraction, using commercially available P4 EIA kits (Enzo Life Sciences, Ann Arbor, MI). Both assays were performed following the manufacturers protocol. EIAs were read at 405nm on a Bio-Tek EL800 absorbance reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Urine and serum samples were run in triplicate, and averaged to determine the concentration of urinary E2 or circulating P4. The sensitivities of the assays were 28.5 pg/ml and 8.57 pg/ml for estrogen and progesterone EIA kits, respectively. The intra-assay and inter-assay coefficient of variation for the urinary E2 EIA was 8.1 and 10.0 %, respectively. The intra- and inter-assay coefficient of variation for P4 was ≤ 5.1% and ≤ 4.0%, respectively. All concentrations of E2 were expressed as ng per mg creatinine (Cr) to correct for variation in urine concentration and/or kidney excretion rate (Taussky & Kurzmann, 1954). Creatinine concentration was assayed using a commercially available kit according to manufacturer protocol (QuantiChrom, BioAssay Sysystems, Hayward, CA), a modified Jaffe method at sample dilutions ranging from 1:2 to 1:10, on a Bio-Tek EL800 absorbance reader at 510 nm. Urine collection and corresponding E2 EIAs were performed by T. Spady. Serum sampled collections were performed by the vets at the field site. P4 extraction and P4 EIAs were performed by L. Stewart for all study years.

Statistical analyses

Student's t-tests, assuming equal variance, were used to detect differences in the duration of physiological estrus and differences in how quickly females exit physiological estrus between females observed mating and females where mating was not observed. To make a conservative estimate for our comparison, day five of estrus was considered the day where females not observed mating were expected to mate. We chose day five of estrus as the day of expected mating because we found that this was the average day that mating first occurred in mated females.

An F test was used to determine if P4 concentrations during late-September and mid-October differed between females not observed mating and isolated females. This was done because coitus did not occur in isolated females and females not observed mating. Because we were looking at P4 concentrations between female groups that had coital stimulation versus females without coital stimulation, isolated females and females not observed mating were combined for this statistical analysis. Equality of variance was verified using an F test (DF = 1, F stat = 0.8, p = 0.5) prior to pooling data from isolated females and females not observed mating prior to analyses. Student's t-tests, assuming equal variance, were then used for P4 analyses.

We used an alpha error significance threshold of 0.05 in all statistical analyses. All statistical analyses were performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

Results

Duration of estrus and decline in vulvar swelling after mating

Average duration of physiological estrus did not differ between mated females and females not observed mating, including isolated females (DF = 29, t-stat = 1.3, P = 0.1; Figure 1). The average day that females were mated during physiological estrus was day five (4.6 ± 0.9 days; mean ± SEM). The average difference in how quickly females exit physiological estrus did not differ between mated females and females not observed mating, including isolated females (DF = 23, t-stat = -0.7, P = 0.2; Figure 2). As previously stated, in females not observed mating, including isolated females, how quickly females exit physiological estrus was defined by the number of days the female maintained a vulva score of 2.5 or greater after day five of physiological estrus. How quickly females exit physiological estrus in mated females was considered the time from the first successful mating to the end of estrus (vulva score less than 2.5).

Urinary estrogen pattern in isolated females

During the mating season, urinary E2 concentrations were determined daily for five estrous cycles in four isolated females in 2004 and 2005 (Figure 3). We observed a gradual increase of urinary E2 concentration leading up to physiological estrus (6 days prior). Peak urinary E2 concentrations were observed one day before physiological estrus. This peak was then followed by a rapid decline in E2 concentration by the 1st day of physiological estrus (day zero). This pattern observed in isolated females would be similarly observed in mated females.

Serum progesterone concentrations in study females

During late-September to mid-October, P4 concentrations in the isolated females and females not observed mating were found equal (DF = 1, F-stat = 0.8, P = 0.5). As a result of the F-test, isolated females and females not observed mating were combined for statistical analysis. The average concentration of serum P4 was equally elevated in both mated females and females not observed mating during July (DF = 4, t-stat = -0.9, P = 0.2; Figure 4) and late-September to mid-October (DF = 11, t-stat = -0.3, P = 0.4; Figure 5).

CL identified in females not observed mating

In 2011, ovaries were harvested from five females, three mated and two not observed mating. All females in 2011 had evidence of ovulation. Among the mated females, one had two CL in one ovary only, another had one CL in each ovary, and the third had one CL in one ovary and one CH in the second ovary. In females not observed mating, one female had three CL in one ovary and one CL in the second ovary; the second female had one CL present in both ovaries. Histological analysis was performed on the ovaries from the females not observed mating, and confirmed that these structures were CL (Figure 6).

Discussion

It is currently a widely held belief that American black bears are coitus-induced ovulators (Okano et al., 2006; Czetwertynski et al., 2007; Reinwald and Burr, 2008; Swenson and Haroldson, 2008; Yamane et al., 2009; Kohira and Mori, 2010; Steyaert et al., 2012; Frederick et al., 2013; Striling et al., 2016), based primarily on the Boone et al. (2004) study. However, the present study clearly indicates that coitus is not the primary, or only, stimulus that induces ovulation in the American black bear, contradicting the conclusions made by Boone et al. (2004) that the American black bear requires vaginal stimulation to induce ovulation. This conclusion was made despite one nonmated female out of seven nonmated females having two CL present after the mating season, as determined via laparoscopy. Since Boone et al. (2004), no other empirical studies have been published supporting that American black bears are coitus-induced ovulators. The present study indicates that more research is needed to determine what type of male stimulus induces ovulation in American black bears.

We hypothesized that if American black bears are coitus-induced ovulators, then the average duration of physiological estrus and how quickly females exit physiological estrus after mating should be shorter in mated females than in females not observed mating because mating would terminate estrus earlier. Early termination of estrus would result because mating would trigger ovulation, and destruction of the follicle would cause a rapid decline of estrogen and end both physiological and behavioral estrus (Nelson, 2011). This hypothesis was not supported by the present study (Figures 1 and 2), suggesting that coitus is not required to induce ovulation, terminating estrus. If coitus was required for ovulation, the follicles in females not observed mating would degrade slowly through the process of atresia, causing estrus to terminate at a slower rate than observed (Uilenbroek et al., 1980; Schatten and Constantinescu, 2007). In this study, the durations of physiological estrus in non-mated and isolated females are similar to those previously observed by our lab for mated females (Himelright et al., 2014).

Furthermore, females physically isolated (with no coital stimulus) from males should not exhibit a temporal E2 pattern indicative of ovulation if coitus induces ovulation. The pattern of urinary E2 concentration observed in isolated females was strongly indicative of ovulation (Figure 3), with a gradual increase of urinary E2 peaking one day before estrus, followed by a rapid decline, as would be expected during an ovulation event. Again, if only coitus was required to induce ovulation, then the ovaries of isolated females should have undergone follicular atresia, which would cause the concentration of urinary E2 to decrease at a much slower rate than what was observed. Atresia characterized by slowly decreasing E2 concentrations has been documented in several species (Mead, 1968; Uilenbroek et al., 1980; Carson et al., 1981; Ravindra and Mead, 1984). Tsubota et al. (1998) reported serum estradiol concentrations from days 0-52 of estrus in 12 mated females from the same semi-free ranging population as the current study. In their study, elevated serum E2 concentrations were observed during early estrus followed by ~50% decline in E2 following estrus, similar to what we observed with urinary E2. It is important to note that in the present study, physically isolated females were still exposed to other male stimuli that may induce ovulation, such as visual, olfactory, and auditory cues. Only coitus and touch stimuli were eliminated in physically isolated females.

