

Characterization of the Reproductive Cycle in Female Bharals (*Pseudois nayaur*) Based on the Changes in Serum Progesterone Concentrations and Parturition Season

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ABSTRACT. The purpose of present study was to determine annual changes in serum progesterone (P₄) concentrations and to clarify basic reproductive characteristics, such as breeding season, estrous cycle, and puberty in female bharals (*Pseudois nayaur*). Blood was collected from 9 female bharals once or twice weekly for approximately one year. Serum P₄ concentrations were determined by radioimmunoassay. Serum P₄ concentrations showed remarkable and cyclic changes between November/December (winter) and May/June (late spring). The mean estrous cycle was 24.9 ± 0.5 days. Chasing insistently to other females and discharge of mucus from the vulva were observed around the time when the serum P₄ concentrations began to increase. The chasing behavior and discharge of mucus were considered to be external indicators of estrus in female bharals. Serum P₄ concentrations of a pregnant female had non-cyclic changes, and the values remained high. In this study, all 37 deliveries were between April and September, and about 70% of these were concentrated in May and June. The conception month determined on the day of birth was between October and April for all animals, and the most common month was in December (54%). This month corresponded to an early stage of the period when the serum P₄ concentrations changed cyclically. These results indicate that many female bharals become pregnant at the beginning of the breeding seasons and, if they do not become pregnant, the estrous cycle, about 25 days in length, is repeated.

KEY WORDS: bharal, breeding season, estrous cycle, progesterone, sexual maturation.

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Bharal (Blue sheep: *Pseudois nayaur*) belong to the family Bovidae (Subfamily Caprinae), and inhabit an undulating montane zone from the Himalayan region to Inner Mongolia [6, 10]. In structure and habit, the bharal is intermediate between genera *Ovis* and *Capra*, but is morphologically closely related to genus *Capra* [10]. Genus *Pseudois* includes two species, bharal (*P. nayaur*) and dwarf bharal (*P. schaeferi*) [6]. Wild populations of both species have decreased because of decreasing habitats and the introduction of livestock, etc. Particularly, *P. schaeferi* and a part of the regional populations of *P. nayaur* are in a critical situation because of excessive hunting for meat that occurred before hunting was legally banned in 1989 and because of poaching after the ban. According to the 2004 IUCN Red List of Threatened Species [4], *P. nayaur* is classified as low risk (near threatened) and *P. schaeferi* is classified as endangered, so it has become more important to conserve wild and captive populations.

It is known that the bharal is a seasonal breeding animal. The birth season is in May–July in their natural habitat [6]. In wild species of the related genera *Ovis* and *Capra*, such as mouflon (*O. gmelini musimon*) [9] and Spanish ibex (*C. pyrenaica hispanica*) [8], there are some reports concerning the breeding season, estrous cycle, and puberty based on

changes in the serum progesterone (P₄) concentrations of the females. However, the endocrinological data of genus *Pseudois* has not been reported, and thus its reproductive physiology has yet to be clarified.

In Japan, bharals are currently kept at only 4 zoo facilities, Gunma Safari Park, Gunma, Kanazawa Zoological Gardens of Yokohama, Kanagawa, Kyoto Municipal Zoo, Kyoto, and Himeji Central Park, Hyogo. There are increasing fears about a possible decrease in genetic variability by inbreeding due to the limited number of facilities in which bharals are kept. Therefore, semen collection and artificial insemination has been planned for animals in captivity with the aim of appropriate genetic and reproductive management.

The purpose of the present study was to determine the annual changes in serum P₄ concentrations and the parturition season in captivity, and thus clarify basic reproductive characteristics of the breeding season, estrous cycle, and puberty in female bharals.

MATERIALS AND METHODS

Animals and blood collection: Nine female bharals reared in Himeji Central Park were studied (Table 1). They were usually kept in an approximately 14,000 m² outdoor area of the safari style park with another 11 bharals, 21 Barbary sheep (*Ammotragus lervia*), 15 mouflons, 83 blackbucks

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Table 1. Age and body weight of the female bharals included in this study

Individual number	Name	Age ^{a)}	Body weight (kg) ^{b)}
No. 1	Wurong	14.1	26.4
No. 2	Sirong	11.2	30.5
No. 3	Puer	5.2	30.6
No. 4	Kei	3.1	37.8
No. 5	Yanlong	2.1	30.0
No. 6	Mouf	2.1	24.4
No. 7	Celong	2.0	27.3
No. 8	Ryoku	1.3	24.4
No. 9	Sheets	1.3	21.5

a) At start of the experiments. b) In January 2004.

(*Antelope cervicapra*), 2 Sika deers (*Cervus nippon*), 2 Himalayan gorals (*Nemorhaedus goral*), and 9 addax (*Addax nasomaculatus*) throughout the year. During this study period, which lasted from July 2003 to July 2004, the nine female bharals were kept in an outdoor paddock that was approximately 210 m² in size. From March 18 to July 15, 2004, 2 females (Nos. 1 and 4) were kept with three males in an outdoor paddock that was approximately 45m² in size. The bharals were fed a daily diet of approximately 500 g/head/day of alfalfa and timothy hay and approximately 500 g/head/day of pellet ZC (Oriental Yeast). Drinking water was available *ad libitum*.

