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Study on Reproduction of Captive Marine Mammals

Etsuko KATSUMATA[1)]

[1)]*Kamogawa Sea World, International Marine Biological Research Institute, Chiba 296-0041, Japan*

Abstract. The reproductive endocrinological characteristics of beluga, killer whale, spotted seal and bottlenose dolphin were evaluated and used in conjunction with applied reproductive research to enhance captive breeding programs. Results from 8 y of biweekly serum progesterone determination in a female beluga indicated that sexual maturity occured at approximately age 13, two to seven estrous cycles, lasting 37 ± 3.9 days, per yr began in April-May every year. Rectal temperature was positively correlated with serum progesterone levels and negatively associated with behavioral estrus. In five cases of pregnancy of two female killer whale, positive relationship was found between serum progesterone concentration and temperature during the first period of 18 month-gestation. In the normal parturitions (n=4), rectal temperature decreased 0.8 C lower than average rectal temperature during pregnancy. Sexual maturity of female killer whales occurred at age nine. Yearly contraception in the mono-estrus captive spotted seals (n=10) using a single dose of the progestagen (proligestone^{TM}; 5 or 10 mg/kg s.c.) was achieved in 94% (33/35) of the attempts over 5 yr when the hormone was administered two months prior to the breeding season. Artificial insemination trials (n=4) were conducted in female bottlenose dolphin (n=3) using fresh and frozen-thawed semen. Estrus synchronization using regumate (27 days) resulted in ovulation occurring 19 to 24 days post withdrawal. Conception was confirmed in 75% of the attempts, with two females successfully delivering calves.

Key words: Artificial insemination, Estrous synchronization, Marine mammals, Progestational hormone, Temperature

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Housing dolphins in Japanese aquariums first began in the1950's, and only recently the era of promoting captive breeding has arrived [1]. Traditional research with marine mammals in Japan has come from post-mortem analysis of animals collected during whaling operations. However, many questions concerning biology and physiology of these animals cannot be learned using these methods. As a result, more emphasis is being placed on using captive animals for non-invasive research programs. Utilizing captive colonies of marine species has been possible to determine growth rates, sexual maturation, breeding period, copulatory behavior, gestation period, labor, parenting, and weaning. The breeding season and the estrous cycle of female bottlenose dolphins [2] were described with endocrine studies of captive animals, and it was found that some delphinids were spontaneous ovulators because ovulation occurred without males [3–5]. Analyzing serum progesterone concentrations, early pregnancy diagnosis was established and reproductive performance was improved [6, 7]. For the male bottlenose dolphin, the seasonality of spermatogenesis and sperm characteristics were described through analysis of yearly changes in spermatozoa production and testosterone excretion [8–11]. For pinnipedia, it has been demonstrated that they have a period of delayed implantation, which are regulated by photo period and can be affected by nutritional variations [12, 13]. In addition, shortly after giving birth, spotted seal quickly returns to estrus, and thus gives birth about the same time every year [14–16]. Artificial insemination attempts with bottlenose dolphins in aquarium was first attempted in conjunction with ovulation induction protocols using PMSG and hCG [9, 17]. Despite these early attempts, the first successful calves to be born after AI did not occur until 25 years later when a indo-pacific bolltenose dolphin in Hong Kong and a killer whale in the US were born in 2001 [18–20].

The objectives of this study were to evaluate the reproductive endocrinology of four species of captive marine mammals in Kamogawa Sea World (Kamogawa, Chiba): beluga, killer whale, bottlenose dolphin in cetaceans and spotted seal in pinnipeds, and evaluate the application of reproductive management tools, artificial insemination and contraception, for development of the breeding programs.