We hypothesized that the concentration of serum P4 during July and late-September to mid-October should be higher in mated females than in females not observed mating and isolated females because only mated females should have CL present. CL form after ovulation producing P4 to support pregnancy (Schatten and Constantinescu, 2007). P4 in mated females and females not observed mating during July were elevated to levels that were not significantly different (Figures 4). Similarly, P4 during late-September to mid-October were elevated and did not significantly differ between mated females, females not observed mating, and isolated females (Figure 5). Similar elevated P4 concentrations observed in all female groups during July and late-September to mid-October suggests that ovulation was stimulated by a male stimulus other than coitus alone. The P4 concentrations observed in the current study are comparable to the P4 concentrations observed by Tsubota et al. (1998) during the early-delay period (3.4 ± 0.2 ng/ml). Similar concentrations, which indicated the presence of CL, were observed in the present study for mated females, females not observed mating and isolated females (Figure 4 and 5). In addition, the present study showed higher concentrations of P4 in mated females, females not observed mating, and isolated females during late-September to mid-October than in July, indicative of active luteal function (Figure 5).

The final prediction was that females not observed mating should not have any CL present in the ovaries following the mating was not supported by the current study. CL structures were identified via *in vivo* laparoscopy in three out of three mated females and two out two females not observed mating. The total number of CL reported in females not observed mating was confirmed via histological analysis (Figure 6A and 6B). In the Boone et al. (2004) study, one of seven isolated females was found to have ovulated based on identification of CL via *in vivo* laparoscopy. This method of analysis may have limited the ability of Boone et al. (2004) to report the full number of CL as some CL may have not been visible on the exterior of the ovary. Although gross morphological laparoscopic analysis is useful, histological analysis is superior for analysis of ovarian morphology. Histological analysis eliminates the possibility of structures being misidentified as luteal cysts (still containing an unfertilized egg). Tsubota and Kanagawa (1993) found large vacuoles present in the cytoplasm of the luteal cells in brown bears between October and December. The sizes of vacuoles observed in the CL of the present study (Figure 6A and 6B) varied between large and small. Canivenc and Bonnin (1981) described that CL during the stage of delayed implantation in the European badger had smaller secretory vacuoles which secrete less P4. Erickson, Nellor, and Petrides (1964) also documented smaller secretory vacuoles just following ovulation in wild American black bears. Similar to the discussions of Erickson, Nellor, and Petrides (1964) and Canivenc and Bonnin (1981), the CL observed in late July 2011 all contained small, irregular secretory vacuoles. The connection between P4 production and CL morphology explains why P4 was lower during the serum sample collection that occurred in July of 2011, during the period corresponding to early embryonic diapause. Therefore, these observations suggest that female American black bears ovulate due to stimuli other than coitus.

Conclusions

This paper challenges the dogma that American black bears are coitus-induced ovulators, and highlights the general need for basic research on reproductive biology in bears. Given that most bear species are considered threatened or endangered, it is crucial to obtain a better understanding of bear reproduction through empirical study rather than acceptance of dogma. Gaining more knowledge concerning bear reproductive biology will lead to better informed management decisions for captive breeding programs. For example, a change in most artificial insemination (AI) protocols, which currently include physical stimulation of the vaginal region (assumes coitus-induced ovulation), may be changed to a much less invasive olfactory stimulation of ovulation to increase AI success.

Acknowledgments

We would like to thank Stephanie Bauman and Alexia Gee for their contributions in aiding with the enzyme immunoassays. We would also like to give a special thanks to Ryan

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Figures

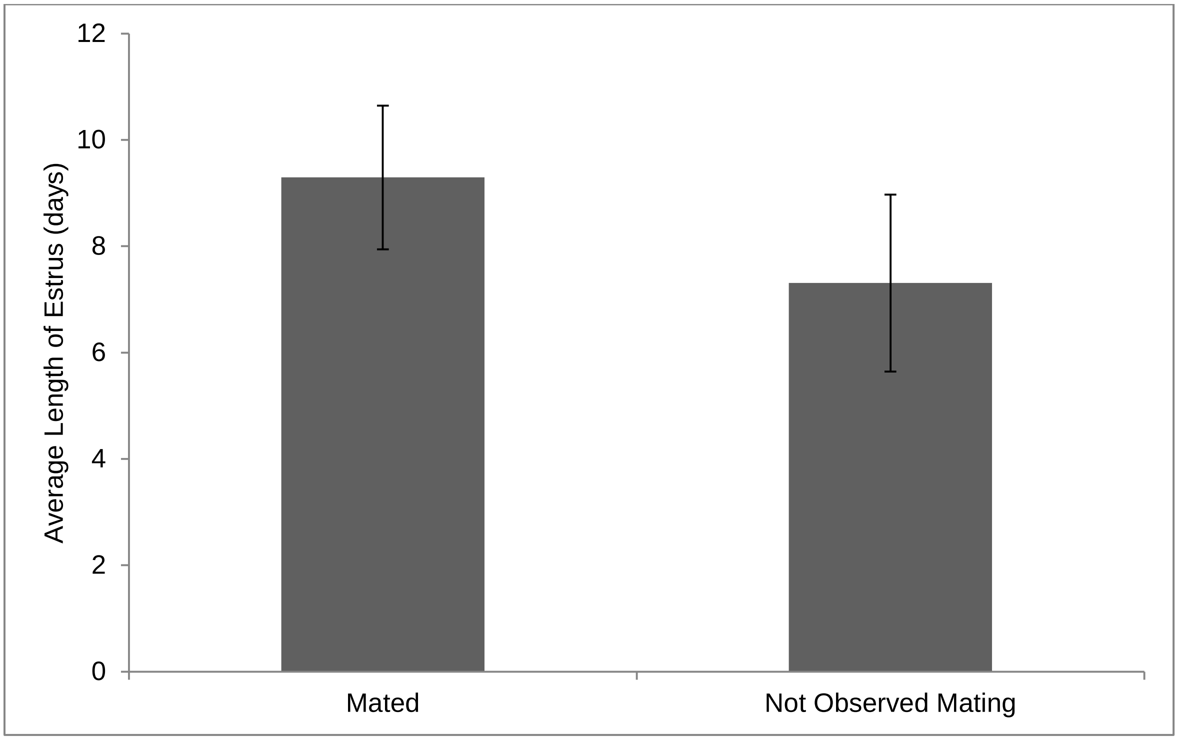

Figure 1: The average duration of physiologic estrus (± SEM) was determined for mated (n= 17) and females not observed mating (n = 13), including isolated females. Physiologic estrus is determined by a vulva score ≥ 2.5. N represents the number of estruses for each group. DF = 29, t-stat = 1.3, P = 0.1.

