Studies on the ovarian dynamics of brown bears to establish artificial insemination protocol

（人工授精技術確立に向けたヒグマの卵巣動態に関する研究）

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Contents

Abbreviations

ARTs	assisted reproductive technologies
AI	artificial insemination
BSA	bovine serum albumin
CLs	corpora lutea
CMO	carboxymethyloxime
EDTA	ethylenediaminetetraacetic acid
E_2	estradiol-17β
GnRH	gonadotropin releasing hormone
GPS	Global Positioning System
hCG	human chorionic gonadotropin
HCL	hydrochloric acid
IUCN	International Union for Conservation of Nature
LH	luteinizing hormone
P_4	progesterone
P450arom	aromatase cytochrome P450
SD	standard deviation

Notes

The contents of Chapter I have been published in Theriogenology.

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Preface

The International Union for Conservation of Nature's Red List of Threatened Species assessed that about 800 out of around 5,800 mammals were threatened with extinction. To save these endangered species, a lot of reproductive research has been done. Assisted reproductive technologies (ARTs), such as artificial insemination (AI), in vitro fertilization or cloning could help to overcome the problems in the management of small, isolated populations of species [1]. AI is the most applicable ART in nondomestic species and can facilitate offspring production between animals that are behaviorally incompatible or in different locations. For example, the population of black-footed ferrets (*Mustela nigripes*) decreased to 18 individuals at one point, but have been successfully reintroduced (release of a captive species into the wild) following reproductive research and an AI project in captivity [2,3]. Regarding giant pandas (*Aliuropoda melanoleuca*), reproductive research led to an improved reproductive rate and higher captive population [4,5]. As a result, the category of both animals on the red list changed to less threatened from "Extinct in the wild" to "Endangered", and from "Endangered" to "Vulnerable", respectively [6,7]. These animals, in which ART has been applied successfully, have features of tractability and trainability in captivity. Therefore, repeated blood samplings and monitoring of estrous cycle or ovarian activity by ultrasonography can be done without anesthesia; these techniques are necessary for intensive research into reproductive physiology and close monitoring when using ARTs in these animals.

In Ursids, eight species are living in the world: giant panda, Andean bear (*Tremarctos ornatus*), sloth bear (*Melursus ursinus*), sun bear (*Helarctos malayanus*), American black bear (*Ursus americanus*), Asian black bear (*Ursus thibetanus*), polar bear (*Ursus maritimus*) and brown bear (*Ursus arctos*). Except for the American black bear and brown bear, the other six species are classified as vulnerable on the red list, which means they are facing the risk of decreasing populations or extinction. Giant pandas are the only species in which AI has become a part of reproductive management [5]. Giant pandas have a single estrus of a few days once a year and show spontaneous ovulation [8]. Estrus can be detected using urine hormones and vaginal cytology [9–11]. Therefore, AI can be performed at natural estrus without any control of ovarian activity by hormonal treatment. Polar bears, an important icon for global warming, perform most mating activity between March and May [12]. They are assumed to be an induced ovulators [13]; although some females show spontaneous ovulation [13] and experience pseudopregnancy in captivity [14–16]. In polar bears, planned breeding has attempted to increase genetic diversity worldwide, but only a few successful cases have

been reported. In most trials, copulation has been confirmed, but causes of failure are not identified [14]. AI could be an option for assisted breeding in polar bears [17]; however, no cubs have been born using AI after eight times trials [18]. Because sample sizes of polar bear studies are small, available information is limited and most AI trials have not been done with an understanding of reproductive physiology and close monitoring of ovarian activity and/or the stage of estrus. For successful AI in polar bears, the reproductive physiology should be understood, but the limited number of animals and their untamable characteristic have prevented detailed studies of this animal's reproduction. Brown bears are taxonomically in the same genus and a related species to polar bears [19], which means their reproductive physiology is expected to be similar to polar bears. Since they are relatively easier to access for reproductive studies owing to their larger population, it may be feasible to collect detailed information on reproductive physiology and develop an AI protocol in brown bears, then adapt this information and experience to polar bears. A similar strategy of using related species as a model has been used in an effort to conserve endangered animals; domestic ferrets and polecat as models for black-footed ferrets and domestic cats as a model for non-domestic felids including the clouded leopard, cheetah and leopard cat [20].

Brown bears have the breeding season from early spring to summer: May to July in Hokkaido, Japan [21]. During the breeding season, males and females spend around 20 days together and copulation occurs at irregular intervals (3-20 days) [21,22]. They are assumed to be an induced ovulators [23], although spontaneous ovulations have been confirmed under captive conditions [24,25]. Implantation usually occurs from November to December after six months of embryonic diapause. Then, pregnant females give birth during their denning period approximately 60 days after implantation [23]. Fecal estradiol or urinary estrogen concentrations are reported to increase during or after mating [26,27]. Serum progesterone (P_4) is slightly elevated after mating, followed by a gradual increase during embryonic diapause, and further elevated in early winter [23,24,26]. However, the ovarian dynamics during breeding season is still unclear.

Ultrasonography can evaluate the reproductive organs non-invasively; therefore, it has become used widely for examinations of genital organs for clinical and research purposes in the medical and veterinary fields. Ultrasonography has also been applied in non-domestic animals: elephants [28], rhinoceroses [29], giraffes [30] and felids [31,32]. In Ursids, several studies used ultrasonography to monitor fetal growth in brown bears [23,33] to monitor follicle size to prepare for AI in giant pandas [34] and to describe the yearly ovarian changes in Asian black bears [35]. Repeated ultrasonography is necessary to characterize follicular development, ovulation and subsequent corpus luteum (CL) formation. In most zoo animals,

ultrasonography for monitoring of detailed ovarian activity needs to be conducted under anesthesia. To develop an AI protocol in zoo animals, including polar bears, a research strategy has to be established to find the minimal and/or optimal frequency for ovarian monitoring. This will help to conduct research to develop an AI protocol in animals with limitations in terms of frequent anesthesia due to concerns about risks and the heavy workload.

The present study was conducted in Noboribetsu bear park, where about 70 brown bears are housed for exhibition and expertise in anesthesia by a park veterinarian is available. The purpose of this study was to elucidate the ovarian dynamics in brown bears for establishing an AI protocol. In Chapter I, the author examined the weekly ovarian activity during the breeding season, from May to July, and described the follicular development pattern and growth rate. In Chapter II, the author continued the monitoring ovarian activity to confirm the findings in Chapter I and extend understanding of ovarian dynamics before and after the breeding season to clarify the type of estrus, *i.e.*, monoestrus or polyestrus.

Chapter I

Monitoring follicular dynamics using ultrasonography in captive brown bears (*Ursus arctos*) during the breeding season

1. Introduction

Brown bears (*Ursus arctos*) are seasonal breeders with a breeding season from May to July and they are assumed to be polyestrous animals [21]. Although they are categorized as "least concern" in the 2017 IUCN Red List Category [36], some subpopulations are classed as having vulnerable status, such as the Himalayan brown bears (*Ursus arctos isabellinus*) and Cantabrian brown bears (*Ursus arctos cantabricus*), due to their scattered habitats and illegal hunting [37–39]. For conservation, semen preservation for later artificial insemination (AI) in brown bears has been well documented [40–42]. However, the success of AI has never been reported in brown bears.

The giant panda (*Ailuropoda melanoleuca*) is the only bear species in which the birth of cubs following AI has been successful. In giant pandas, vaginal cytology has predictive chromic shifts prior to ovulation that can be used as an indicator for AI timing [11]. With well-developed animal husbandry practices in this species, daily examination can be performed without general anesthesia. However, in other bear species including brown bears, it is difficult to perform examinations (e.g., ultrasonography, blood hormone measurements, and vaginal cytology) to determine the timing of AI because anesthesia is required. In addition, female giant pandas show spontaneous ovulation [5], but brown bears are generally assumed to be induced ovulators; therefore, I needed to develop an ovulation induction protocol to perform AI in brown bears. In polar bears (*Ursus maritimus*), which is a closely related species, an AI trial was reported; however, cubs were not born [17]. This previous study performed hormonal treatment to stimulate follicular development using exogenous gonadotropins. However, the treatment was not designed based on an understanding of the follicular dynamics of polar bears. For successful AI, understanding follicular development and ovulation, along with endocrine changes, is essential. However, my knowledge of follicular development and ovulation in bear species is limited; therefore, more information, including follicular growth rates and ovulatory follicular size in the target species, is needed.

Ultrasonography has been used as a tool for monitoring follicular development and ovulation in domestic animals [43] and non-domestic animals including elephants [28], rhinoceroses [29], giraffes [30], and Asian black bears (*Urusus thibetanus*) [35]. Kang et al. [35] performed monthly transrectal ultrasonography in Asian black bears focusing on changes

in ovarian structure. However, more frequent observations are necessary to monitor follicular development and determine the timing of ovulation induction.

Therefore, in the present study, ultrasonography was performed weekly to investigate follicular growth rates and ovulatory follicular size using captive female Hokkaido brown bears (*Ursus arctos yesoensis*). Further, two bears were included in an ovulation induction trial with exogenous gonadotropin treatment.

2. Materials and Methods

2.1 Animals

A total of six female Hokkaido brown bears housed in the Noboribetsu Bear Park (Noboribetsu, Hokkaido, Japan) were used for the experiments (Table I-1). All animals were fed a diet consisting of vegetables and commercial concentrations for bears (ZOO FOOD bear, Nosan corporation, Kanagawa, Japan). Water was provided *ad libitum*. They were housed separately from males but Bears 4 and 5 were in close proximity to adult males. Bear 5 delivered a cub in January 2018, but the cub died in March at 54 days old. The others did not have any cubs within the year before the study. All bears were apparently healthy throughout the study period with no abnormal values on hematological or biochemical examinations before and after the study period. Experimental procedures followed the ethical guidelines of Hokkaido University Animal Care and Use Committee (No. 18-0108).

2.2 Ultrasonography of the ovaries

Transrectal ultrasonography of the ovaries was performed under anesthesia. For anesthesia, I administrated xylazine HCl (1 mg/kg; Selactar; Bayer, Tokyo, Japan) and a 1:1 mixture of zolazepam HCl and tiletamine HCl (2.0 to 4.0 mg/kg; Zoletil 100; Virbac, Carros, France) via intramuscular injection using blow darts. A B-mode ultrasonography device (HS-2100V, Honda Electronics, Aichi, Japan) equipped with a linear transducer (5 to 10 MHz, HLV-475, Honda Electronics) was used for all examinations. The transducer was attached to a hand-made carrier of a polyvinylchloride pipe (49 cm long, 2.6 cm outside diameter) and inserted into the rectum along with conductivity gels with the bear in a lateral recumbent position. All visible antral follicles (>1.5 mm) and corpora lutea (CLs) in the right and left ovaries were counted, and the diameter and relative positions of follicles and CLs in the ovaries were recorded. After examination, atipamezole HCl (Atipame; Kyoritsu, Tokyo, Japan) at the same volume as xylazine was injected and recovery from anesthesia was observed.

2.3 Measurements of steroid hormone concentrations in peripheral blood

Blood samples were collected via the medial saphenous vein into EDTA-loaded vacuum tubes. Blood samples were centrifuged at 1,200 *g* for 15 min, and plasma was separated in plastic tubes and stored at -20˚C until assayed. Plasma progesterone (P_4) and estradiol-17β (E_2) concentrations were determined using competitive double-antibody enzyme immunoassays, as previously described [44]. The primary antibodies used for the E_2 and P_4 assays were anti-estradiol-17β-6-carboxymethyloxime (CMO)-BSA (FKA204, Cosmo bio, Tokyo, Japan) and anti-progesterone-3-CMO-BSA (KZ-HS-P13, Cosmo bio), respectively. Goat anti-rabbit serum (111-005-003, Jackson Immuno Research, West Grave, PA, USA) was used as the secondary antibody. The inter- and intra-assay coefficients of variation were 3.8 and 4.0% for E_2, and 2.4 and 2.2% for P_4, respectively. The parallelism confirmed between the reference standard curves and serial dilution of plasma extractions containing known amounts of steroid hormones in brown bears is shown in Figure I-1.