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Correspondence: E Katsumata (e-mail: etusko_katsumata@granvista.co.jp)

Blood Sampling, Endocrine Analysis and Rectal Temperature Measurement

The blood samples from the beluga, killer whale, bottlenose dolphin were collected voluntary from the tail fluke between 0830 to 0900 h every 2 to 4 weeks. Progesterone measurement was requested to SANRITSU. (Chiba). Serum concentration of progesterone was determined by radioimmunoassay (RIA) system with ^{125}I-labeled radioligands. The spotted seals were placed in a squeeze cage for restraint at an unspecified time, and the blood samples were collected from vascular network of hind limb. The samples of spotted seals were determined by EIA in Nippon Veterinary and Life Sciences University [21]. Rectal temperature for beluga and killer whale were measured voluntary with an electronic

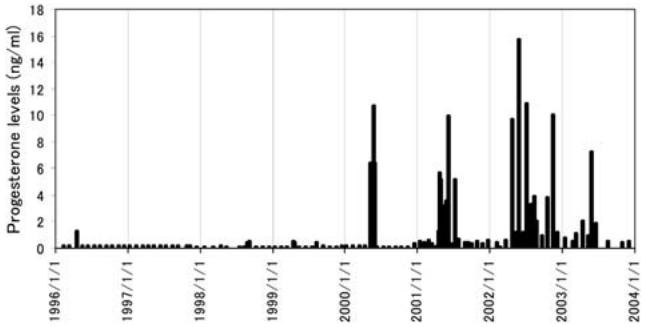

Fig. 1. Serum concentrations of progesterone during a 8 years period from Feb. 10, 1996 through Nov. 27, 2003 in a Beluga, Marsha.

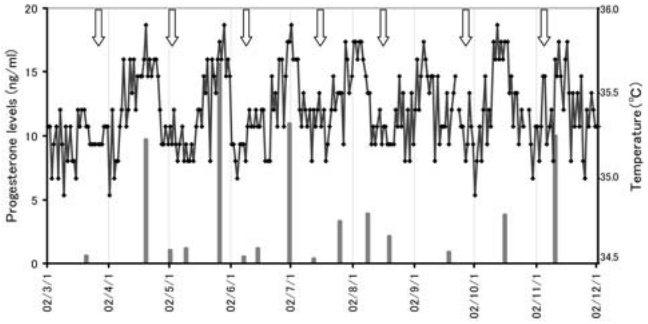

Fig. 2. Representative body temperature (line), serum concentration of progesterone (solid bar) and mating behavior (arrow) during the estrous cycle of a female beluga in 2002. The body temperature was measured daily at 0800 h before active exercise. Concentrations of serum progesterone were determined every 2 to 4 weeks.

thermometer (Terumo Finer CTM-303, Terumo, Tokyo) by inserting a probe (PK-K04, temperature range: 0–50 C).

Estrous Cycle and Change of Body Temperature in Female Beluga

One female beluga from Okhotsk (name: Marsha, brought in October 24, 1990, length of 280 cm, weighting 418 kg, estimated to be born in 1987 [22]) was used to examine the changes in concentrations of serum progesterone and body temperature during the estrous cycles of 8 years from 1996 to 2003. In this period of time, Marsha was kept with two males from Hudson Bay of Canada and Okhotsk sea. Serum progesterone concentrations remained low (0.1–1.3 ng/ml) from Jan 1996 to March 2000, and increased in May 2000 to 6.4 ng/ml, when it was considered that she had reached sexual maturity at 13 yr (length 382 cm, weight 635 kg)(Fig.1). Cyclic changes in serum progesterone concentrations (range 0.1 to 15.7 ng/ml) occurred once in May 2000, four times from April to July in 2001, seven times from April to November in 2002, and two times from April to June in 2003. This data suggest that that beluga can be seasonally polyestrous. A positive correlation was observed during 7 estrous cycles between temperature and

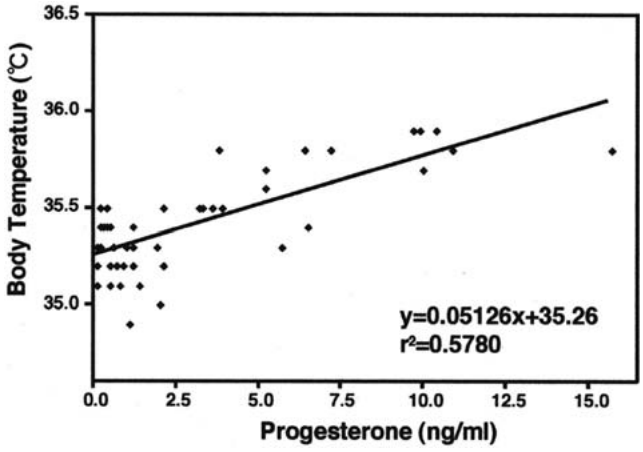

Fig. 3. Relationship between serum progesterone levels and body temperature in a female beluga.