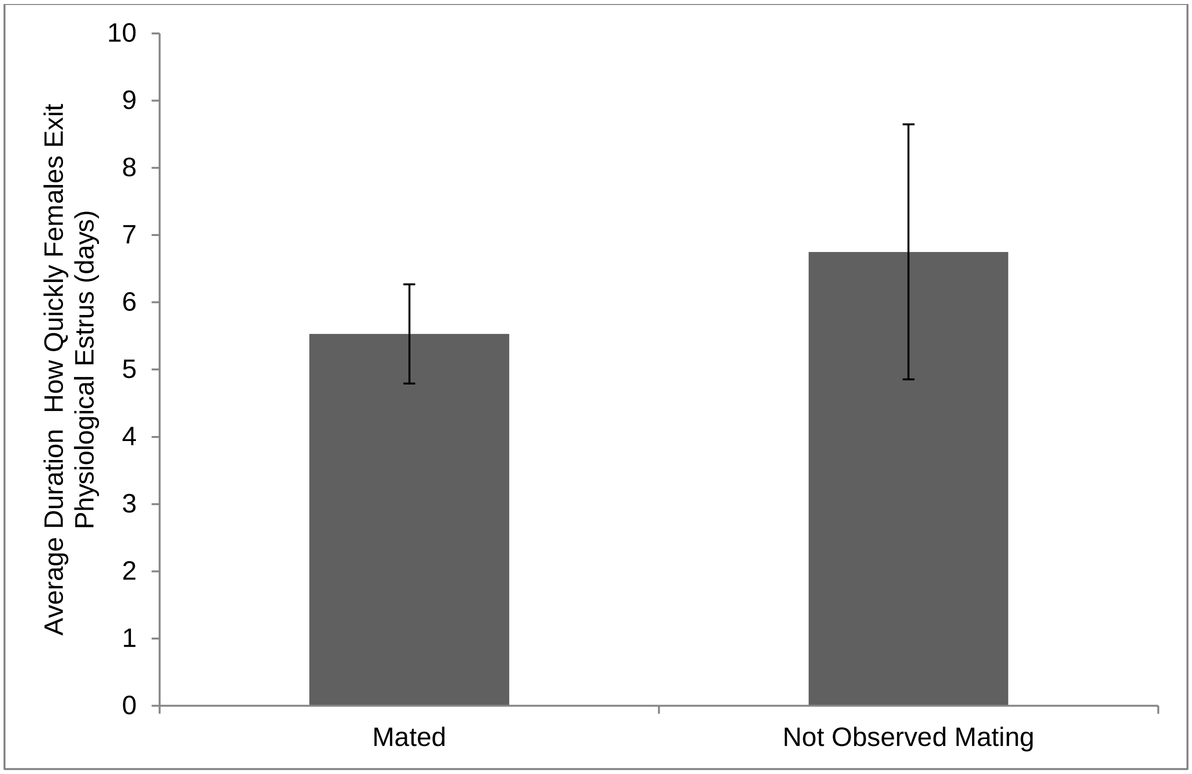

Figure 2: The average duration of how quickly females exit physiological estrus (± SEM) was determined for mated females (n = 17) and females not observed mating (n =8). How quickly females exit physiological estrus was considered to be from the first day of mating through the end of estrus (vulva score < 2.5). For the purpose of comparison to mated females, this measure was calculated in females not observed mating by determining how long vulvar swelling persisted above 2.5 after the fifth day of estrus, which was the average day of physiological estrus at which mated females were first mated. N represents the number of estruses for each group. DF = 23, t-stat = -0.7, P = 0.2.

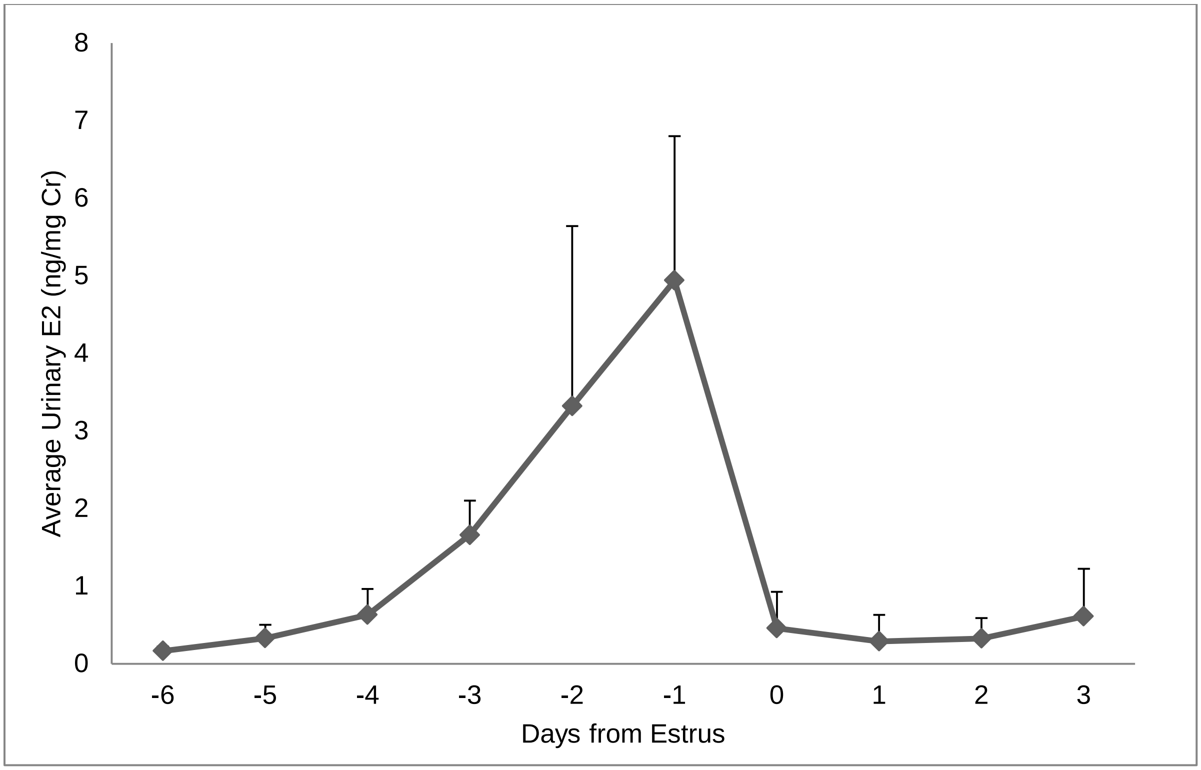

Figure 3: The concentration of urinary estrogen (E2) (+ SD) was observed during five estruses from four isolated females during the 2004 and 2005 mating seasons. The sharp spike in E2 concentration, followed by a rapid decline, is indicative of ovulation.

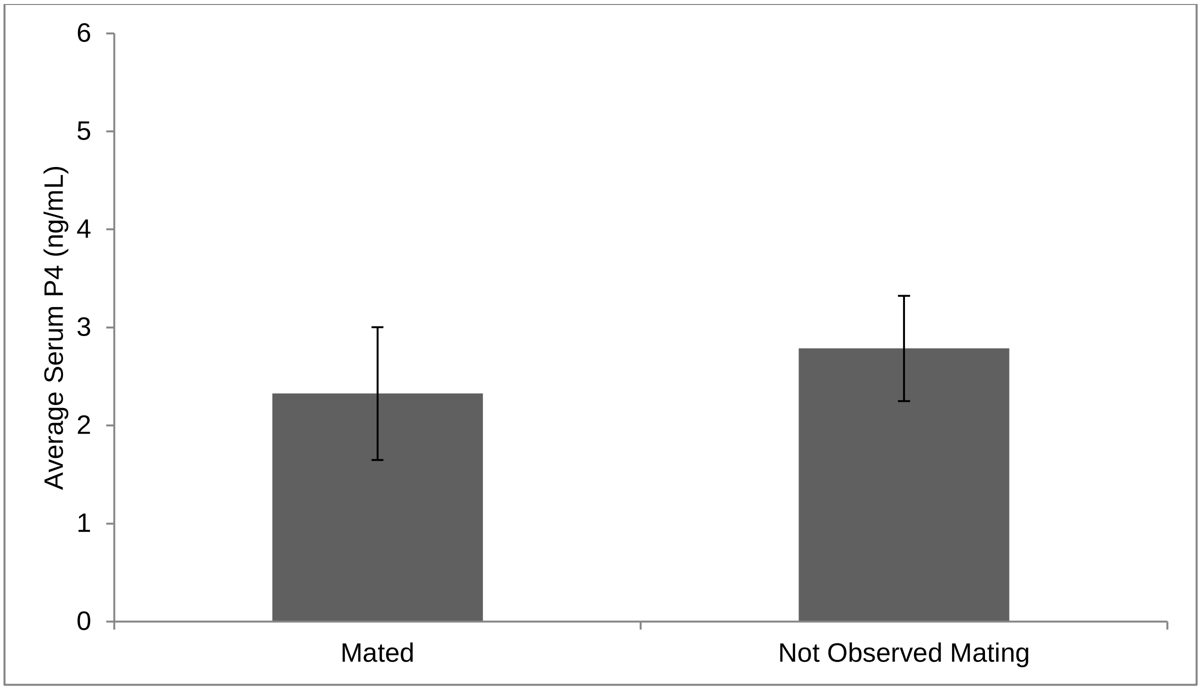

Figure 4: Serum progesterone (P4) concentration (± SEM) during July (early embryonic diapause) was observed in mated females (n = 3) and females not observed mating (n = 3). N represents the number of females sampled for each group. DF = 1, F-stat = 0.8, P = 0.5.