2.4 Experimental design

Information regarding the bears and observation schedule are shown in Table I-1. Monitoring of the ovaries using ultrasonography and blood collections were principally performed weekly. In 2017, two bears (Bears 1 and 2) were used for monitoring, four and three times in Bear 1 and 2, respectively, from June to July. In 2018, Bear 2 and an additional three bears (Bears 3 to 5) were used for monitoring nine to 12 times from May to July. In addition to monitoring the ovaries, I performed preliminary trials to induce ovulation with exogenous hormone treatment. In 2018, Bears 2 and 3 were administered a gonadotropin releasing hormone (GnRH) agonist, buserelin acetate (20 μg/head, IM; Estomal; Intervet, Ibaraki, Japan), to induce ovulation on the first observation day on which the largest follicle detected was greater than 10.0 mm in diameter. The timing of ovulation induction was determined according to a previous study together with observation of spontaneous ovulation in the first year of the present study. In Asian black bears, follicles of 7.4 mm in diameter have ovulated [35]. On the other hand, a 13.1-mm follicle ovulated spontaneously in first year of the present study. Therefore, I speculate a follicle more than 8.0 mm in diameter might have the potential for ovulation, and then I considered a 10.0 mm diameter follicle as the starting point for treatment. Further, I followed the manufacturer's instructions and determined to use the highest dose recommended for cattle ovulation induction as the GnRH dosage for brown bears.

2.5 Statistical analysis

Results are expressed as mean ± SD. The follicular development was divided into two periods, an early and a late period based on the appearance of follicles with a diameter larger than 6.0 mm. The mean number of total follicles in both ovaries on each examination between the early and late periods were compared using Student's *t*-test. To analyze the follicular growth rate, the day on which the largest follicle of each follicular wave showed the maximum size was defined as Day 0, and the previous diameter data for the same follicle were plotted before Day 0 in a scatter diagram. Growth rates between the early and late period were compared using multiple regression analysis. All statistical analyses were performed using JMP software version 14. (SAS Institute, Cary, NC, USA). The values were considered significant at $P < 0.05$.

3. Results

3.1 Follicular dynamics

The changes in the ovarian structure of Bears 1 and 2 in 2017 are shown in Figure I-2. In 2017, there were already large follicles that had grown to more than 6.0 mm in diameter in Bears 1 and 2 (13.1 mm and 8.2 mm, respectively) on the first day of observation, June 16th. In Bear 1, the largest follicle persisted at a similar size for the following two observations; however, no follicles or CLs were observed on July 30th, which was 28 days after the 3rd observation. In Bear 2, spontaneous ovulation was observed; namely, the largest follicle, 13.1 mm in diameter, observed on June 16th (Fig. I-3A) had disappeared by June 24th and a CL was found with a cavity inside (Fig. I-3B). On July 2nd, the CL had developed fully within eight days (Fig. I-3C).

The changes in ovarian structure of the four bears (Bears 2 to 5) used in 2018 are shown in Figure I-4. The follicular development was classified into two periods based on the follicular development during the breeding season from May to July. In the early period (May to mid-June), one to three wave-like follicular growths with a maximal diameter of 5.3 ± 0.4 mm (range 4.7 mm to 6.0 mm, n = 8) were observed in each animal. For example, in Bear 2, a small follicle which was 3.3 mm in diameter (Fig. I-3D) developed to 5.2 mm by six days later (Fig. I-3E) but disappeared in subsequent observation. In the late period (mid-June to July), at least one follicle reached a diameter greater than 6.0 mm. In Bear 2, two follicles larger than 6 mm existed, one in each ovary, and in Bear 3 and 4, two follicles exceeded 6 mm in diameter, both in the same ovary (Fig I-3F). However, in Bear 5, a large follicle 10.9 mm in diameter was observed at the first observation on May 6th and persisted for 12 days at a similar size. Subsequently, new follicles emerged, and the largest follicle persisted at a

diameter greater than 6.0 mm until the end of the study period. Although some wave-like follicular development was observed, the data from Bear 5 were excluded from further analysis.

The largest follicle size in each follicular wave during the late period including observations in 2017 was 11.2 ± 2.0 mm (range 9.3 mm to 14.0 mm, n = 5) and this was larger than that (5.3 ± 0.4 mm, range 4.7 to 6.0 mm, n = 8) in the early period (Table I-2). The total numbers of follicles observed in the pair of ovaries at each examination were higher in the early period than in the late period ($P < 0.01$, Table I-2). Figure I-5 shows the relationship between follicular diameter and days as a scatter diagram. The day on which the largest follicle reached the maximum size in each follicular wave was defined as Day 0. A moderate linear relationship was found between days and follicle diameter during the early ($r^2 = 0.62$, $P < 0.01$) and late period ($r^2 = 0.59$, $P < 0.01$). The growth rate of the large follicles in each wave in the late period (0.25 mm/day, n = 6) was greater than that in the early period (0.15 mm/day, n = 8, $P < 0.01$).

As shown in Table I-1, Bears 2 and 3 were used for ovulation induction in 2018, and were given GnRH on the first day on which a follicle of greater than 10.0 mm in diameter (14.0 mm and 10.2 mm, respectively) was observed. In Bear 3, the largest follicle on the day of GnRH treatment disappeared six days later and a CL was detected in the ipsilateral ovary 15 days after GnRH treatment (Fig. I-4). In Bear 2, the largest follicle on the day of GnRH treatment decreased in size but did not ovulate (Fig. I-4). In Bears 2 and 3, the second largest follicles were a similar size to the largest follicles on observation prior to GnRH treatment. These secondary follicles then decreased in size on the day of GnRH treatment, although the largest follicle developed in size. However, these secondary follicles grew again after GnRH treatment.

3.2 Hormonal changes

Peripheral hormonal changes in individuals in 2017 and 2018 are shown in Figures I-2 and 4, respectively. In Bears 2, 3, and 4 in 2018, E_2 concentrations increased and showed a peak on the last day monitoring (five to seven days) immediately before the largest follicles reached their maximum sizes. Plasma P_4 concentrations remained at base levels during follicular development. P_4 concentrations increased with CL formation in Bear 2 in 2017 (Fig. I-2) and Bear 3 in 2018 (Fig. I-4).

4. Discussion

A better understanding of follicular dynamics is essential for developing assisted reproductive technologies. In the present study, the follicular development in brown bears was monitored using ultrasonography during the breeding season and I attempted to induce ovulation. To the best of my knowledge, this is the first report describing follicular development during the breeding season in brown bears.

Follicular development was classified into two periods based on the largest follicle size of 6.0 mm in diameter during the observation period from May to July. In the early period (May to mid-June), the largest follicles grew to 6.0 mm or less in diameter and then regressed. In domestic cats, a seasonal breeding carnivore, peripheral luteinizing hormone (LH) concentrations increase toward postpartum estrus [45]. In other seasonal breeders, like mares and ewes, follicles grow and regress without ovulation during the transition phase from the non-breeding season to the breeding season due to insufficient LH pulse frequency [46,47]. In a previous study employing immunohistochemical observation of the ovaries in brown bears, aromatase cytochrome P450 (P450arom) was detected in follicles greater than 6.0 mm in diameter, but not in follicles between 2.0 mm and 6.0 mm in size [48]. Since estrogen synthesis depends on the expression of P450arom, follicles smaller than 6.0 mm during the early period may not produce enough estrogen to develop and mature follicles to ovulatory size in brown bears. In fact, relatively low plasma E_2 concentration levels persisted during the early period of the breeding season.

In the late period of the breeding season (mid-June to July), the largest follicles developed to greater than 6.0 mm in diameter. The follicular growth rate (0.25 mm/day) was approximately 1.6-fold that in the early period (0.15 mm/day, $P < 0.01$). Ewes showed an increase in follicular growth rate of the largest follicles toward the end of the anestrus period because of changes in follicular gonadotrophic responsiveness, especially to LH [49]. In mares, large follicles show increased enzyme activity, including P450arom activity, toward the ovulatory phase [50]. Moreover, it is known that a gradual LH increase due to gonadotropin secretion from the pituitary gland toward the breeding season upregulates P450arom expression in the follicles [51]. Therefore, I assumed that LH levels in brown bears may increase toward the late period of the breeding season and that follicles grow to greater than 6.0 mm in diameter with P450arom expression. In the present study, the plasma E_2 concentration increased in the late period of the breeding season. This increase in E_2 may reflect that follicles greater than 6.0 mm in diameter develop dependent upon LH. Production of E_2 probably leads to the selection of follicles. This is supported by the decrease in the total number of follicles at each observation after reaching a follicular size of 6.0 mm.

Many reports have discussed ovarian dynamics in spontaneously ovulating ungulates, but only two studies have described the ovarian dynamics in detail in induced ovulating carnivores (i.e., minks and domestic cats). In minks, a smaller cohort of follicles is selected to become the dominant follicles (about 10 follicles) at a diameter of 0.5 mm between day 4 and 6 after ovulation. These follicles acquire the ability to ovulate at a diameter of 0.7 mm [52]. In domestic cats, the LH receptor in antral follicles are expressed at a diameter of 0.8 mm [53], and E_2 production becomes apparent when follicles reach 1 mm in diameter [54]. On the first day of estrus, a previous study found approximately five follicles with a mean diameter of 2.3 ± 0.01 mm that developed to an ovulatory size within 3.8 ± 0.3 days at a growth rate of 0.2 mm/day [55]. In brown bears, the number of dominant follicles (two to three) is different from that in minks and domestic cats. The follicular growth rate of bears in the late period of the breeding season (0.25 mm/day) was similar to that of domestic cats; however, the preovulatory follicle size differs between brown bears and domestic cats (3.5 mm vs. 10.0 mm). Consequently, the duration of follicular development becomes longer in brown bear (more than two weeks) than domestic cats (seven to nine days).

The present study shows that spontaneous ovulation could occur under captive conditions in the presence of male brown bears, even though brown bears are generally assumed to be induced ovulatory animals. Boone et al. [56] reported that female American black bears housed close to males (approximately 100 m) ovulated spontaneously, probably due to olfactory and auditory cues from the males. Similarly, both domestic cats and wild felids that are classified as induced ovulators, show spontaneous ovulation under captive conditions [57].

For ovulation induction, human chorionic gonadotropin (hCG) has been used in American and Asian black bears [58,59]. hCG is a heterologous protein which can develop antibodies following frequent use, resulting in an impairment of hormonal action. Therefore, I used a GnRH agonist. In my study, GnRH was administered when the largest follicle reached a size more than 10.0 mm in diameter. One follicle of 10.2 mm in diameter ovulated after GnRH injection, and another animal spontaneously ovulated after a follicle developed to 13.1 mm in diameter. My results suggest that a follicle size of 10.0 mm in diameter or larger has the potential to ovulate in brown bears. However, one dominant follicle that grew to 14.0 mm in diameter did not ovulate after GnRH injection. I assumed that this follicle was already atretic and could not respond to an LH surge as reported in cows [60]. In cows, atretic-dominant follicles may have non-functional LH receptors on the granulosa cells. Another possibility in the present study is that the dose of GnRH used was not enough to induce an LH surge and

subsequent ovulation in brown bears. In further studies, I should examine the follicle size and responsiveness to different doses of GnRH.