progesterone levels in 2002 (Fig. 2). The body temperature changed from 34.9–35.9 C. All mating behaviors occurred during the low-temperature phase, with the body temperature increasing 11.0 ± 1.5 days after mating behaviors. A significant difference was found between the temperature of the day when mating behaviors were observed and the highest temperature measured (Fig.3). The estrous cycle, as defined by the number of days between mating behaviors was 36.7 ± 3.9 days (n=6). These results suggest that, similar to humans [23], progesterone can affect body temperature and thus the beluga ovarian cycle can be determined by measuring body temperature. These results demonstrate a simple, rapid and inexpensive way to track beluga estrus activity and could be a practical tool for breeding management of captive female beluga.

Pregnancy Period, Periparturient Body Temperature and Concentration of Serum Progesterone of Killer Whale

Individual profiles for the two female killer whales (name: Maggie, Stella) were shown in Table 1. During the sampling period, five pregnancies were monitored from two females from 1997 to 2003. Changes in body temperature and serum progesterone levels during pregnancy were shown in Figs. 4 and 5. The serum progesterone levels sharply increased (increasing phase) during the first phase of pregnancy, and decreased slowly (decreasing phase) until parturition. Positive correlation between serum progesterone levels and body temperature were only observed during the increasing phase of early pregnancy (Fig. 6). Gestation lasted 545 ± 3.7 days (n=4) and with the rectal temperature 5 days prior to parturition becoming 0.3 C lower than the mean rectal temperature during pregnancy. Moreover, the temperature on the day before parturition decreased 0.8 C as compared with the mean temperature during pregnancy. The rectal temperature returned to baseline after parturition (Fig. 7). For Maggie and Stella, the first significant serum progesterone concentrations indicative of sexual maturity occurred at age 9. Christensen (1984) reported that wild north

Table 1. Profiles of two female killer whales

	Date into KSW	Birth year	Length at capture (cm)
Maggie*	03/29/1988	1983	401
Stella **	03/29/1988	1986	274

Birth year was estimated from length-growth curves according to the method of Duffield and Miller [24]. KSW, Kamogawa Sea World. * First offspring died 30 min after birth. Second offspring was delivered as a premature birth (225 cm in length) at 16 months of pregnancy and Maggie died 3 days later. ** Stella gave three births successfully.

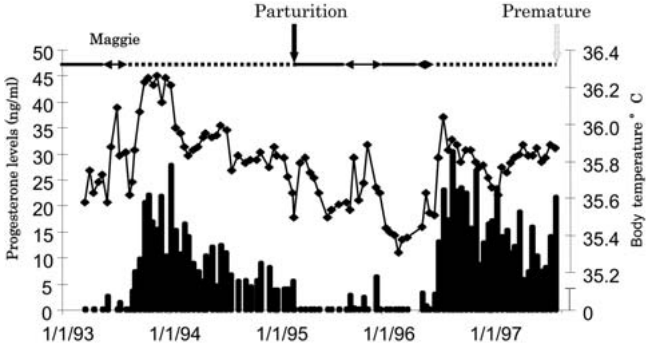

Fig. 4. Serial changes in the serum progesterone levels (closed bars) and the body temperature (closed squares) in killer whale (Maggie) during the period of anestrous (—), estrous cycle (◂▸) and pregnancy (▪▪▪). For killer whale (Maggie), mating was observed in August 1993, and first birth occurred in March 1995. For this parturition, delivery was normal but the calf died 30 min after parturition. After parturition, there was a 6 month anestrous period, 3 subsequent estrous cycles, a 3-month anestrous period, and then one estrous cycle. Mating was observed again in June 1996, and conception was subsequently confirmed; however, premature birth occurred in 16th month of pregnancy. This animal died 3 days after premature birth occurred.