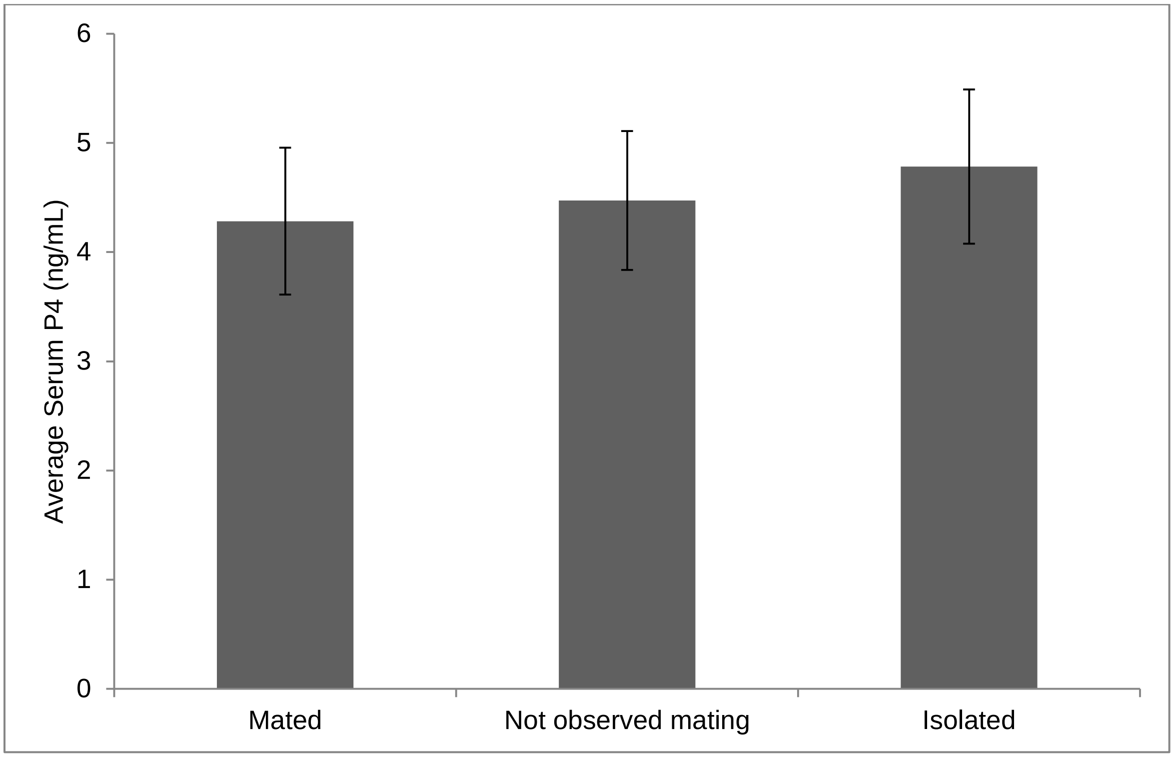

Figure 5: Serum progesterone (P4) concentration (± SEM) during late-September to mid-October (late embryonic diapause). P4 concentrations were measured in mated females (n = 9), females not observed mating (n = 2), and isolated females (n =2). Isolated females were females that were physically isolated from males by a fenced barrier and had no opportunity for coitus. N represents the number of females sampled for each group. DF = 11, t-stat = -0.3, P = 0.4.

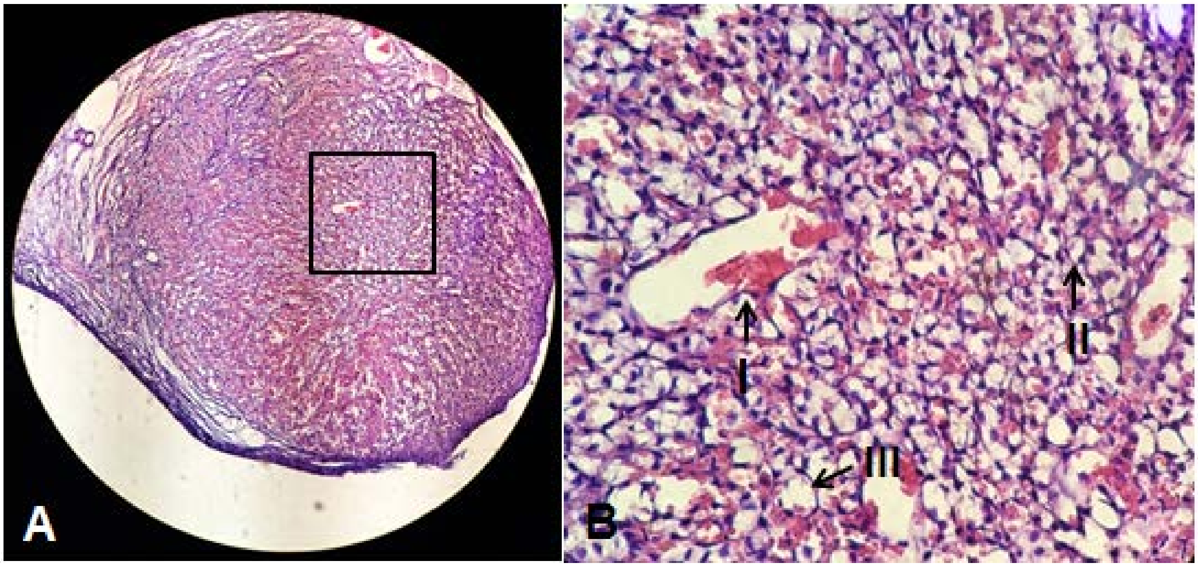

Figure 6: Histoarchitecture of a corpus luteum from an adult female that was not observed mating during the 2011 mating season. Figure 6A illustrates a prominent CL, superficial on the ovary (X40). Figure 6B is a x400 magnification of the boxed area (6A) uniformly representative of the CL. In Figure 6B, note the hypervascularity (I) and abundance of small and large secretory vacuoles (II and III, respectively) that can be observed in the cytoplasm of the luteal cells.

Table 1 – Female ID, year(s) observed, types of data collected

F ID – Year(s)	Mating Status	Time Sampled	Vulva Score	P4 Conc. (ng/mL)	Urine Sampled	CL #	Histology
F1-04/05	Isolated	June-July	No	n/a	Yes	n/a	No
F2-04/05	Isolated	June-July	No	n/a	Yes	n/a	No
F3-04/05	Isolated	June-July	No	n/a	Yes	n/a	No
F4-04/05	Isolated	June-July	No	n/a	Yes	n/a	n/a
F1-09	Mated	Late-Sept.	Yes	6.4	No	n/a	n/a
F2-09	Mated	Late-Sept.	Yes	4.4	No	n/a	n/a
F3-09	Mated	Late-Sept.	Yes	3.3	No	n/a	n/a
F4-09	Mated	Late-Sept.	Yes	5.8	No	n/a	n/a
F5-09	Mated	Late-Sept.	Yes	3.6	No	n/a	n/a
F1-11	Mated	July	Yes	3.1	No	2	No
F2-11	Non-mated	July	Yes	2.5	No	n/a	Yes
F3-11	Non-mated	July	Yes	2.4	No	2	No*
F4-11	Mated	July	Yes	2.1	No	2	No
F5-11	Non-mated	July	Yes	3.4	No	3	Yes
F6-11	Mated	July	Yes	1.8	No	2	No
F1-13	Mated	June-July	Yes	n/a	No	n/a	n/a
F2-13	Mated	June-July	Yes	n/a	No	n/a	n/a
F3-13	Mated	June-July	Yes	n/a	No	n/a	n/a
F4-13	Mated	June-July	Yes	n/a	No	n/a	n/a
F5-13	Non-mated	June-July	Yes	n/a	No	n/a	n/a
F6-13	Mated	June-July	Yes	n/a	No	n/a	n/a
F1-15	Mated	Mid-Oct.	Yes	7.6	No	n/a	n/a
F2-15	Non-mated	Mid-Oct.	Yes	3.9	No	n/a	n/a
F3-15	Non-mated	Mid-Oct.	Yes	5.1	No	n/a	n/a
F4-15	Mated	Mid-Oct.	Yes	3.4	No	n/a	n/a