Interestingly, the second largest follicles grew again after GnRH treatment, even though their size decreased one week before GnRH treatment, when the follicles began deviation. These results suggest that the second largest follicles have the potential to ovulate following hormonal stimulation. In contrast, the plasma E_2 concentration showed a peak prior to follicular deviation. Considering ovulation induction timing, it is possible that follicles could acquire the ability to respond to a stimulus of a smaller size than I expected.

In conclusion, the present study found for the first time that brown bears show multiple follicular waves without dominant follicle selection in the early period of the breeding season. In the late period of the breeding season, a few follicles developed to 6.0 mm in diameter or greater as dominant follicles with a faster follicular growth rate. Before the follicles developed to more than 10.0 mm, plasma E_2 concentrations tended to show a peak. Based on the results of this study, further research of ovulation induction timing is needed to develop an AI program in brown bears.

5. Tables and Figures

Table I-1. Bears used for observation of follicular dynamics and induction of ovulation in 2017 and 2018.

ID	Age (Y)	Parity	Estimated BW (kg)	Year	Dates of observaton	Number of observations	Ovulation induction	Remarks
Bear 1	22	0	120	2017	June 16, 24 July 2, 30	4	—	
Bear 2	18	0	120		June 16, 24 July 2	3	—	Spontaneous ovulation was observed.
Bear 2	19	0	120	2018	May 6, 12, 18, 25 June 1, 7, 17, 22, 29 July 4, 10, 19	12	GnRH (20 μg, IM) June 29	
Bear 3	5	0	100		May 3, 10, 16, 24, 31 June 7, 15, 21, 30	9	GnRH (20 μg, IM) June 15	
Bear 4	12	1	150		May 3, 10, 16, 24, 31 June 8, 17, 22 July 3, 10, 19	11	—	
Bear 5	19	2	150		May 6, 12, 18, 25 June 1, 8, 14, 21, 30 July 4, 10, 19	12	—	One cub was born in January and died in March in 2018.

Table I-2. Characteristics of follicular development identified from ovarian ultrasonography in the early and late periods of the breeding season.

Follicle characteristics	Early period	Late period
Total number of follicles at observation	14.4 ± 7.5^{a} (4-25)	6.2 ± 4.4^{b} (1-13)
Maximum diameter (mm)	5.3 ± 0.4 (4.7-6.0)	11.2 ± 2.0 (9.3-14.0)
Growth rate (mm/day)*	0.15^{a}	0.25^{b}

Early period: May to mid-June, data from Bears 2 to 4 in 2018

Late period: Mid-June to July, data from Bears 1 and 2 in 2017 and Bears 2 to 4 in 2018

*Growth rate: Data from Bears 2 to 4 in 2018

Means with different superscripts are different (a, b: $P < 0.01$).

Data are shown as mean ±SD.

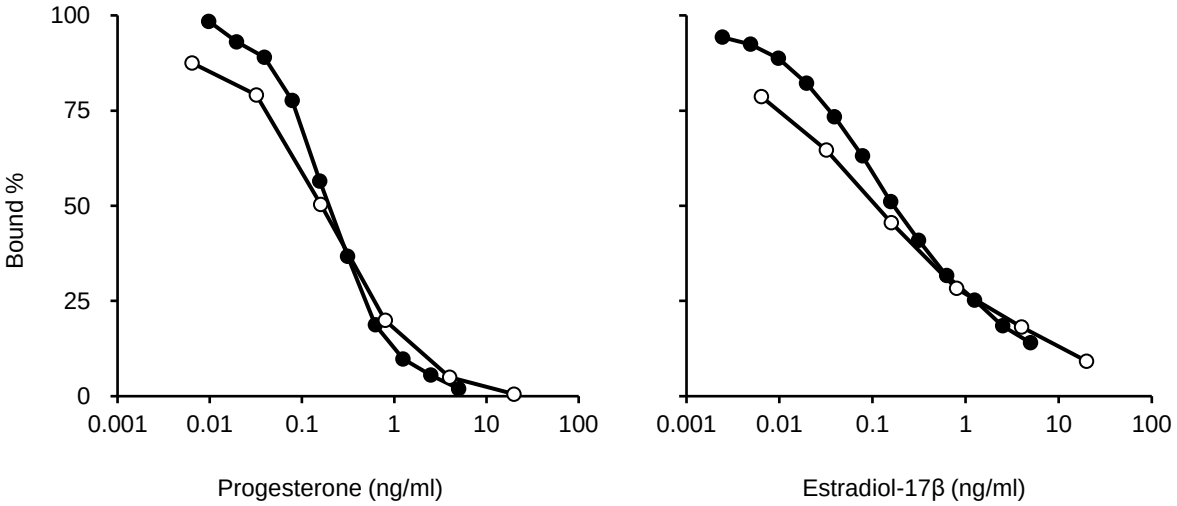

Figure I-1
Parallelism between curves for reference standards (solid circle) and those for serial dilution of plasma samples with known concentration of progesterone and estradiol-17β (open circle).

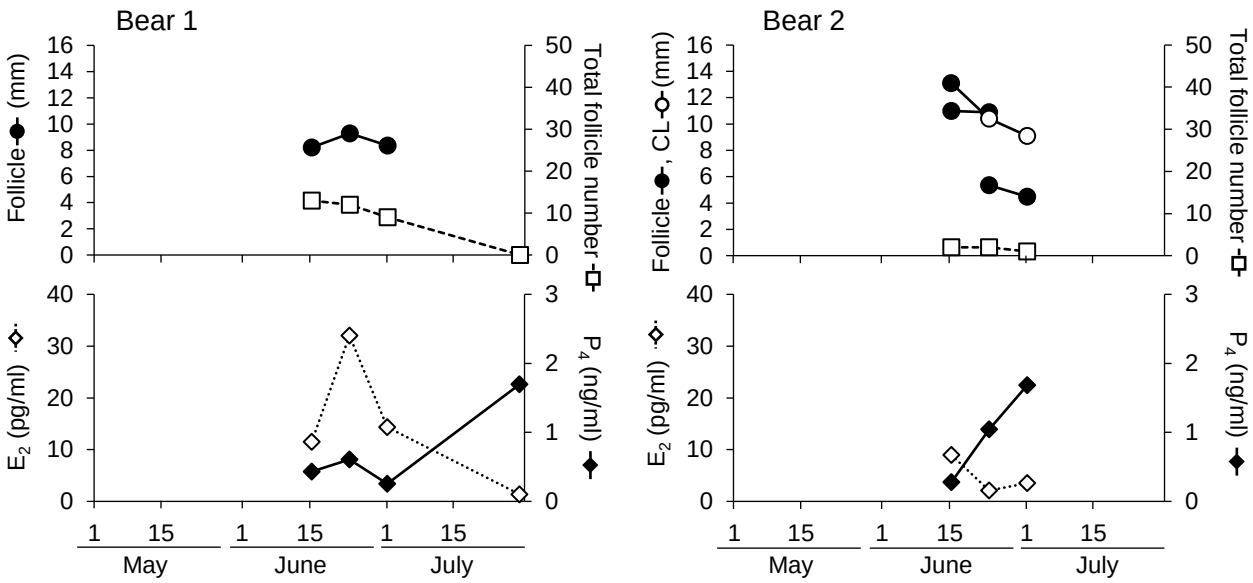

Figure I-2
The follicle growth profiles in non-treated bears in 2017, along with plasma steroid hormones. Follicles (●) and corpora lutea (CLs: ○) are presented using connecting symbols. In Bear 1, the largest follicle remained the same size. In Bear 2, the largest follicle (13.1 mm in diameter) ovulated spontaneously and the CL was detected eight days after the follicle disappeared. E_2: estradiol-17β, P_4: progesterone.

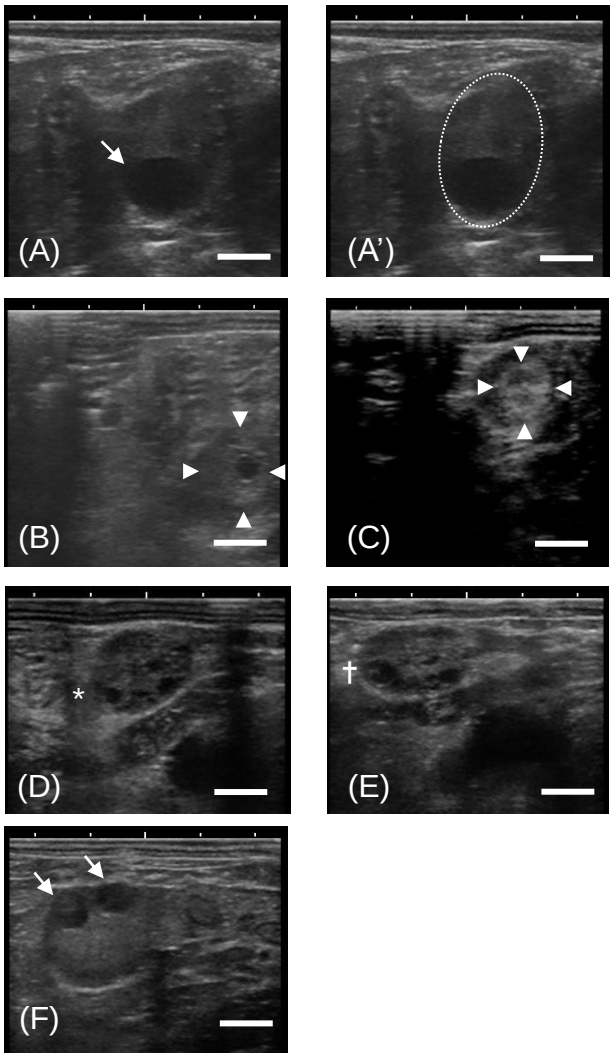

Figure I-3
Ultrasonographic images of the ovaries in brown bears using a transrectal 7.5 MHz transducer. (A) The dominant follicle of 13.1 mm (arrow) was observed on June 16th in Bear 2, 2017, as an anechoic structure. (A') Same images as panel A, with the ovarian border indicated by a dashed line. (B) Same ovary eight days after panel A (June 24th). Corpus luteum (CL: arrowhead), which was 10.4 mm in diameter, with a cavity inside, was observed as a hypoechoic structure. (C) Same ovary eight days after panel B (July 2nd). Cavity inside the CL was filled with luteal tissue and became a fully developed CL. (D) Ovarian image from the early period of breeding season at the emergence of the wave-like development. A small follicle of 3.3 mm (*) and several other follicles of a similar size were observed (June 1st). (E) Same ovary as panel D, six days later (June 7th). The 3.3-mm follicle (*) in panel D had developed to 5.2 mm (†). (F) Ovarian image from the late period of the breeding season on July 10th in Bear 4. Two follicles exceeded 6 mm in diameter (arrow) were seen in the ipsilateral ovary. Scale bar represents 10 mm.