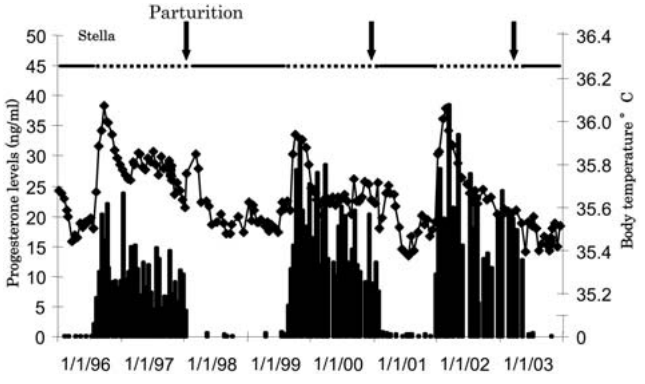

Fig. 5. Serial changes in the serum progesterone levels (closed bars) and the body temperature (closed squares) in killer whale (Stella) during the period of anestrous (—) and pregnancy (▪▪▪). Killer whale (Stella) gave birth three times between 1998 and 2003. All parturitions were normal, and the three calves matured normally. First mating was observed in July 1996, and first birth occurred in January 1998. Second mating was observed in August 1999, with second birth occurring in February 2001. Third mating was observed in December 2001, and third birth occurred in May, 2003.

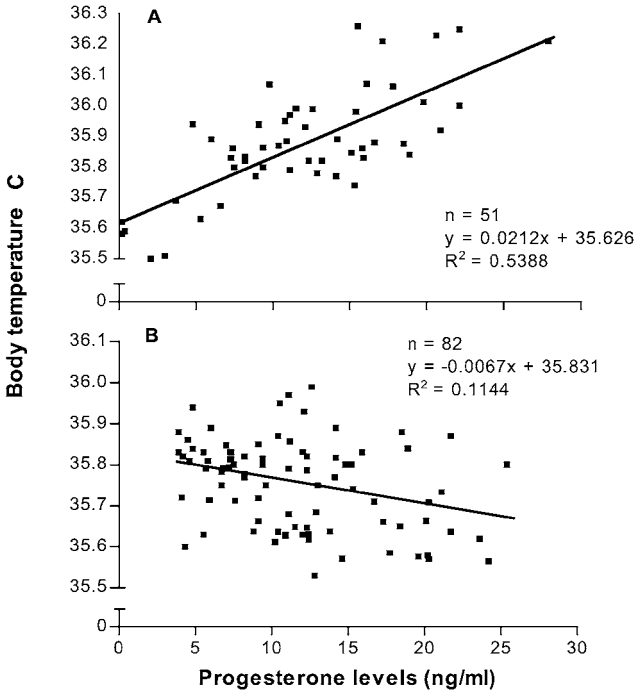

Fig. 6. Correlation between serum progesterone levels and body temperature in two killer whales during pregnancy. Body temperature was measured between 0800 and 0830 h before activity, and the serum progesterone levels were determined every 2 to 4 weeks. The values for the body temperature of each animal represent the average of every two weeks. A significant correlation between serum progesterone concentration and body temperature was observed in the increasing phase (A) during the first period of pregnancy. In contrast, no statistically significant correlations were found between body temperature and serum progesterone pattern in the decreasing phase (B) during the second period of pregnancy.

Atlantic female killer whales were pregnant at from age six to eight [25]. Additionally, Robeck *et al*. (1993) found the first estrous cycle of six captive female killer whales from North Atlantic was 8–9 years old [26]. Our females showed similar ages for sexual maturity as described in these wild and captive female killer whales. Female Stella produced three normal calves that nursed from 22 to 25 months during the sampling period. After the first and second calf, for the 2nd and 3rd pregnancy, during the first estrus cycle after a 10–20 months of anestrus. This period of anestrus occurred during the nursing period and was considered a lactation-

ally (suckling) induced anestrus. Lactational anestrus is thought to be due to suppression of hypothalamic-pituitary-ovarian function [27, 28]. The phenomenon described herein where the body temperature decreases before parturition, indicates that body temperature measurements can be useful for predicting of parturition, similar to other domestic species [29–34]. Of note, this decreased body temperature was not observed in Maggie's second abnormal parturition with a stillborn calf. This suggest that the decrease in mother body temperature before parturition might be an important mechanism in the process of normal delivery in this species. The present study was the first report to detail changes in body temperature of pregnant killer whales during pregnancy and parturition.