F ID – Year(s)	Mating Status	Time Sampled	Vulva Score	P4 Conc. (ng/mL)	Urine Sampled	CL #	Histology
F5-15	Mated	Mid-Oct.	Yes	0.9	No	n/a	n/a
F6-15	Non-mated	Mid-Oct.	Yes	3.1	No	n/a	n/a
F7-15	Isolated	Mid-Oct.	Yes	5.5	No	n/a	n/a
F8-15	Isolated	Mid-Oct.	Yes	4.1	No	n/a	n/a

**Histology analysis was not performed on F3-11 because this female's ovaries were not found in storage.*

Chapter 3: Fecal progestins indicate cyclicity and pseudopregnancy in a female polar bear

**The data from this chapter are included in a manuscript in preparation.*

Abstract

The process of determining fertility, pregnancy, and pseudopregnancy in polar bears, like other bears, is difficult due to the lack of indicators or measures of pregnancy. For this case report, fecal samples were collected by zoo keepers at the Milwaukee County Zoo at opportune times throughout four years, 2010, 2011, 2014, and 2015. The samples were collected from a singly-housed, captive adult female polar bear. Progestin metabolites were extracted from the feces and the concentrations were measured via an enzyme immunoassay. It was found that the study female exhibited signs of cyclicity and pseudopregnancy during 2010 and 2014. The results from the current case report will help inform captive management decisions in regards to whether this female will be included in the pool of fertile adult female polar bears. Furthermore, methods outlined in this case report can be used by wildlife managers to inform management of wild populations of polar bears.

Introduction

Polar bears (*Ursus maritimus*) are considered a vulnerable species (IUCN Redlist, 2015). Curry et al. (2014) described that despite recommendations from the Species Survival Plan® (SSP) that all viable individuals should be bred, only four females have given birth in the United States between 2008 and 2013. Attempts to describe the reproductive mechanisms of polar bears are few (Rosing-Asvid et al., 2002; Stoops et al., 2008; Curry et al., 2012; Curry et al., 2014; Gustavson et al., 2015). This is likely due to their solitary nature in the wild and the limited reproduction success in captivity (Linke, 1999). Like many bears, monitoring pregnancy in polar bears is difficult (Curry et al., 2012; Stoops et al., 2012).

Polar bears are seasonal breeders, where mating takes place in March through May, followed by delayed implantation of the embryo (Spady et al., 2007). Pregnant females den in October and November, before giving birth in December and January (Lentfer and Hensel, 1980). Polar bears also exhibit pseudopregnancy, where behaviors and hormone concentrations are indistinguishable between pregnant bears and non-pregnant bears (Stoops et al., 2012). Finally, polar bears, like most bears, are considered to be coitus-induced ovulators though no empirical data is available in the literature (Rosing-Asvid et al., 2002; Curry et al., 2014). Due to the limited literature available on reproduction in polar bears, and the need for higher reproduction rates in captive situations, any and all information on reproduction in polar bears will improve captive breeding programs. Furthermore, techniques developed in captive situations may be translated into the wild to help better manage wild populations.

Zoo keepers at the Milwaukee County Zoo (MKE) noticed behavioral changes similar to what would be expected in pregnant polar bears, in their singly-housed female. This female, named Snow Lily, and identified by studbook accession number 5155, is approximately 30 years old. During the years of study, Snow Lily was completely isolated from male polar bears and her keepers were unsure of her fertility status due to her age and duration of isolation (T.Spady, personal communication). Due to the SSP need for all fertile polar bears to mate, the keepers at the MKE Zoo wanted to confirm whether Snow Lily was exhibiting pseudopregnancy and was therefore likely cycling.

Several studies of captive polar bears have been published where fecal samples were used to determine the reproductive status in females (Stoops et al., 2008; Curry et al., 2012; Stoops et al., 2012). Stoops et al. (2008) observed progesterone and progesterone metabolites (PdG) in feces. Analysis of steroid metabolites in fecal samples is a minimally-invasive way to monitor steroids. In March, Stoops et al. (2008) reported elevated PdG concentrations (282.5 ± 25.8 ng/g feces) with a spike in mid-October (1025.5 ± 142.6 ng/g feces), likely during peri-implantation. Curry et al. (2012) suggested that pregnancy-related proteins may be more useful than steroid metabolites when determining pregnancy status. Stoops et al. (2012) observed a rise in P metabolites between 60-90 days post-mating and then again at 120-150 days post-mating in female polar bears that resulted in births of cubs. In females that did not give birth, P metabolite concentrations remained relatively constant, yet were ~200 ng/g feces, above what Stoops et al. (2012) considered baseline (218.99 ± 8.36 ng/g feces).

The current case report aimed to make a determination of Snow Lily's reproductive status by monitoring concentrations of progestin (P) metabolites in feces. Knowledge gained from this case report will be used to make captive management decisions as to whether Snow Lily will be incorporated into the next mating pool. Finally, the current case report aimed to provide clear evidence to question whether polar bears are coitus-induced ovulators.

Methods

Subject, condition and study duration

The subject of the present case report is a 30 year old, female polar bear, held at the Milwaukee (MKE) Zoo. The female, called Snow Lily by her keepers, can be identified by accession number 5155. Fecal samples were collected approximately three times per month during 2010, 2011, 2014 and 2015. During these years, Snow Lily was housed singly, and completely isolated from males, with no olfactory, tactile, visual, or auditory stimulation from a male.

Fecal sample collection, storage and processing

Fecal samples were collected, in whole, by Snow Lily's keepers whenever possible. Fecal samples were transferred into freezer bags and kept frozen at the MKE Zoo. Keepers sent fecal samples through the mail in large batches and samples were kept frozen throughout the shipping process. Fecal samples were kept frozen at the CSUSM laboratory until they could be processed.

Fecal samples were thawed at room temperature until the entire sample could be manipulated. Using the freezer bags for containment, the fecal samples were homogenized. The homogenizing process required that the thawed fecal samples be mixed via a firm kneading motion. Once homogenized, a small dollop of feces was removed from the sample and placed into a weigh boat. The samples were allowed to dry for approximately 48 hours at 65^0C in a

Precision™ Compact oven (Thermo Fisher Scientific, Waltham, MA, USA). The dried fecal samples were ground into a fine powder using a mortar and pestle. The powder was sifted and then placed into plastic bags for long-term storage in a -20^{0}C freezer.

Steroid extraction

A small portion (0.100g) of dried fecal sample was placed into a clean test tube with 3.3mL of 20% methanol extraction buffer and vortexed for 30 minutes, continuously. The samples were then placed on a Rugged Rotator (Glas-Col, Terre Haute, In, USA) at a speed of 35% for continuous mixing overnight. Tubes on the rotator were protected from light. After a minimum of 24 hours, the samples were removed from the rotator, vortexed continuously for 10 minutes, and centrifuged at 2500 rpm for 20 minutes. The supernatant was confirmed to be free of particulate matter. If particles were present, that sample would be placed in the -20 C freezer for 15 minutes and centrifuged an additional time to ensure separation. The supernatant was collected and stored in the 20^{0}C freezer until hormone analysis could be performed.