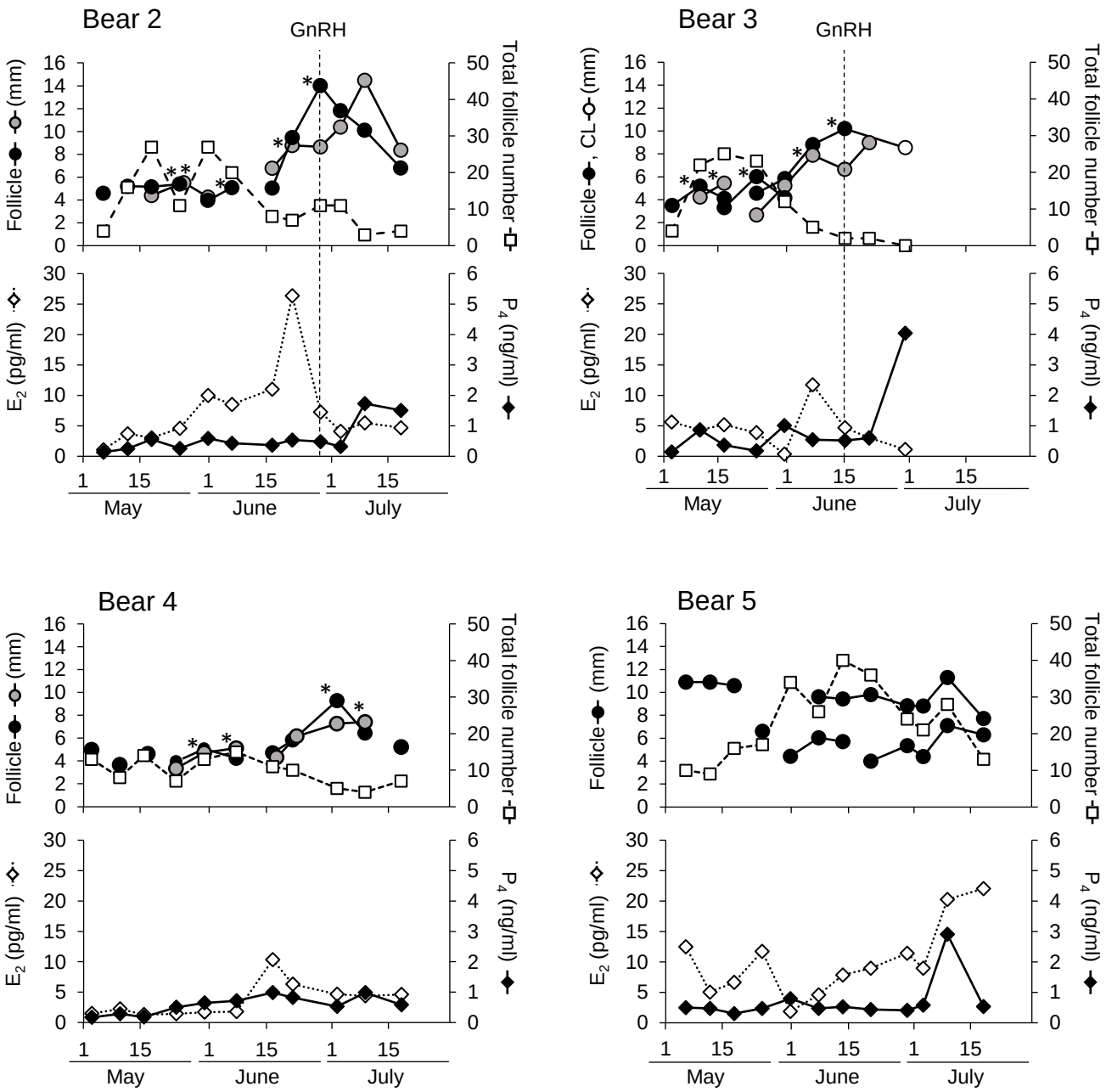

Figure I-4

The follicle growth profiles of Bears 2 to 5 in 2018, along with plasma steroid hormones. Follicles and corpora lutea (CLs) are shown. Follicles of the 2nd wave in the early period and the secondary large follicles in the late period are shown by gray circles, and the other follicles by black circles. Bears 2 and 3 were administrated gonadotropin-releasing hormone (GnRH) to stimulate ovulation. The broken lines indicate the date of GnRH administration. E_2: estradiol-17β, P_4: progesterone. The follicles with asterisks indicate the day on which follicles showed the largest diameter and this information was used to calculate the follicular growth rates in Figure I-5.

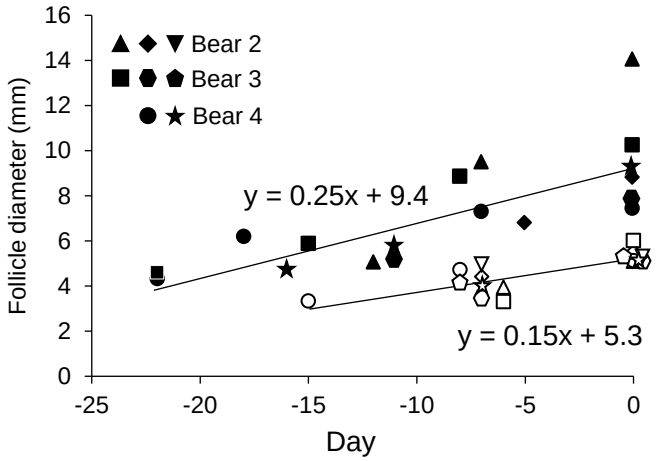

Figure I-5
Scatter diagram and linear relationship for follicular diameter during the follicular wave in Bears 2, 3, and 4 during the early (open, n = 8) and late (solid, n = 6) period. The day on which each follicle showed the largest diameter (asterisk in Figure I-4) was defined as Day 0.

6. Summary

Artificial insemination (AI) may be a useful tool in the reproductive management of endangered animals, including bears. To establish an AI program for bears, I investigated follicular dynamics using weekly transrectal ultrasonography in six captive brown bears. Along with ultrasonography, I monitored plasma progesterone (P_4) and estradiol-17β (E_2) concentrations. Furthermore, two bears were administered a gonadotropin releasing hormone (GnRH) agonist to induce ovulation on the first day on which the largest follicle reached more than 10.0 mm in diameter. Brown bears showed two patterns of follicular development in the early and late periods of the breeding season. In the early period (May to mid-June), multiple follicular waves were observed; namely, many follicles developed, and the largest follicles grew to less than 6.0 mm in diameter then regressed. In the late period (mid-June to July), one or two follicles grew to greater than 6.0 mm in diameter and developed as dominant follicles. Moreover, the growth rate of the largest follicle in the late period was faster than that in the early period of the breeding season. One bear with a follicle of 13.1 mm ovulated spontaneously, and one bear ovulated when the follicle was 10.2 mm in diameter after GnRH agonist treatment. Plasma E_2 concentrations increased and showed a peak on the last day monitoring (five to seven days) immediately before the largest follicles reached their maximum sizes. Plasma P_4 concentrations increased on the day the corpus luteum could be detected using ultrasonography. This is the first study that showed there are two patterns of follicular development in brown bears. Furthermore, the largest follicle reaching more than 10.0 mm in diameter could be an indicator of the appearance of ovulatory follicles.

Chapter II

Monitoring follicular dynamics to determine estrus type and timing of ovulation induction in captive brown bears (*Ursus arctos*)

1. Introduction

Six out of eight bear species are classified as endangered on the IUCN Red List [61]. Brown bears (*Ursus arctos*) are categorized as least concern; however, there are concerns about the extinction of some subpopulations due to their small size [36]. The development of assisted reproductive technology is necessary to promote breeding, and semen preservation for future artificial insemination (AI) has been well documented in brown bears [42,62,63]. Development of an AI protocol relies on a thorough understanding of female reproductive physiology, especially the follicular development and size of mature follicles for optimal timing of AI.

Brown bears are seasonal breeders with the breeding season from late spring to early summer [64]. They have a polygamous mating system and are assumed to be polyestrous [65,66]. They are also assumed to be induced ovulators [66], although spontaneous ovulations have been confirmed under the captive condition [24,25]. Implantation usually occurs after six months of embryonic diapause, then pregnant females give birth during their denning period approximately 60 days after implantation [23]. Several studies have investigated hormonal changes in brown bears. These have shown that fecal estradiol or urinary estrogen concentrations increase during or after mating [26,27]. Additionally, serum progesterone (P_4) is slightly elevated after mating; in captive females, this increase is greater in early winter [23,24,26]. Annual progesterone profiles are similar in both unmated and mated females [24]. However, no detailed information about ovarian activity is currently available.

Recently, I reported morphological changes in the ovaries of brown bears using transrectal ultrasonography to investigate follicular development during the breeding season. This study revealed that follicles developed in a wave-like manner [25]. The size of the largest follicles was less than 6.0 mm (minor wave) in the early period (May to mid-June), while one or two follicles were selected as dominant follicles and exceeded 6.0 mm (major wave) late in the breeding season (mid-June to July). Furthermore, I observed only one major wave in each animal between May and July [25].

The type of estrus (polyestrus or monoestrus) in bear species is not well understood. Previous studies have classified bears as monoestrous animals [64,67]; however, the evidence for this is not clear. Based on behavioral observations of both captive and wild brown bears,

they are assumed to be polyestrous animals [21]. In monoestrous animals, the major wave leading to ovulation occurs only once during the breeding season; thus, there is only one chance to perform AI. In polyestrous animals, there are multiple chances to perform AI in the breeding season. Therefore, it is important to confirm estrus type when developing a breeding plan.

In addition to estrus type, ovulatory follicle size also needs to be determined for AI. Because it is generally assumed that brown bears are induced ovulators, hormonal treatment for ovulation induction is necessary in any AI protocol. At present, the size of mature follicles is undetermined in this species, although my previous study suggested that follicles in the range of 10.0 to 13.0 mm in diameter have the potential for ovulation [25]. For AI preparation, it is desirable to predict when follicles will reach their preovulatory size.

In the present study, to clarify the estrus type in brown bears, I observed follicular development between April and October, before the start and after the end of the breeding season. In the previous study, one bear already had a dominant follicle on the first day of observation in early May [25]; I therefore started observations in April, before the breeding season, in the present study. When a follicle was estimated to have reached 10.0 mm in diameter (the likely mature follicle size in brown bears based on observations noted in Chapter I), I administered a gonadotropin releasing hormone (GnRH) agonist to induce ovulation.

2. Materials and Methods

2.1 Animals

Six female Hokkaido brown bears (Bears A to F) housed in the Noboribetsu Bear Park (42° N, 141° E, Noboribetsu, Hokkaido, Japan) were used for the study (Table II-1). All animals were kept, as described in Chapter I. All bears were apparently healthy throughout the study period with no abnormal hematological or biochemical values during examinations before and after the study period. All experimental procedures were approved by the Hokkaido University Animal Care and Use Committee (No. 18-0108).

2.2 General anesthesia

For anesthesia, I administered xylazine HCl (0.8 to 1.2 mg/kg; Selactar; Bayer, Tokyo, Japan) and a 1:1 mixture of zolazepam HCl and tiletamine HCl (2.0 to 4.0 mg/kg; Zoletil 100; Virbac, Carros, France) via intramuscular injection using blow darts. After examination, atipamezole HCl (at the same volume as xylazine; Atipame; Kyoritsu, Tokyo, Japan) was injected and recovery from anesthesia was observed.

2.3 Ultrasonography of the ovaries

Transrectal ultrasonography of the ovaries was performed under anesthesia, as described in Chapter I. A B-mode ultrasonography device (HS-2100V; Honda Electronics, Aichi, Japan) equipped with a linear transducer (5 to 10 MHz; HLV-475; Honda Electronics) was used for all examinations. The transducer was attached to a hand-made carrier (polyvinylchloride pipe, 49 cm long, 2.6 cm outside diameter) to observe the ovaries via the rectal wall. All visible antral follicles (>1.5 mm) and corpora lutea (CLs) in both ovaries were counted, and the relative positions of follicles and CLs in the ovaries were recorded. The follicle and CL diameters were calculated by averaging the major and minor axes. During each observation, the ultrasonography was recorded using a video recorder (VR570; Toshiba Teli Corporation, Tokyo, Japan) to confirm follicle positions over time. Figure II-1 shows example ultrasonography pictures from an ovary in Bear C.

2.4 Blood collection

Following ultrasonography examinations, blood samples were collected via the medial saphenous vein into EDTA-loaded vacuum tubes. Blood samples were immediately centrifuged at 1,200 *g* for 15 min, and plasma was separated in plastic tubes and stored at -20˚C until assayed.