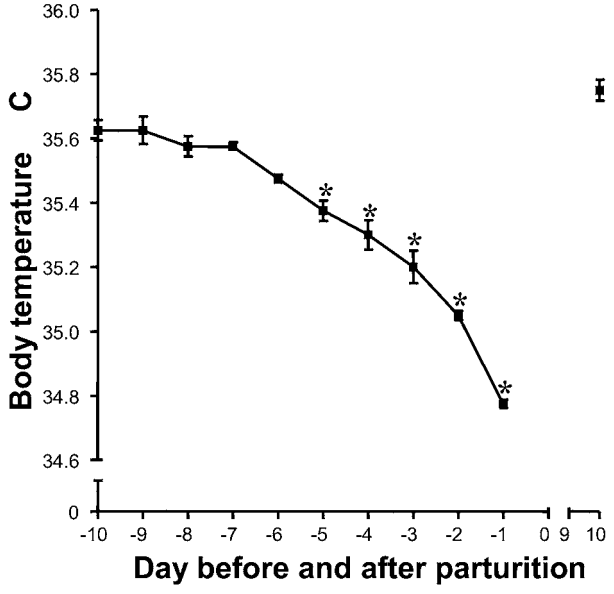

Fig. 7. Changes in body temperature before and after parturition. Day 0 is the day of parturition. Values are means ± SEM of four normal pregnancies. *P<0.01 *vs*. day-1.

Contraception of Female Seals with Synthetic Progesterone Agent

Seals typically breed quite readily in captivity, thus for space and genetic management purposes, the breeding program at Kamogawa Sea World required the development of a contraceptive plan. Ten female spotted seals and crossbreeds of spotted seals and harbor seals were used (Table 2) and proligestone (PRG) [35, 36] used in this experiment was a commercial estrus prevention agent for dogs (Kobinan®, Sankyo, Tokyo). A total of 35 trials in 10 animals was made over five years. Of these trials, 32 animals used 5 mg/kg PRG and the other 3 animals used 10 mg/kg. PRG was administered in one month before the estimated starting time of the breeding season, in 1994, 1995 and 1996. For estrus in 1997 and 1999, PRG was administered in December of the previous year, 1996 and 1998, respectively, which was two months before the estimated starting time of the breeding season. Serum progesterone concentrations in the untreated pregnant and non-pregnant animals were shown Fig. 8. Serum progesterone concentrations in pregnant

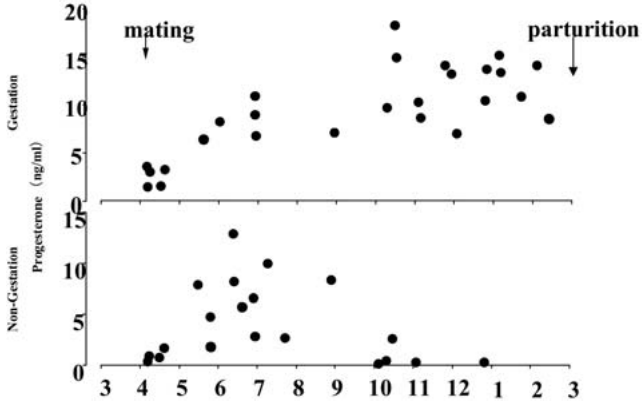

Fig. 8. Concentrations of serum progesterone of pregnant and non-pregnant spotted seals and crossbreds of spotted seals and harbor seals. To obtain reference values for the experiment, blood was collected from three pregnant animals and five non-pregnant animals between 1982 and 1994. Blood was collected 1–8 times from each animal.