Hormone analysis

Extracted progestin (P) metabolites were measured using a commercially available P4 enzyme immunoassay (EIA) kit (Enzo Life Sciences, Ann Arbor, MI). The assays were performed following the manufacturers protocol. EIAs were read at 405nm on an EPOCH 2 Microplate Spectrophotometer (BioTek, Winooski, VT, USA). Samples were run in triplicate and averaged to determine the concentration of progestins. The sensitivity of the progesterone assay kit is 8.57 pg/mL. The intra- and inter-assay coefficient of variation for the assay was ≤ 5.8% and ≤ 3.9%, respectively.

Data analysis

Baseline concentrations of progestins in the current study were defined as concentrations less than 25 pg/mg. This baseline level was determined from the yearly average of fecal P concentrations plus standard deviations in an immature female polar bear. Rosing-Asvid et al. (2002) found that some of their female polar bears were able to give birth by age five. This baseline concentration of P is indicative of inactive ovaries. The female that was used to determine the concentration of P was two years old and housed at the San Diego Zoo. The determination of baseline P concentrations was performed by T.S. in an unpublished preliminary study.

Results

During 2010, elevated P concentrations were observed from mid-September to late-December (Figure 1). During 2011, variable P concentrations were observed from March to October (Figure 2). In 2011, few points fell above baseline in late-April and September (Figure 3). During 2014, elevated P concentrations were observed during May and then again from October to late-December. During 2015, elevated P concentrations were observed from early-January to late-August (Figure 4).

Discussion

The goal of the current case report was to determine if this captive female polar bear showed signs of cyclicity and pseudopregnancy. By monitoring when and at what magnitude P concentrations increase in the female's feces, we can determine whether the female was exhibiting symptoms of pseudopregnancy.

During 2010 and 2014, elevated concentrations of P observed from mid-September and October to late-December suggest that the female was exhibiting an active luteal phase (Figure 1 and Figure 3). P concentrations in 2010 were never observed to drop below baseline levels. The elevated P concentrations observed during May 2014 may suggest the presence of a luteal cyst being present on the ovaries during the mating season. The P concentrations observed from September 2010 to January 2011 and October 2014 to January 2015 were similar to P concentrations that a pregnant polar bear should exhibit.

In 2011, over half of the samples collected fell below baseline (n = 12 of 22 samples) P concentrations (Figure 2). Because there was no trend observed between the samples elevated and below baseline, it is likely that this female was not cycling in 2011. The elevated P concentrations observed in 2011 may be a result of a luteal cyst, which would cause elevated P concentrations. During 2015, elevated P concentrations were observed in January to late-August (Figure 4). In no case was a peak that dropped below the baseline threshold observed. This suggests that during 2015, Snow Lily may have exhibited a luteal cyst, which caused increased P concentrations year-round. It is possible that Snow Lily was cycling in 2015, but without samples during fall and early-winter, this conclusion cannot be fully supported.

The current case report provides hormonal evidence to suggest that Snow Lily was cycling during some of the periods in which samples were collected. Evidence of ovarian activity was observed in 2010 and 2014, where active luteal phases were observed. Due to active luteal phases being exhibited by Snow Lily during 2010 and 2014, it is likely that Snow Lily also exhibited pseudopregnancy during these years. For pseudopregnancy to occur, ovulation must also occur. As a result, we suggest that it is also likely that Snow Lily ovulated in 2010 and 2014, without the presence of any male stimuli. This is contradictory to conclusions made by Rosing-Asvid et al. (2002) who concluded that polar bears are coitus-induced ovulators. Furthermore, it is important to note that even at 30 years of age, Snow Lily was considered to be cycling, which means that it may also be possible for older female polar bears to contribute to captive populations. This information is being used by zoo managers to determine whether Snow Lily should be incorporated into the breeding population of polar bears in North America.

Acknowledgements

Thank you to the zoo keepers at the Milwaukee County Zoo, especially Dawn Fleuchaus, who collected, froze, stored, and shipped the fecal samples. Additional recognition must be provided to Alexia Gee and Stephanie Baumann who performed a large majority of the fecal processing, extraction, and EIA. Students from the Spring 2016 semester of BIOL 380 at CSUSM assisted with re-analyzing samples from the 2010 and 2011 study years.

Figures

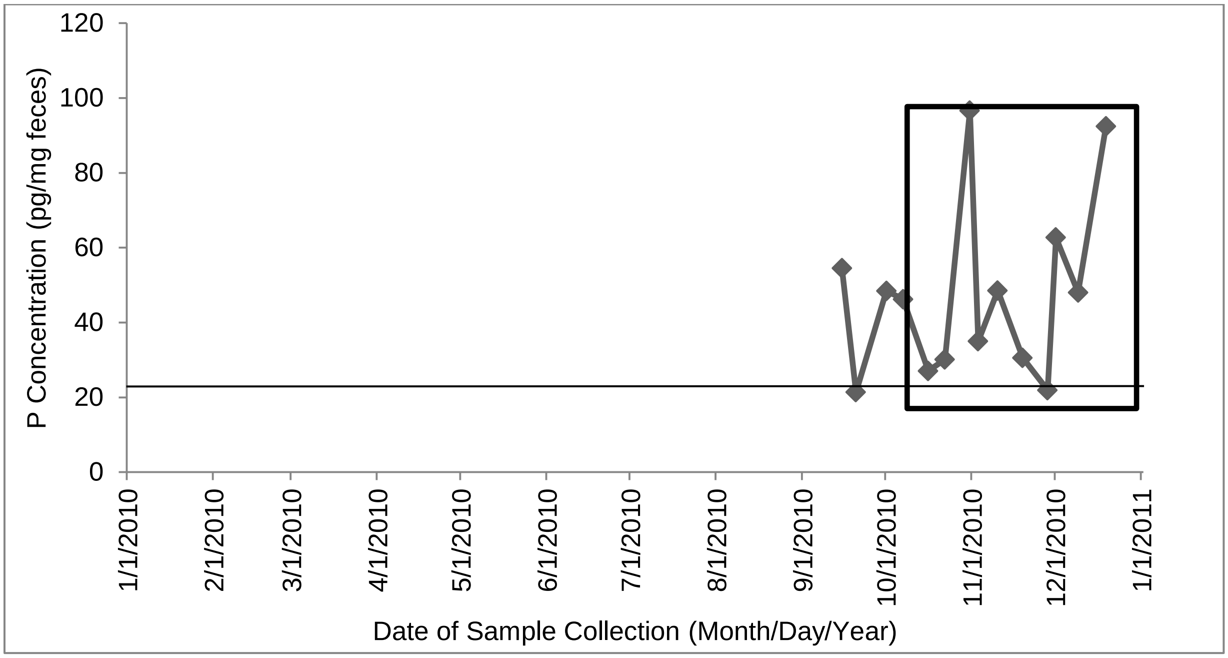

Figure 1 Fecal progestin concentrations when isolated from all male contact. Samples were collected from September 15, 2010 to December 19, 2010. Line at 25 pg/mg feces demarcates the 25 pg/mg feces baseline threshold. The boxed region from mid-October through late-December is indicative of an active luteal phase.