2.5 Steroid hormone concentrations

Plasma estradiol-17β (E_2) and progesterone (P_4) concentrations were determined using competitive double-antibody enzyme immunoassays, as described in previous studies [25,44]. The primary antibodies used for the E_2 and P_4 assays were anti-estradiol-17β-6-carboxymethyloxime (CMO)-BSA (FKA204; Cosmo Bio, Tokyo, Japan) and anti-progesterone-3-CMO-BSA (KZ-HS-P13; Cosmo Bio), respectively. Goat anti-rabbit serum (111-005-003; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used as the secondary antibody. The inter- and intra-assay coefficients of variation were 7.3 and 4.0% for E_2, and 8.2 and 2.2% for P_4, respectively.

2.6 Vaginal cytology and vulva score

A plastic pipe (17.5 cm long, 1 cm in diameter) was inserted into the vagina, and a sterile cotton swab (4.8 mm in diameter) moistened with physiological saline was inserted through the tube until it stopped. Each cotton swab was gently rotated 360°, then removed and stamped on a glass slide. The slide was stained using a differential quick stain kit (Diff-Quick

staining kit; Sysmex Ins., Kobe, Japan). Vaginal cells were classified according to the criteria for domestic dogs [68]. A minimum of 100 cells in at least four fields per slide were counted under a light microscope and classified as parabasal, intermediate, superficial, or anucleate cells. The cornification rate was calculated as the ratio of superficial to anucleate cells.

The vulval appearance (swelling and color) was evaluated by opening the vulva at the time of ultrasonography; longitudinal and transverse lengths were also measured. Vulval changes were scored using a ranking scale of 0–3 based on the vulva score for giant panda [11] and sun bear [69]. This was adapted here for brown bear: 0 = no swelling, pale color; 1 = slight swelling, pale color; 2 = increased swelling, moderate pink color; 3 = swelling, pink color.

2.7 Experimental design

Four bears (Bears A to D; 5 to 20 years old) were monitored for ovarian activity from April to October. Ultrasonography of the ovaries and blood collections were performed every other week in April, and every week from May until ovulation [25]. After confirming CL formation, observations were performed once a month until October. Two bears (Bears E and F; 5 and 20 years old), were used to obtain information on ovulatory follicle size. Their ovarian observations started from June, when the major wave was expected to appear, until ovulation was confirmed by detecting CLs. Ovulation was induced in three out of six bears by administering 20 or 40 μg of buserelin acetate, a GnRH agonist (Estomal; Intervet, Ibaraki, Japan). The 20 μg GnRH dose is the maximal dose indicated for cattle. Anesthesia for ovulation induction was carried out on the day when the largest follicle was estimated to exceed 10.0 mm in diameter. This was calculated based on the follicular growth rate (0.25 mm/day) reported in my previous study [25]. The terms related to ovarian dynamics are based on this previous study and are defined in Table II-2.

2.8 Data analysis

Results are expressed as mean ± SD. Ovulated follicle diameters at the last observation before ovulation were compared between ovulation type (spontaneous and induced) using the Student's *t*-test, as was the CL diameter on the first day I could detect it by ultrasonography. The follicular growth rate was estimated as described in Chapter I. In brief, the day on which the largest follicle of each follicular wave showed the maximum size was centralized as Day 0, and the previous diameter data for the same follicle were placed before Day 0 on a scatter diagram. To show estimates were reasonable, follicular growth data from Chapter I were included in the present analysis. Growth rates of the minor wave and major wave were

compared by multiple regression analysis. The study period was divided into three periods: minor wave, major wave, and post-ovulation. The minor wave period was from the beginning of observation to the day prior to when the major wave was first detected. The major wave period began from the day of its first detection to either the last day an ovulatory follicle was observed prior to CL detection or to the day GnRH treatment began. The post-ovulation period was from the day of CL detection to the end of the study period. Plasma E_2 and P_4 concentrations in the three periods were compared by Kruskal-Wallis one-way analysis of variance, with the Steel-Dwass test used as a post-hoc test.

All statistical analyses were performed using JMP software, version 14 (SAS Institute, Cary, NC, USA). Significant difference was determined at $P < 0.05$.

3. Results

3.1 Follicular dynamics

The changes to the bears' ovarian structures are shown in Figures II-2 (Bears A to D) and 3 (Bears E and F). Small follicles (range 2.7 to 5.4 mm) were observed in April. From May to June, several wave-like follicles developed; however, they were less than 6.0 mm and regressed. Thereafter, follicles developed to more than 6.0 mm and became dominant follicles. In all bears except for Bear A, a major wave was observed once during the breeding season. Bear A repeatedly showed minor waves and did not show dominant follicles throughout the study period. Therefore, Bear A was excluded from further analysis. The total follicle numbers were highest in May, and then decreased sharply at the beginning of the major waves.

The growth rate of the dominant follicles in the major waves (0.19 mm/day, n = 9) was greater than that in the minor waves (0.13 mm/day, n = 13). Including the results of the previous study [25], the growth rate was 0.13 mm/day in the minor waves (n = 21) and 0.21 mm/day in the major waves (n = 15; Figure II-4). After CL formation, a few small follicles (2.0 to 3.7 mm) were observed; however, dominant follicles did not appear throughout the study period.

Bears B and F showed spontaneous ovulation before GnRH treatment. However, I did not observe ovulatory signs by ultrasonography, such as dominant follicle disappearance or fibrin-filled follicles [70], in Bears C to E on the day of GnRH treatment; CLs were confirmed in subsequent observations. In Bear B, three out of four dominant follicles disappeared just before the CL was first confirmed. When the CL was first confirmed (June 20), I could only observe the right ovary due to a technical problem (Figure II-2, asterisk). In Bear D, the largest dominant follicle ovulated and formed a CL nine days after GnRH

treatment, while an ipsilateral follicle (7.6 mm in diameter) regressed. On the other hand, a contralateral follicle (5.5 mm in diameter) grew to 11.7 mm after nine days of GnRH treatment and CL was confirmed four days later.

3.2 Ovulation induction

Dominant follicle diameter at the last observation before ovulation and CL diameter at the first detection by ultrasonography were 9.4 ± 1.1 mm (range: 8.2 to 11.2 mm, n = 5) and 10.3 ± 2.7 mm (range: 7.6 to 14.3 mm), respectively, in treated bears (C, D, and E). In contrast, these diameters were 7.7 ± 1.2 mm (range: 5.8 to 8.8 mm, n = 6) and 7.2 ± 0.9 mm (range: 6.1 to 8.5 mm), respectively, in spontaneously ovulating bears (B and F). There was no significant difference in the diameters of follicles and CLs between induced and spontaneous ovulation. In Bear D, there were central cavities in the CLs on the first day they were confirmed, and the size of CLs decreased gradually as the central cavities disappeared.

3.3 Hormonal changes

Hormonal changes in the bears are shown in Figures II-2 (Bears A to D) and 3 (Bears E and F). Additionally, plasma E_2 and P_4 concentrations relative to follicular development in each period are shown in Table II-3. Except Bear A, E_2 concentrations fluctuated in the presence of minor waves, and then showed an increase before spontaneous ovulation or on the day of ovulation induction (maximum value: 11.4 ± 3.8 pg/ml; range: 7.0-16.1 pg/ml). After ovulation, E_2 concentrations remained at the baseline levels. Plasma E_2 concentration was higher during follicular development (minor and major wave periods) than post-ovulation ($P < 0.05$). In Bear D, E_2 concentration slightly increased when the CL was first detected after ovulation induction, which coincided with follicle development after GnRH treatment. P_4 concentrations stayed at baseline levels during April, but fluctuated from May onwards, starting to increase before ovulation or on the day of ovulation induction in most cases. Plasma P_4 was higher in the major wave periods than the minor wave periods, but it was the highest in post-ovulation periods (luteal phase, $P < 0.05$). Concentration of P_4 when the CL was first detected was 1.6 ± 0.4 ng/ml (range: 1.0-2.0 ng/ml), increasing gradually from July to October in Bears B to D (2.7 ± 0.6 ng/ml; range: 2.9-3.5 ng/ml).

3.4 Changes of vaginal cytology and vulva score

The cornification rate showed a similar change to the total follicle number. Although the changes in the cornification rate differed between individuals, it was less than 25% at the beginning of April but increased to more than 75% during follicular development. After CLs

formation, the cornification rate dropped to less than 20%. The changes to the vulva score were relatively high from May to June.

4. Discussion

The present study showed that brown bears showed one major follicular wave from which several follicles were selected. Furthermore, one to three follicles could ovulate during the breeding season. This suggests that brown bears are monoestrous animals.

A few studies have suggested that brown bears are polyestrous animals because of multiple distinct estrous observations and multiple paternities [65,71]. In captive observations, some females mate continuously for more than two weeks and the others mate for up to 20 days with 3-20 days interval [21]. Also, during another field observation, males and females remained together for two weeks to allow them a chance to breed [65,71]. Using GPS location data during the mating season, Stenhouse et al. [72] reported that the duration of associations between males and females in the wild ranged from 4 to 468 hours. Although they suggested that more than one estrus may have occurred because of this long duration, the mean duration of associations was 59.9 hours and 72% of associations were less than 72 hours. It is unlikely that brown bears show multiple estrus in such a short period. Instead, these previous results seem to indicate that brown bears have a long and variable estrus period.

My results show that it takes about two weeks for follicles to develop to the preovulatory size in the major wave. Considering the field observations, I suggest that females may show receptivity to males when a major wave commences. This is because follicles of 6.0 mm in diameter have aromatase cytochrome P450 activity and sexual receptivity may be related to E_2 synthesis [48]. Therefore, the long and variable period of receptivity to males may correspond to the follicular phase.

There is further evidence in the literature suggesting that brown bears have multiple estrus. In ursids, inactivation of CLs (low P_4 production) following ovulation is related to delayed implantation or embryonic dormancy. It has been suggested that low progestin during a delayed implantation period may allow females to re-enter estrus [64]. However, the present study indicates that the major wave appears only once during the breeding season, with no other major wave occurring after CL formation and subsequent to ovulation. Therefore, it is considered unlikely that brown bears experience multiple estrus via this mechanism. However, the domestic cats, which are seasonal polyestrous animals, occur the multiple major waves without ovulation [73]. In the present study, I have monitored ovaries after the major wave only in two bears without ovulation (Bears 1 and 4 in Chapter I), therefore, I

should investigate whether the similar phenomena happen in brown bears to conclude the number of major waves.

The mating system of the brown bear is generally classified as polygamous [66]. One to three cubs are born per litter and multiple paternity in litters has been documented by genetic studies in wild bears [65,71]. The present study confirmed that two to three follicles ovulate spontaneously within four to seven days after GnRH treatment. In brown bears, estrous females could copulate with a number of different males until ovulation. In canines, spermatozoa may remain motile while they are stored in the utero-tubal junction and the uterine glands [74]. These numbers noticeably diminished six days after copulation, but motile spermatozoa were found as long as 11 days after copulation [75]. Dogs are monoestrous animals that ovulate spontaneously, but multiple paternity has also been reported in wild dogs [76,77]. The duration of spermatozoa survival in the female reproductive tract in bears is unknown. Assuming multi-ovulation following a single luteinizing hormone (LH) surge, it is possible that the sperm derived from different males could survive for a certain period, allowing polygamous mating. In Bear B, ovulations were observed over two successive observations within eight days. This ovulation gap may also contribute to multiple paternity.