Table 2. Animals tested

No.	♂ × ♀ (Breed)		Birth day (age) [a]	Body weight (kg) [b]	Parity [c]
1.	Spotted Seal	(Wild)	1971 (25)	116	9
2.	Spotted Seal	?	1979.3.5 (15)	104	2
3.	Crossbreed	Spotted Seals × Harbor Seal	1979.3.7 (15)	120	2
4.	Crossbreed	Spotted Seals × Harbor Seal	1982.2.26 (12)	117	1
5.	Crossbreed	Crossbreed × Crossbreed	1984.2.14 (10)	90	1
6.	Crossbreed	Crossbreed × Harbor Seal	1984.3.5 (10)	101	1
7.	Spotted Seal	?	1987.3.18 (7)	86	0
8.	Crossbreed	Crossbreed × Crossbreed	1991.2.24 (4)	75	0
9.	Crossbreed	Crossbreed × Crossbreed	1991.3.7 (4)	80	0
10.	Crossbreed	Crossbreed × Harbor Seal	1995.2.19 (3)	64	0

[a–c] Age, body weight, and past delivery at the time the experiment was initiated.

Table 3. Conceptive effect of the administration on proligestone in seals

Day of administration	No.1	No.2	No.3	No.4	No.5	No.6	No.7	No.8	No.9	No.10	Conception rate
1994.1.30	–	○	○[a]	○[a]	○[a]	○	○	–	–	–	6/6 (100%)
1995.1.23	–	○	○	○	○	○	○	○	○	–	8/8 (100%)
1996.1.20	○	○	×	○	○	○	○	×	○	–	7/9 (77.8%)
1996.12.27	○	○	–	–	○	○	○	○	○	–	7/7 (100%)
1998.12.27	–	–	○	–	○	○	–	○	–	○	5/5 (100%)

[a] 10 mg/kg PRG. ○: Successful contraception. ×: Unsuccessful contraception (pregnant). –: Not used in the experiment.

Table 4. Concentrations of serum progesterone (ng/ml) in seals in which the contraception succeeded

Date of blood sampling		No.1	No.2	No.3	No.4	No.5	No.6	No.7	No.8	No.9
1994	2/28		0.08		0.17	0.28	0.40	0.33		
	4/7		1.00		0.59	0.15	1.00	5.66		
	8/11						0.21			
	11/4							0.32		
	11/26		0.27	0.64	0.34	0.12	0.69	0.02		
1995	6/18							0.67		
	7/31		0.10							
	8/20		0.59					0.43		
	9/9							0.22		
	10/13		0.03							
	11/27		0.63	0.39	0.69	0.36	0.69	0.10	0.17	0.21
1996	7/1							0.32		
	7/10		0.10							
	7/16	0.69	0.10							
	8/21			Gestation				0.43	Gestation (abortion)	
	9/20				0.10					
	9/27				0.10					
	12/2	0.98	0.59			0.79		0.85		0.10
1997	4/22	1.01				0.37	1.00	0.02	0.67	0.42
1998	11/19									
	11/26									
1999	11/9					0.80				
	11/11			0.60			5.30			
	12/11						3.00			

groups was 1.5–3.5 ng/ml in April, and increased thereafter and the mean level was 8.9 ± 1.1 ng/ml in June, and it showed a high level of 7.1–18.2 ng/ml between October and immediately before delivery. The time-course of serum progesterone level in non-pregnant animals was similar to that of pregnant animals between April and July, but the level after October was 0.1–2.6 ng/ml with a mean level of 0.8 ± 0.5 ng/ml. Prior to administration of PRG, the blood progesterone concentrations of the seals were all less than 1.0 ng/ml, thus confirming none was pregnant [14, 15]. Determination of contraceptive effect was made by the presence of delivery of the test animals. The contraceptive effects were shown in Table 3. Among 23 trials treated with 5 mg/kg PRG between 1994 and 1996, 2 trials were pregnant, and the other 21 were successful in contraception (91.3%). Additionally, contraception was successful in all 12 trials (100%) treated with 5 mg/kg PRG in December. The overall success rate of contraception was 94.3%. Of 2 animals, in which contraception failed, one delivered a normal neonate, but the other aborted at six months of gestation. It was estimated that the reason was the timing of the administration of PRG, but not a lack of dose. The side effects were not observed with PRG treatment. Determination of the suppression of ovulation were shown in Table 4, in which ovulation was determined to be suppressed in individuals whose concentrations of progesterone were lower than 1.0ng/ml during April to September. It has been reported that the conception rate in wild seals was almost 100% [37]. Therefore, the results of this study demonstrate adequate contraceptive effects. No adverse effects on fertility was noted when one female (No.7) was able to deliver a normal pup when the PRG administration was discontinued.