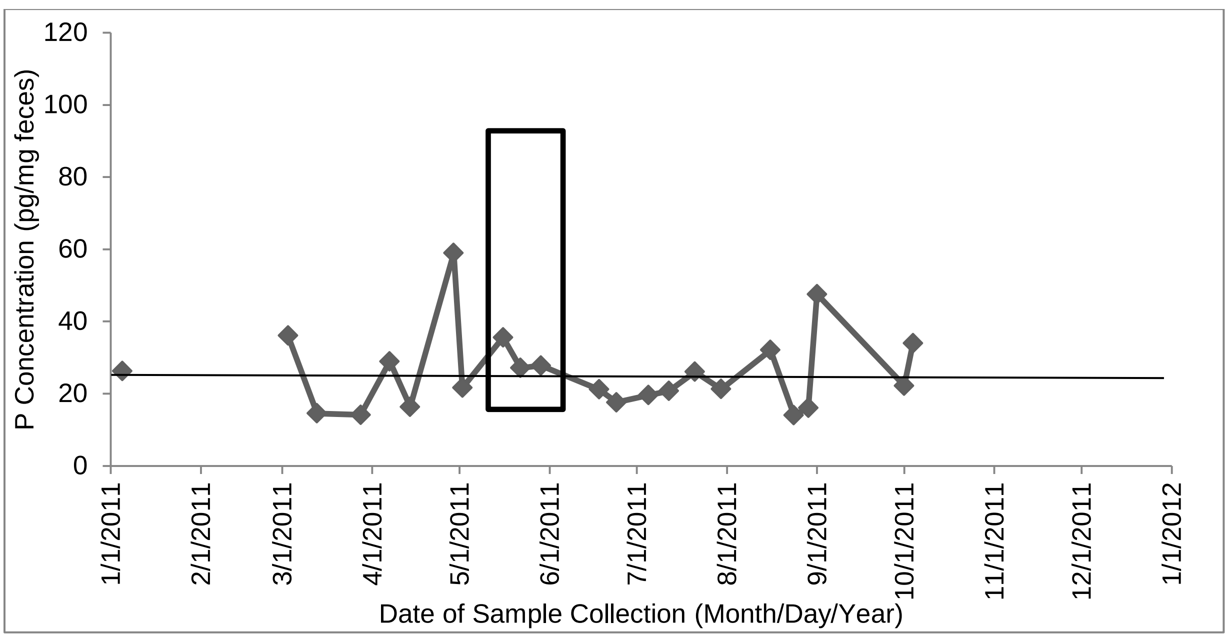

Figure 2 Fecal progestin concentrations when isolated from all male contact. Samples were collected from March 3, 2011 to October 4, 2011. Line at 25 pg/mg feces demarcates the 25 pg/mg feces baseline threshold. The boxed region from mid-April through late-June is indicative of a luteal cyst.

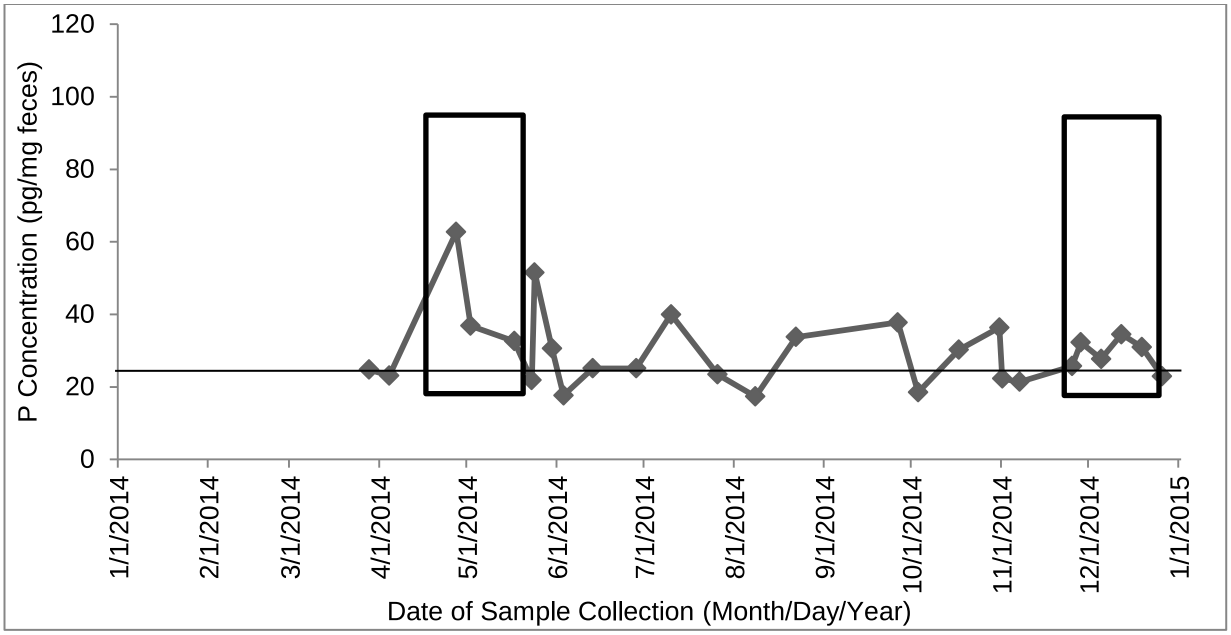

Figure 3 Fecal progestin concentrations when isolated from all male contact. Samples were collected from March 28, 2014 to December 26, 2014. Line at 25 pg/mg feces demarcates the 25 pg/mg feces baseline threshold. The boxed region from mid-April through mid-June may be indicative of a luteal cyst, the boxed region from late-November through late-December is indicative of an active luteal phase.

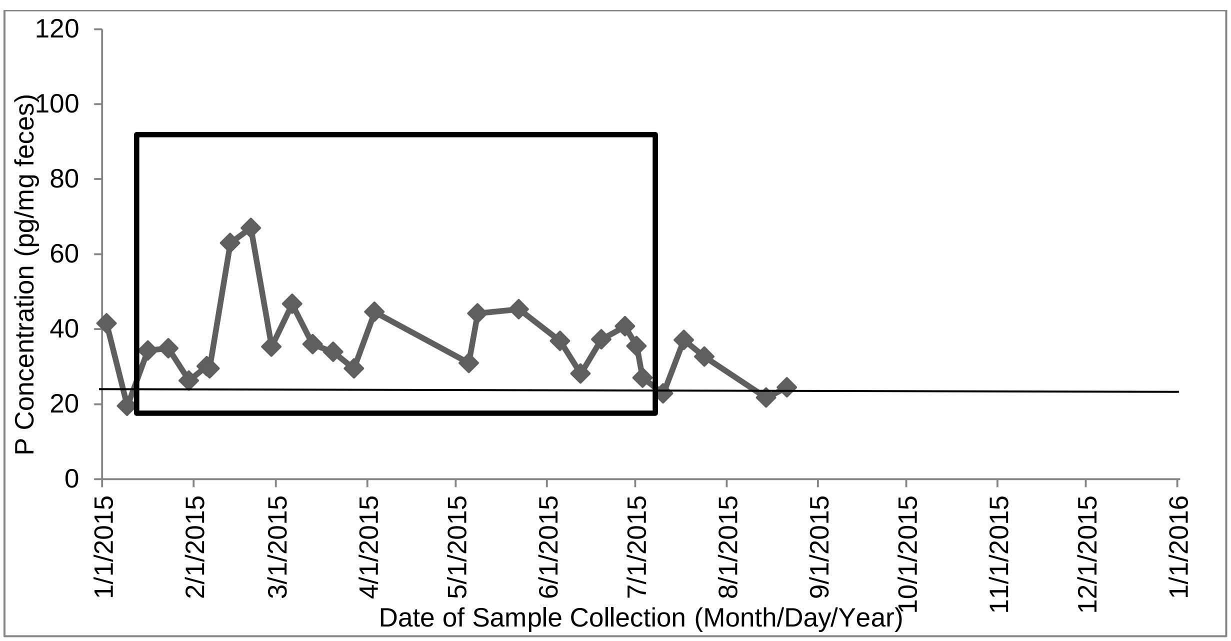

Figure 4 Fecal progestin concentrations when isolated from all male contact. Samples were collected from January 2, 2015 to August 21, 2015. Line at 25 pg/mg feces demarcates the 25 pg/mg feces baseline threshold. The boxed region from early-February through late-July is indicative of a luteal cyst.

Overall conclusions

In Chapter 2, we presented physiological, hormonal, and histological data that clearly indicates that coitus is not the only male stimulus inducing ovulation in American black bears. The general idea that all bears are coitus-induced ovulators was also suggested in Chapter 3. In Chapter 3, we showed via hormone analysis that in at least one polar bear, no male stimuli was required to induce ovulation and cause pseudopregnancy in polar bears. Due to the similarities in reproduction among most bear species (Spady et al., 2007), it is possible to use the information gained in this thesis to better inform reproduction management in both captive and wild situations.