Bears B and F, kept in a female group without male contact, showed spontaneous ovulation. In brown bears, spontaneous ovulation has previously been reported in captivity [66]. Okano et al. [78] showed that female Asian black bears with no male contact did not form CLs, while females with male contact via a fence did form CLs. Domestic cats and some nondomestic felids classified as induced ovulators have also been reported to show spontaneous ovulation in captivity due to female social interaction or the presences of males (domestic cats [79,80], lions [81], cheetah [82], and clouded leopard [83]). Given this evidence, the phenomenon of spontaneous ovulation may be easily induced in captivity.

From my results of spontaneous and induced ovulation, follicles with a size between approximately 6.0 to 11.0 mm have the potential to ovulate if a LH surge occurs. In Bear F, a follicle approximately 6.0 mm in size ovulated spontaneously, followed by two 8.8 and 6.6 mm follicles at the next observation seven days later. A follicle about 6.0 mm is possibly responds to the LH surge from dominant follicles, thus 6.0 mm follicles may have acquired LH receptors. However, a 6.0 mm follicle may not induce an LH surge by itself. Additionally, plasma P_4 production increased within nine days prior to ovulation, with the largest follicle size being 8.8–9.9 mm in Bears B to D. An increase in P_4 prior to ovulation is a common phenomenon in animals with a long estrous period, a gradual LH increase, and extended LH surge. In dogs [84], horses [85], and pigs [86], LH concentrations gradually increase over a

few to several days and blood P_4 concentrations start to increase after initiation of the LH surge, well in advance of ovulation. In addition, P_4 concentration is an established indicator for determining AI timing in dogs. Generally, it reflects an increase in secretion of LH, the indicator for follicular maturation in animals. Therefore, follicles approximately 6.0 mm in size may be immature. Several reports have revealed hormonal changes without ovarian activity during the breeding season in giant pandas [87], sun bears [88], polar bears [14], and brown bears [26]. However, Kang et al. [35], monitored ovaries by ultrasonography in Asian black bears and showed a plasma progesterone increase before ovulation when the follicle size was between 7.0 to 8.8 mm.

The present study performed weekly ovarian observations because follicular growth occurs in three to ten day intervals at a rate of 0.25 mm/day (1.75 mm/week) [25]. Although I focused on the size of largest follicle in the present study, it may be necessary to consider the size of subordinate follicles, since two or three follicles typically ovulate in brown bears. In Bear D, GnRH induced ovulation of the largest follicle, while simultaneously stimulating the development of a 5.5 mm follicle and resulting in ovulation within a week. This ovulation gap occurred due to the exogenous hormonal treatment. Monitoring follicle development in the major wave needs to be undertaken more frequently to confirm the timing of ovulation induction.

My results suggest that 20 μg of GnRH may be sufficient to induce ovulation in brown bears. In the present study, all three bears ovulated successfully when either 20 or 40 μg of GnRH was administered. In addition, a 10 mm follicle was induced to ovulate by 20 μg of GnRH in the previous study [25]. There was no significant difference in follicle diameters prior to ovulation or CLs at first detection between induced and spontaneous ovulation. Furthermore, P_4 concentrations increased in a similar manner after both induced and spontaneous ovulation.

The vulva score and cornification rate in vaginal cytology have been used as indicators of estrus in giant pandas and sun bears [11,69]. In the present study, cornification rate was high from early May; it then dropped to the baseline rate when dominant follicles disappeared. Vulva score showed similar changes. In general, the cornification rate and vulva score reflect E_2 concentrations. My results show a relationship between these indicators and E_2 concentrations similar to that described in previous reports. These features may be used as indicators of the follicular phase but may not be precise enough to determine the timing of AI.

In conclusion, brown bears may be seasonal monoestrous animals, and follicles greater than 8.0 mm in size are suitable for ovulation induction by hormonal treatment. There

appears to be only one opportunity to perform AI in their breeding season. More accurate growth rates and prediction methods for major follicular waves need to be determined in a future study to confirm the optimal timing of AI.

5. Tables and Figures

Table II-1. Bears used for observation of follicular dynamics and induction of ovulation.

ID	Age (Year)	Parity	Estimated body weight (kg)	Ovarian observation Period (Number of observations)	Type of ovulation	GnRH* dose (μg)	Follicle size before ovulation (mm)	Corpora lutea diameters at the first detection (mm)
Bear A	20	0	130	Apr - Oct (17)	–	–	–	–
Bear B	17	2	180	Apr - Oct (13)	Spontaneous	–	7.6, 8.8, 8.8	6.3, 6.6, 6.1
Bear C	6	0	130	Apr - Oct (14)	Induced	40	8.2, 9.9	7.6, 8.4
Bear D	20	1	150	Apr - Oct (13)	Induced	40	9.1	14.3
Bear E	5	0	130	Jun (3)	Induced	20	11.2, 8.5	12.8, 8.7
Bear F	20	1	150	Jun (3)	Spontaneous	–	8.8, 6.6, 5.8	8.4, 8.5, 7.2
Bear 2	19	0	130	May - July (12)	Induced	20	14.0	–
Bear 3	5	0	130	May - July (9)	Induced	20	10.2	8.5

Bear A did not show any major waves during the observation period.

Bears 2 and 3: data from Chapter I.

*GnRH (gonadotropin releasing hormone): Buserelin acetate

Table II-2. Definition of the terms related to ovarian dynamics

Term	Definition
Dominant follicle	Follicle(s) developed more than 6 mm in diameter
Ovulation	Disappearance of the dominant follicle(s) observed in previous examination or dominant follicle filled with fibrins which confirmed by subsequent CL
Minor wave	Growth of a group of follicles with none of them becoming dominant follicles
Major wave	Growth of a group of follicles with some of them becoming dominat follicles with < 10 follicles in total follicle number

Table II-3. Comparison of plasma estradiol-17β (E_2) and progesterone (P_4) concentrations in three ovarian activity periods

Period	E_2 (pg/ml)	P_4 (ng/ml)
Minor wave	6.8 ± 3.9 [a] (2.2–12.8)	0.3 ± 0.3 [c] (0.1–1.0)
Major wave	7.3 ± 4.4 [a] (2.2–16.1)	1.0 ± 0.6 [b] (0.3–2.3)
Post-ovulation	3.2 ± 1.0 [b] (2.0–5.4)	1.8 ± 0.7 [a] (0.8–3.2)

Values are presented as mean ± SD (range).

[a,b,c] The values with different superscript differ significantly within a column ($P < 0.05$).

The values include data from Bears B to F.

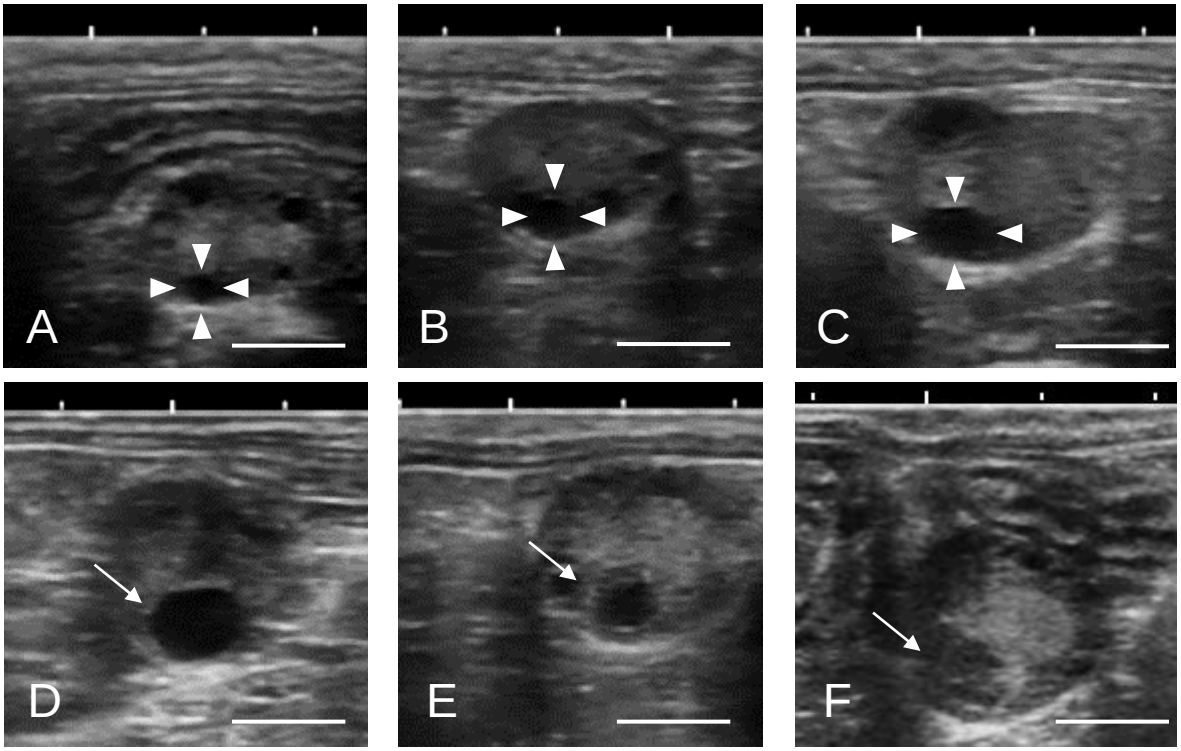

Figure II-1
Images recorded from ultrasonography of an ovary in Bear C. Panels A to C show the same follicle (gradually increasing in size), which was identified and traced by its position in the ovary and location relative to other follicles (arrow head). These observations were done in May, the second and third observations were undertaken four and nine days, respectively, after the first. Panels D to F are images of the same follicle in Panels A to C. The dominant follicle (D) ovulated and a corpus luteum (CL) with cavity was identified in the same location (E; arrow). Later, a CL filled with luteal tissue was identified (F; arrow). Scale bar: 10 mm.