Table 5. Profile of three female bottlenose dolphins

Name	Date into K.S.W.	Estim. birth year	Reproductive history
Norma	1989/11/21	1981	4 calves, 1 abortion
Meru	1989/10/19	1985	Nulliparous
Slim	1971/11/08	1966	7 calves, 2 abortion

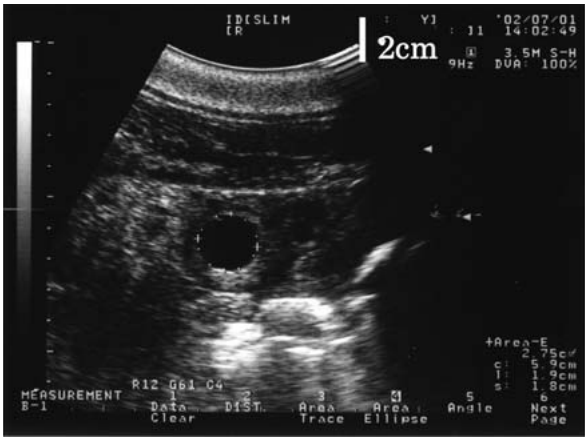

Fig. 9. Ultrasonographic image of ovarian follicles of right side ovary in a bottlenose dolphin (Slim). Round follicle (outlined by caliper) were observed from June 30 to July 5, 2003.

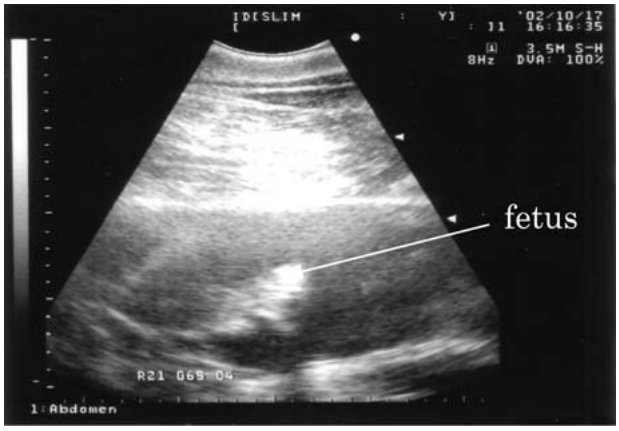

Fig. 11. Ultrasonographic image of dolphin fetus, Slim from 3 months of gestation.

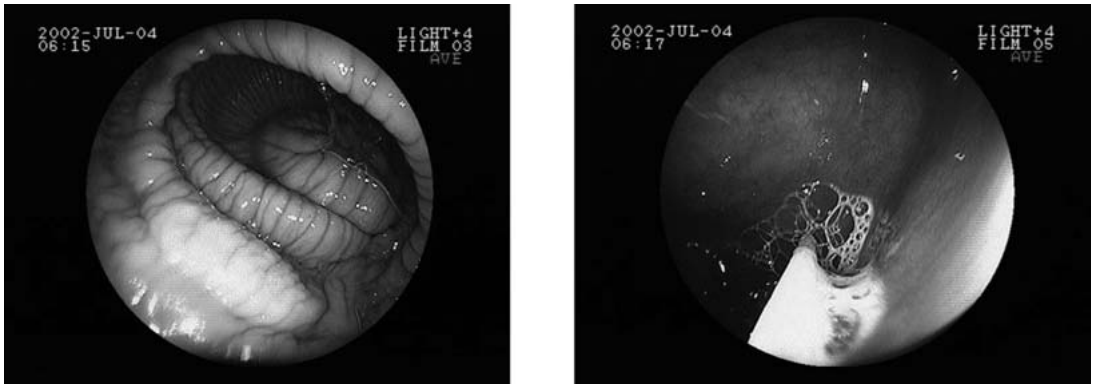

Fig. 10. Pictures of external os of uterus (left) and inside of uterus (right) of bottlenose dolphin, when artificial insemination was done.