Some of the current literature on reproduction in American black bears indirectly suggests that American black bears do not fit the model for exhibiting induced ovulation. Because bears are solitary animals, it is not likely that bears will encounter more than a few mates throughout the breeding season. As a result of this, it seems likely that females would only ovulate near the time of mating, which would give the females the highest chance for fertilization. However, because females are seasonally polyestrus (Gonzales et al., 2013), with each subsequent estrus being fertile (Himelright et al., 2014), then the necessity to "save" their ovulation event for a time when they encounter a mate seems unnecessary. An additional, early argument for why bears were thought to be coitus-induced ovulators is the presence of the baculum in males (Lariviere and Ferguson, 2002). However, when you look at the detail of the baculum in most bears, they appear elongated with no other additional structures to provide added stimulation to induce ovulation (Abella et al., 2013). Even upon phylogenetic analysis, the induced ovulation hypothesis was found not to be correlated to the presence of a baculum in mammals, including the American black bear (Lariviere and Ferguson, 2002). Taking these arguments into consideration on top of the conclusions made in the current thesis, it seems reasonable to believe that most bear species are not primarily coitus-induced ovulators.

The conclusions made in the following thesis are very different from the conclusions made by Boone et al. (2004). Boone et al. (2004) was cited at least nine times in reference to American black bears and other bear species as being coitus-induced ovulators (Okano et al., 2006; Czetwertynski et al., 2007; Reinwald and Burr, 2008; Swenson and Haroldson, 2008; Yamane et al., 2009; Kohira and Mori, 2010; Steyaert et al., 2012; Frederick et al., 2013; Stirling et al., 2016). Despite the small sample size in Boone et al. (2004), and that one female who was not mated had CL, Boone et al. (2004) reached the conclusion that American black bears, and likely all bears, are coitus-induced ovulators. One important difference between Boone et al. (2004) and the current thesis, is that Boone et al. (2004) observed the ovary *in vivo* via laparoscopy, but in the current study, we also performed histology on the ovaries. Histology is a superior method of analysis because we can confirm our morphological identifications and can identify structures present inside the ovary and thus not visible by laparoscopy. In contrast to the Boone et al. (2004) study, other studies on other bear species have suggested that coitus may not be the only stimulus inducing ovulation.

Okano et al. (2006) and Chang et al. (2011) both concluded that Asiatic black bears were ovulating at high rates due to other stimuli, besides coitus. In these studies, females were housed in similar situations to those described in Chapter 2 of this thesis. These studies found that females completely isolated from males did not ovulate, but females housed near males, but unable to mate, ovulated at rates similar to mated females. Even one of the most conclusive studies on brown bear reproduction by Craighead et al. (1995) was unable to provide evidence that brown bears ovulate strictly due to coital stimuli. Taking these observations into account, it is likely that bears as a whole are not spontaneous ovulators, but are ovulating due to male stimuli such as olfactory, auditory, or visual cues. If there is little empirical evidence that bears are coitus-induced ovulators, then it is important to re-examine this issue so that more informed management decisions can be made, especially in regards to species that are either vulnerable or endangered.

In Chapter 3 of the current thesis, evidence is provided that in at least one captive female polar bear, ovulation and subsequent pseudopregnancy were observed in a female that had been completely isolated from males for many years. This observation does not follow the current assumptions that polar bears are coitus-induced ovulators. Although there are no other studies investigating the ovulation mechanism in polar bears, zoos are applying knowledge from the current limited literature to make management decisions that are extremely invasive. For example, Curry et al. (2014) reported their failed attempt to artificially inseminate a female polar bear, following hormonally induced ovulation. In no way do the authors consider whether their induction method is inappropriate or that inducing ovulation in general is unnecessary.

If we find that olfaction stimulates ovulation, placing scents from males in a female's enclosure prior to AI may be sufficient for inducing ovulation. This stimulus is less invasive than artificial stimulation via exogenous hormonal induction. This information may also lead to changes of how and when males are introduced to females for breeding in captive settings. It has been found in both brown bears and polar bears that chemosensation is used for communication between conspecifics (Rossell et al., 2011; Clapham et al., 2012; Owen et al., 2015). Assuming that it is found that olfaction of a male induces ovulation, priming females with a male's scent may ensure that the female ovulates closer to the time that the male and female are allowed in the same enclosure to mate. This would reduce the time that the individuals are housed together, decreasing the risk of injury. This would reduce the amount of stress that individuals may encounter and improve the probability of successful breeding. Due to the vulnerable status of polar bears, and five other bear species, it is necessary that more intensive studies on bear reproduction be performed so that the proper management decisions can be made.

Based on the results presented in Chapter 2 and preliminary results presented in Chapter 3, it is no longer acceptable to consider American black bears and perhaps bear species in general as only coitus-induced ovulators. With this, more research needs to be performed to look at what stimuli, if any, induce ovulation. Preferably, these studies will be performed on species that are not considered vulnerable or endangered, like American black bears, and then can be used to make management decisions on species that have a critical conservation status.

Future directions

There are still many questions that remain regarding bear reproduction that should be investigated in American black bears, and then applied to other bear species that are considered vulnerable or threatened, like polar bears. The following list includes suggested questions (or methodologies) that should be pursued to answer questions related to those included in this thesis:

1. Are American black bears spontaneous ovulators? In this thesis, we determined that coitus is not the only stimulus inducing ovulation in American black bears. Due to limitations of the current study design, we were unable to determine if American black bears exhibit spontaneous ovulation, or if another male stimulus is involved in triggering ovulation. An ideal scenario would be one in which a third study pen can be created, away from the main population pens and the other study pens. If we were able to eliminate all male stimuli, we could then repeat the measures from chapter 2 of this thesis to determine whether these completely isolated females ovulated. If it was determined that these females ovulated, then this would suggest that American black bears are spontaneous ovulators. If these females did not ovulate, this leads us to my next proposed project.
2. Do olfactory, visual, or hearing cues from males induce ovulation in American black bears? This research question could be answered in conjunction to the previous question, but due to limited resources in the field, would likely have to be performed during separate field seasons. In answering this question, having three different study pens for certain groups of females would be ideal. These pens would all need to be separate enough from the main population to ensure confidence that no undesired stimuli would be introduced into the study groups. The first group of females could be the females in which olfaction only is tested. Here, females would be held in a study pen in which the females could not see or hear other males. Stations with scents from

mature, dominant males could then be placed into the pen. Another female group could test the visual cues hypothesis for inducing ovulation. Here, the females would be placed into a pen and males that are mature, but sterile, would be placed into a pen next to the study pen. If these males were made sterile at a young age, it is likely that they would not develop any courting behavior, including vocalizations, which would likely be the hearing male cue that would induce ovulation. Our third group of females would be the group testing the hearing hypothesis of induced ovulation. This group of females could also be held in a study pen adjacent from a group of sterile males, but in this scenario, a visual barrier would have to be placed between the two pens. This male group would likely have to be one in which the males were sterilized later in life and were therefore able to develop the mating calls and courting behavior.

3. A third future direction is in relation to the third chapter of my thesis. Because we observed evidence of an active luteal phase in an isolated polar bear, this suggests that polar bears may exhibit some amount of spontaneous ovulation. This leads to my next suggested study question: are polar bears spontaneous ovulators? Due to the very small sample size (n = 1) included in Chapter 3, conclusions about the species as a whole cannot be sufficiently supported. I suggest that the sample size should be increased to answer this question. By locating other singly housed female polar bears in the United States and asking the keepers to collect bi-weekly fecal samples, we would be able to determine whether the evidence of an active luteal phase are being observed in other isolated female polar bears.

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