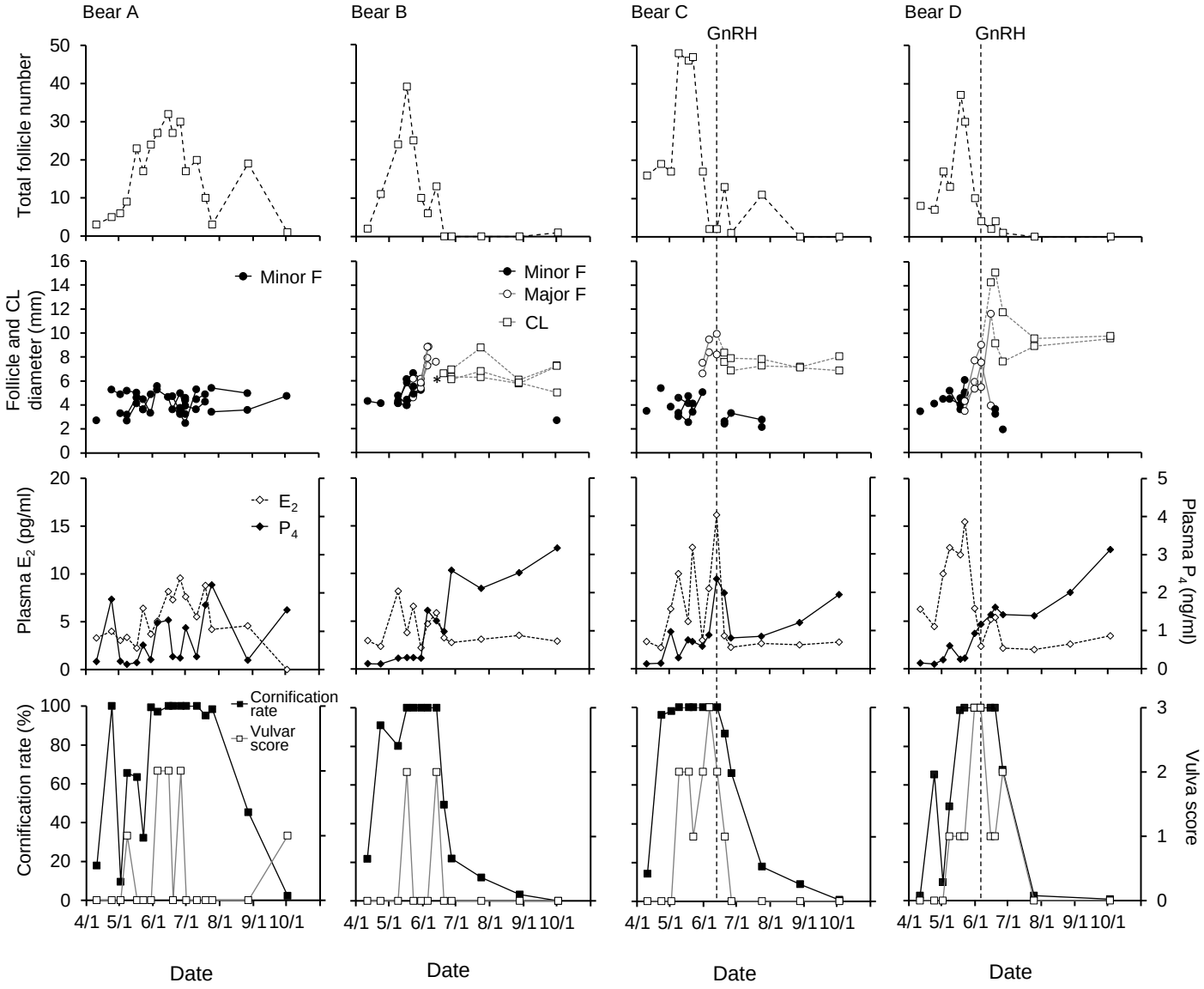

Figure II-2

The follicle growth profiles of Bears A and D (those observed from April to October), along with changes in plasma steroid hormones, vaginal cytology, and vulva score. The top panels show the total follicle number in each animal. Follicles and corpora lutea (CLs) diameters that could be traced by ultrasonography and shown in the second row of panels. Minor wave follicles (Minor F) and major wave follicles (Major F) are follicles that developed to less than 6.0 mm and those that greater than 6.0 mm in diameter, respectively. The changes in plasma estradiol-17β (E_2) and progesterone (P_4) are shown in the third row of panels. The bottom panels show the cornification rate and the vulva score. Bears C and D were administered gonadotropin releasing hormone (GnRH) to induce ovulation. The broken lines indicate the date of GnRH administration. The asterisk for Bear B indicates unilateral (right side) observation of the ovary due to a technical problem.

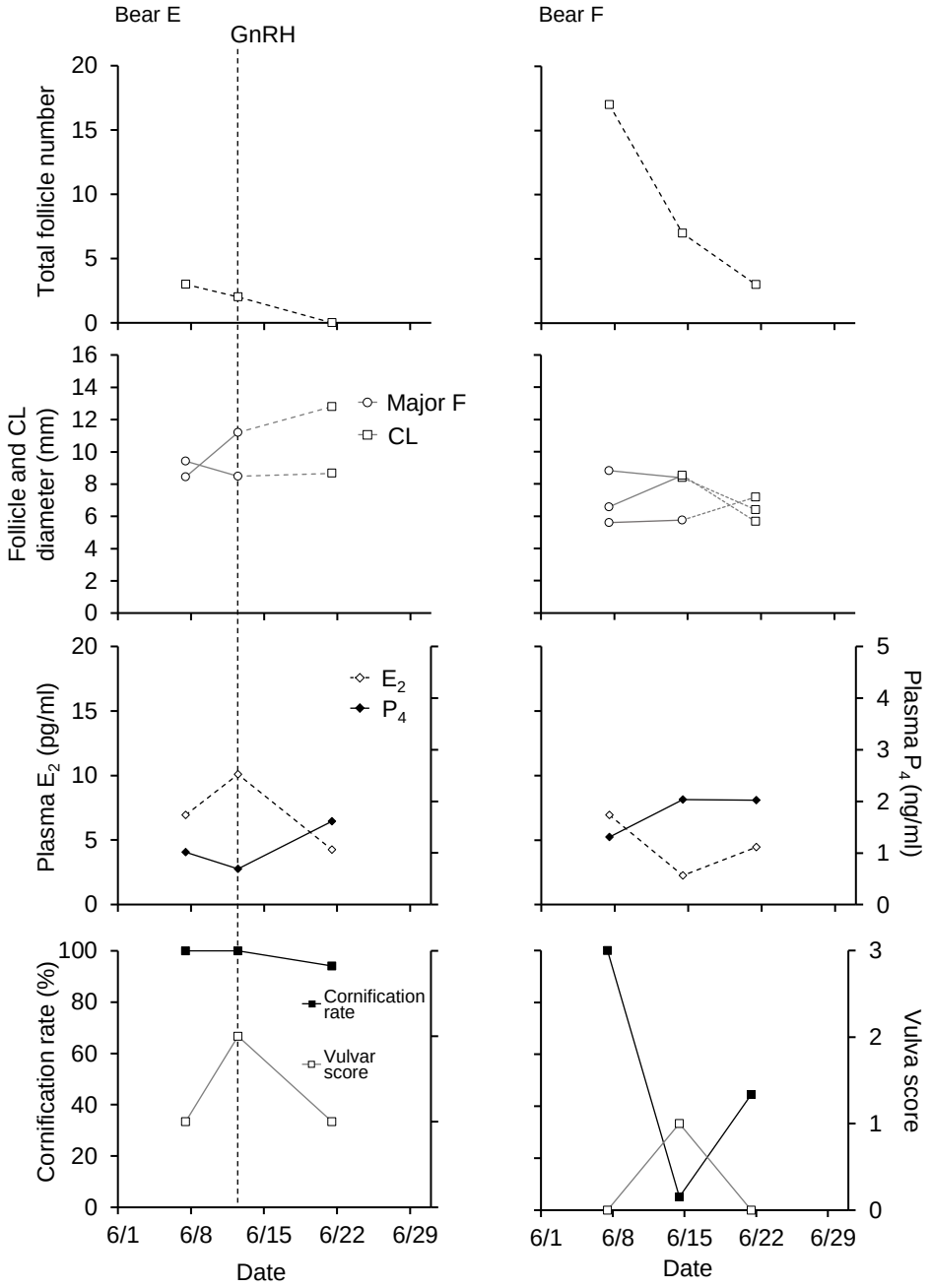

Figure II-3

The follicle growth profiles of Bears E and F (those observed in June), along with changes in plasma steroid hormones, vaginal cytology, and vulva score. The top panels show the total follicle number in each animal. Follicles and corpora lutea (CLs) diameters that could be traced by ultrasonography and shown in the second row of panels. Major wave follicles (Major F) are those that developed to greater than 6.0 mm in diameter. The changes in plasma estradiol-17β (E_2) and progesterone (P_4) are shown in the third row of panels. The bottom panels show the cornification rate and the vulva score. Bear E was administered gonadotropin releasing hormone (GnRH) to induce ovulation. The broken lines indicate the date of GnRH administration.

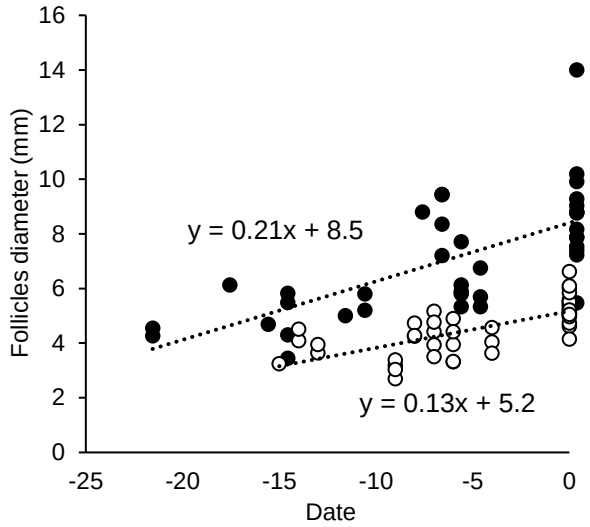

Figure II-4
Scatter diagram and linear relationship for follicular diameter in the minor wave (open circles, n = 21) and major waves (closed circles, n = 15). Data from previous observations (n = 3) were combined with those obtained in Chapter 1. The day on which each follicle had the largest diameter was defined as Day 0.

6. Summary

It is important to understand ovarian physiology when developing an artificial insemination (AI) protocol. Brown bears (*Ursus arctos*) have a breeding season from May to July, although the type of estrus (polyestrus or monoestrus) is still contested. The present study aimed to define the ovarian dynamics, including follicular waves and ovulatory follicle size, and estrus type in brown bears. Six brown bears were used for ovarian ultrasonography; four were observed between April and October (before the start and after the end of the breeding season) and two in June (breeding season). In addition, I attempted to induce ovulation by administering a gonadotropin releasing hormone (GnRH) agonist. I observed follicular development in April in four bears, but follicles did not develop to greater than 6.0 mm in diameter until May. Thereafter, a group of follicles developed to more than 6.0 mm and grew as dominant follicles, except in one bear. After ovulation and subsequent corpus luteum (CL) formation, the follicular waves disappeared. Furthermore, in three bears treated with GnRH, follicles between 8.2 to 11.2 mm in diameter at the time of treatment ovulated and formed CLs. In two bears, follicles between 5.8 to 8.8 mm ovulated spontaneously within the observation interval. My results suggest that brown bears are monoestrous animals. Therefore, AI can only be performed once during the breeding season. My results also suggest that dominant follicles larger than 8.0 mm are a suitable size for inducing ovulation.

Summary and Conclusions

The present study was conducted to characterize the ovarian dynamics in brown bears in order to discover the follicular developmental pattern, identify the ovulatory follicle size, further confirm the estrus type and subsequently develop an AI protocol.

In Chapter I, the ovarian dynamics of six bears were monitored weekly by ultrasonography during June in 2017 and from May to July in 2018. Two bears were also included in an ovulation induction trial using a gonadotropin releasing hormone (GnRH) agonist. Follicular development was classified into two periods based on the largest follicle size of 6.0 mm in diameter during the observation period from May to July. In the early period (May to mid-June), the largest follicles grew to 6.0 mm or less in diameter and then regressed. In the late period of the breeding season (mid-June to July), the largest follicles developed to greater than 6.0 mm in diameter. The follicular growth rate (0.25 mm/day, n = 6) was approximately 1.6-fold that in the early period (0.15 mm/day, n = 8). Plasma estradiol-17β (E_2) concentrations increased and showed a peak on the last day monitoring (five to seven days) immediately before the largest follicles reached their maximum sizes. GnRH was administered when the largest follicle reached a size more than 10.0 mm in diameter. The follicle of 10.2 mm in diameter in one bear ovulated after GnRH treatment, while another bear with 14.0 mm failed to ovulate. Further, one animal spontaneously ovulated after a follicle developed to 13.1 mm in diameter. The largest follicle reaching more than 10.0 mm in diameter could be an indicator of the appearance of ovulatory follicles.

In Chapter II, the ovarian dynamics of six brown bears were monitored and four bears were observed between April to October (before the start and after the end of the breeding season) and two in June (breeding season). A GnRH agonist was administered to three bears to confirm the ovulatory follicle size. Following the observations noted in Chapter I, since one bear already had a greater than 10.0 mm follicle in early May, it was necessary to confirm the appearance of follicular development from April. Moreover, the results of Chapter I did not elucidate the estrus type of brown bears. In Chapter II, monitoring of ovarian activity was continued to confirm the findings in Chapter I. To identify the ovulatory follicle size, ovulation induction was done when follicles greater than 10.0 mm were detected. The number of days of GnRH treatment for ovulation induction were determined based on the follicular growth rate in Chapter I.