Estrous Synchronization and Artificial Insemination of Bottlenose Dolphin

For this research, 3 mature female bottlenose dolphins (Table 5) were used in artificial insemination trials in 2002 (AI test 1) and 2003 (AI test 2). These female individuals were kept separately from mature males 4 months to one year before artificial insemination test. The synthetic progestin agent, altenogest (Regu-Mate, Intervet, DE, USA, 1ml, containing 2.2 mg of altenogest) were orally administered for artificial control of estrous cycle [38, 39]. Prostaglandin F2α (puronarugon F, Upjohn, Tokyo, 1ml containing 5 mg of Dinoprost) was administered (0.08 mg/kg) to a female Bottlenose dolphin (name: Helen, length 301 cm, weight 315 kg, 22 years old) as a preliminary experiments to determine the dose required to cause luteolysis during the estrous cycle. The luteolytic effect of prostaglandin F2α was confirmed when Helen's blood progesterone concentration of 12.9 ng/ml decreased sharply to 1.3 ng/ml at 8 h, then to 0.6 ng/ml at 24 h post administration.

In the AI test 1, the female bottlenose dolphins were administered orally with altenogest, 2.2 mg/ 50 kg (5–6 ml/individual), once per day for 27 days. The last day of treatment with altenogest, each individual was injected into the back muscle at a dose of 25 mg prostaglandin F2α in order to determine the period of ovulation ensured by luteolysis function. In the AI test 2, the female were administered altenogest, 2.2 mg/ 50 kg (5–6 ml/individual), once per day for 27 days without Prostaglandin F2α. Ovarian follicular

development was monitored by transabdominal ultrasound (SSD-900, Electronic Convex Probe UST-979-3.5, Aloka, Tokyo). Ultrasound proved an affective tool to monitor follicular activity on the ovaries [40, 41] (Fig. 9). Once the growing ovarian follicle were observed, their rate of growth could be determined and ovulation confirmed by the disappearance of the pre-ovulatory follicle. Fresh or frozen-thawed semen was artificially inseminated every 12 h until the ovulation occurred (until the pre-ovulatory follicle disappeared). For Slim AI trial 1, Her pre-ovulatory follicle was 2.1cm in diameter on day 23 and ovulation was confirmed on July 5, 2002, 24 days after the regumate withdrawal. For the AIs, semen was deposited into the uterus by injecting thought a 6.3 FR, 4m catheter that had been thread though the biopsy channel of the endoscope (Fig.10). The serum progesterone concentration increase, and reached 41.8 ng/ml, and a 16 cm-length fetus was confirmed by the ultrasound imaging for three months of pregnancy (Fig.11). Serum progesterone concentration maintained high level even after that, and a female calf was born on July 17, 2003. The gestation period was 377 days.

Norma of AI trail 2, her pre-ovulatory follicle was 1.9 cm in diameter on day 18 and ovulation was confirmed 19 days after the regumate withdrawal. She was inseminated artificially two times with frozen-thawed semen 10 h after ovulation on September 12, 2003. A male was born naturally on September 21, 2004. The gestation period was 375 days.

It has been observed that the fertilizing capacity of the egg decreases from 6 to 12 h after ovulation, and in most species is absent after 20 h [42]. Therefore, it is important to identify the timing of ovulation and inject sperm intrauterine to ensure fertilization. In this study, the diameter of the follicle was measured over time using ultrasound imaging device, and the proper time of artificial insemination was determined by identifying ovulations the loss of pre-ovulatory follicle.

Artificial insemination is an important technique to preserve the genetic diversity of cetaceans in captive breeding. If captive cetaceans throughout the world are considered one population, then genetic management via international cooperation can only be accomplished with this husbandry tool.

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