Ultrasonography of the ovaries and blood collection were performed every other week in April, and every week from May until ovulation. After confirming CL formation, observations were performed once a month until October. Small follicles (2.7 - 5.4 mm) were

observed in April. From May to June, several wave-like follicles developed; however, they were smaller than 6.0 mm and regressed. Thereafter, follicles developed to more than 6.0 mm and became dominant follicles. After ovulation and subsequent CL formation, the follicular waves disappeared, but a few small follicles (2.0 - 3.7 mm) were observed. Furthermore, in three bears treated with GnRH, follicles between 8.2 to 11.2 mm in diameter at the time of treatment ovulated and formed CLs. In two bears, follicles between 5.8 to 8.8 mm ovulated spontaneously within the observation interval. These results suggest that brown bears are monoestrous animals. Including the results in Chapter I, the follicle growth rate was 0.13 mm/day in the minor waves (n = 21) and 0.21 mm/day in the major waves (n = 15).

Ovulation was induced in three out of six bears by administering 20 or 40 μg of GnRH. The 20 μg GnRH dose is the maximal dose indicated for cattle. All three bears ovulated successfully when either 20 or 40 μg of GnRH was administered. These results suggest that 20 μg of GnRH may be sufficient to induce ovulation in brown bears. Regarding the ovulatory follicle size, the largest dominant follicle larger than 10.0 mm was targeted. The dominant follicle diameter at the last observation before ovulation was 9.4 ± 1.1 mm (mean ± S.D, 8.2 - 11.2 mm, n = 5) in treated bears. However, two bears showed spontaneous ovulation before the GnRH treatment; the dominant follicle diameter at the last observation before ovulation was 7.7 ± 1.2 mm (5.8 - 8.8 mm, n = 6). Although follicles of around 6.0 mm ovulated seven days after the last observation, it was concluded that follicles greater than 8.0 mm in diameter are more suitable for ovulation induction. In dogs, plasma progesterone (P_4) concentrations are an established indicator for determining AI timing because P_4 concentrations start to increase after initiation of luteinizing hormone surge, well in advance of ovulation. Similarly, P_4 production increased within nine days prior to ovulation, with the largest follicle size being 8.8 – 9.9 mm in three bears. The subordinate follicles also induced ovulation and they were 8.2 and 8.5 mm in diameter. These results indicated that the size of subordinate follicles has to be considered for ovulation induction since two or three follicles typically ovulate in brown bears.

Plasma E_2 concentrations fluctuated in the presence of minor waves, and then showed an increase before spontaneous ovulation or on the day of ovulation induction. Plasma P_4 concentrations remained at base levels during minor follicular waves. The vulva score and cornification rate in vaginal cytology were examined. The cornification rate was high from early May and then dropped to the baseline rate when dominant follicles disappeared. The vulva score showed similar changes. These features may be used as indicators of the follicular phase but may be not precise enough to determine the timing of AI.

This is the first study describing follicular development during the breeding season in brown bears. Brown bears show multiple follicular waves without dominant follicle selection in the early period of the breeding season. In the late period of the breeding season, a few follicles developed and exceeded 6.0 mm in diameter to become dominant follicles with a faster follicular growth rate. The present study showed that brown bears are seasonal monoestrous animals. In addition, the present study suggested that the timing of the largest dominant follicle of greater than 8.0 mm could be the appropriate timing for ovulation induction. The present study provided information necessary to establish an AI protocol with minimal frequency of anesthesia in brown bears. This research strategy may be applicable to develop an AI protocol in polar bears and other endangered species.

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Summary in Japanese

IUCN（国際自然保護連合）で評価されている哺乳類約 5800 種のうち 800 種は、絶滅のおそれがあるとされており、人工繁殖技術は絶滅危惧種の個体群保護・増殖の一助となり得る。人工授精（AI）は、飼育下で繁殖が難しい野生動物における自然交配に代わる有用な技術であると考えられ、実用化できれば交尾行動に問題のある個体や遠隔地で飼育されている個体同士の繁殖が可能となり、遺伝的多様性の維持や個体数増加にも貢献できる。絶滅危惧動物であるホッキョクグマは、遺伝的多様性の低下、飼育個体群の高齢化により自然交配だけでは飼育下個体群を維持することが難しい状況となっている。しかし、同種においては、これまでに 8 度 AI が試みられているものの産子を得るに至っていない。その原因として、AI を実施するのに不可欠な、繁殖生理すなわち発情や卵胞発育の様式および排卵卵胞直径などの情報が得られていないことが挙げられる。超音波検査は、非侵襲的に繰り返し生殖器の観察が可能であることから、繁殖学領域の臨床および研究に広く用いられている。しかし、野生動物および多くの動物園動物では検査実施のために全身麻酔が必要となることから、その利用が制限されている。卵巣動態を解明するためには頻回の超音波検査、つまり麻酔の実施が必要となるため、そのリスクに対する懸念から個体数の少ない動物種では AI 技術確立に必要な基礎情報を収集する調査研究が難しい。そこで本研究は、近縁種であり絶滅危惧種となっていないヒグマをモデル動物として、同種を多数飼育し、かつ麻酔経験が豊富なのぼりべつクマ牧場（登別市）において超音波検査を用いて卵巣動態を調査し、卵胞発育の様式および速度を調べ、さらに発情様式および排卵誘起処置の目安となる卵胞直径を明らかにした。

第 1 章では、ヒグマの卵巣動態を明らかにするため、超音波画像診断装置を用いて卵巣を観察し、さらに、排卵可能な卵胞直径を明らかにするため排卵誘起処置を施した。また、卵胞発育期間中の内分泌学的評価のため、検査日に血液を採取し、性ステロイドホルモン濃度を測定した。のべ 6 頭の成熟雌ヒグマを使い、2017 年 6、7 月、2018 年 5 月から 7 月におよそ週 1 回（3 - 11 日）の頻度で、麻酔下において超音波検査により卵巣内の構造物を観察記録した。超音波検査には、自作のアタッチメントを超音波検査用 I 型リニアプローブに装着して経直腸的に卵巣を描出した。その結果、5 月には直径 6.0 mm 未満の卵胞が発育と退行を繰り返す波状の発育がみられ、6 月から 7 月にかけて 1 - 2 個の卵胞が直径 6.0 mm を超えて大型の卵胞（> 10.0 mm）へ発育することが分かった。卵巣に存在する胞状卵胞数は、5 月の平均 14.4 個（4 - 25 個）から 6 月以降は平均 6.2 個（1 - 13 個）に減少しており、主席卵胞への選抜が起こっていることが示唆された。また、血中エストラジオール-17β（E_2）濃度は、主席卵胞が最大直径を示す約 14 日前から増加し始め、約 7 日前に最大値を示し、その後低下していた。さらに卵胞発育速度は、5 月（0.15 mm/日）に比べて主席卵胞が出現した 6 月以降で加速していた（0.25 mm/日）。6 頭中 1 頭では、約 13 mm の卵胞の排卵が観察されており、一般には交尾排卵動物と考えられているヒグマにおいても飼育環境下では自然排卵する場合のあることが分かった。また、卵胞発育をモニタリングしていた 2

頭に対し、卵胞直径が初めて 10 mm を超えた検査日に排卵誘起のために性腺刺激ホルモン放出ホルモン（GnRH）を筋肉内投与したところ、直径 10.2 mm の卵胞は排卵し、その後黄体の形成が確認できたことから、直径 10.0 mm 以上の卵胞は排卵を誘起できることが示唆された。一方、直径 14.0 mm の卵胞は排卵せず、その後、卵胞サイズが小さくなっていったことから、すでに退行過程にあったと考えられた。

第 2 章では、第 1 章で確認された卵胞発育および排卵可能な卵胞直径に関する情報の精度向上と発情様式の解明を目的として検討を行った。成熟雌ヒグマ 6 頭において繁殖期前である 4 月から排卵後の 10 月まで卵巣の観察を行い、卵胞発育開始時期の確認および発情様式の決定を行った。第 2 章では、血中ホルモン濃度に加え、血中ホルモンの影響を受けて変化する膣上皮細胞中の角化細胞率の割合および外陰部の充血・腫脹の変化を記録した。その結果、4 月には卵胞発育の開始が確認された。5 月から 6 月にかけては、波状の卵胞発育が認められ、6 月以降は直径 6.0 mm を超えて発育して 1 - 3 個の主席卵胞へと選抜されていった。排卵および黄体形成後は主席卵胞の発育は認められなかった。以上の結果から、ヒグマの発情様式は季節性の単発情であることが分かった。第 1 章の卵胞発育動態を含めて計算した卵胞発育速度は、波状の卵胞発育時の卵胞は 0.13 mm/日、主席卵胞の発育速度は 0.21 mm/日であった。一方、血中 E_2 濃度は、卵胞発育が活発となる 5 月および 6 月に高値を示し、排卵後は低値を維持していた。角化細胞率および外陰部の変化を示すスコアは E_2 濃度変動と類似し、5 月初期に増加後、主席卵胞消失後には低下し、その後も低値を維持した。以上の角化細胞率および外陰部徴候は、卵胞期の指標にはなるものの AI 実施時期の決定指標にはならないことが分かった。

排卵誘起処置は、GnRH 製剤を、牛への最大投与量とされる 20 µg またはその 2 倍量である 40 µg を筋肉内に投与して実施した。卵巣観察を行った 6 頭中 3 頭に排卵誘起処置を行い、GnRH 投与量によらず排卵が確認された。排卵または黄体形成が確認される直前の検査日（7 - 9 日前）に確認された卵胞直径は、9.4 ± 1.1 mm（平均 ± 標準偏差, 8.2 - 11.2 mm, n = 5）であった。また 6 頭のうち 2 頭において自然排卵が認められ、排卵する 7 日前に観察された卵胞直径は、7.7 ± 1.2 mm（5.8 - 8.8 mm, n = 6）であった。そのため、自然排卵が生じる可能性を考慮し、AI のための排卵誘起処置時期を決定する卵胞直径は、10.0 mm ではなく 8.0 mm が適切であることが考えられた。

本研究は、定期的な超音波検査によってヒグマの卵胞発育および発情様式と排卵可能な卵胞直径を明らかにした。ヒグマは、繁殖期開始前である 4 月には卵胞の発育が開始され、繁殖期中の 5 月から 6 月にかけて発育と退行を繰り返して波状に卵胞が発育し、交尾活動がピークとなる 6 月以降から卵胞直径 6.0 mm を超えて主席卵胞への発育が 1 回だけ認められることが分かった。また、主席卵胞の発育速度は、0.21 mm/日であり、それ以前の卵胞発育速度（0.13 mm/日）に比べ加速していた。また、繁殖期終了後には主席卵胞の発育が認められなかったため、ヒグマの発情様式は季節性の単発情であり、AI を実施する機会が 1 年に 1 回しかないことが明らかとなった。自然排卵の可能性を考慮すると、年 1 回の AI のチャンスを逃さないためには、排卵誘起処置のタイミングとして、最大卵胞が直径 8.0 mm になる時期が適切であることが分かった。本研究に

より、卵胞の発育速度および排卵可能な卵胞直径を調べることで、必要最低限の麻酔頻度で実施できるヒグマの AI プロトコールの確立が可能となった。また、このプロトコールをホッキョクグマなどの絶滅危惧種に応用することで、繁殖生理学に基づいた AI プロトコールの確立が可能となり、絶滅危惧種の飼育下での個体群および遺伝的多様性の維持に寄与すると考えられる。