Blood sampling was conducted once or twice weekly for approximately 1 year. The bharals were restrained by 2 zoo keepers clutching both the horns and the hind legs, respectively, and blood was collected from the jugular vein under non-anesthesia conditions. Immediately after blood sampling, the blood was put into an evacuated blood collection tube containing a serum-separating medium (Venoject II, Terumo). The tube was left for approximately 30 min at room temperature, and then the serum was separated from the whole blood by centrifugation at 3,000 rpm for 10 min. The serum samples were kept at -20°C until assay.

Radioimmunoassay: Steroids in the serum was extracted twice with diethyl ether. Radioimmunoassay for P₄ was carried out using a modified version of a previous method [2]. Briefly, a 100 µl aliquot of the assay sample, 50 µl of [1,2,6,7,16,17-³H]-labeled radioligand (10,000 dpm/50 µl, TRK641, Amersham Pharmacia Biotech), and 100 µl of antiserum against P₄ (1:60,000, LC-28, Teikoku Hormone Mfg.) were vortex-mixed. After incubation for 24 hr at 4°C, 500 µl of 0.25% charcoal-0.025% dextran PBS was added and vortex-mixed. Then, the antiserum bound hormones were separated from the free hormones by centrifugation at 3,000 rpm for 10 min at 4°C. Six hundred µl aliquot of the supernatant and 5 ml of toluene scintillation solution [2,5-diphenyloxazole and 1,4-bis(5-phenyl-2-oxazolyl) benzene] was vortex-mixed. Radioactivity was measured for 5 min with a Liquid Scintillation Analyzer (Tri-Carb 2900TR, Packard Bioscience). The cross-reactivity of the antiserum in the P₄ assay was as follows: P₄, 100%; 5α-pregnanedione,

62.2%; pregnenolone, 6.26%; 11-deoxycorticosterone, 3.88%; 17α-hydroxyprogesterone, 2.25%; 11α-hydroxyprogesterone, 1.23%; 20α-hydroxyprogesterone, 0.51%; testosterone, 0.18%; 11-deoxycortisol, 0.14%; androstenedione, 0.12%; 5α-dihydrotestosterone, 0.12%; corticosterone, 0.07%; 17α-hydroxypregnenolone, 0.05%; dehydroepiandrosterone, 0.04%; and cortisol, cortisone, aldosterone, estrone, estradiol-17β, estriol, and androsterone, 0.01%. The sensitivity of the assay was 30 pg/ml. The intra- and inter-assay coefficients of variation were 4.5 and 11.6%, respectively.

Investigation of parturition history: To investigate the birth season, all birth dates of the bharals at Himeji Central Park for 12 years within the period of 1992–2004 were totaled for each month. The day of pregnancy or age at pregnancy was determined by counting backwards 160 days from the birth date of each individual [6], 160 days was the mean gestation period. Moreover, sexual maturity was determined based on the age at first conception.

RESULTS

Seasonal changes in serum progesterone: The serum P₄ concentrations showed remarkable changes in November, December, or early January in 8 of the females, all except female No. 7. Remarkable changes in serum P₄ concentrations continued until April, May, or June in 8 of the females, all except one pregnant female No. 4 (Fig. 1). In only one female No. 7, the changes in serum P₄ concentrations started earlier than the other females, in late August. The serum P₄ concentrations in the 9 females, showed repeated cyclic changes during these periods. The length of the estrous cycle was calculated as the number of days from the first P₄ rise to the next P₄ rise in the following cycle. The mean (± SEM) length of the estrous cycle was 24.9 ± 0.5 days (n=64 cycles). The shortest estrous cycle was 21 days and the longest was 35 days. Estrous cycles of 21–28 days made up 89.8% of all cycles. The number of estrous cycles during the one breeding season varied between 4–12 cycles (Fig. 1). Serum P₄ concentrations during July–November did not change and remained at basal levels in 7 of the females, all except female No. 7 and female No. 4, which was pregnant.

Chasing insistently to other females and discharge of mucus from the vulva were observed for females Nos. 3, 4, and 8, and mounting and/or chasing behaviors by males were observed for females Nos. 1 and 4 (Fig. 1). In most cases, the behaviors and discharge of mucus occurred around the time when the serum P₄ concentrations began to increase.

Females Nos. 1 and 4 copulated at the beginning of April. After copulation, the serum P₄ concentrations of female No. 4 had non-cyclic changes and the values remained high (Fig. 1). This female gave birth normally after a gestation period of 168 days. Female No. 1 did not conceive.

Seasonality of parturition and conception: Eight females gave birth at Himeji Central Park over a period of 12 years (April 1992–September 2004). There was a total of 37

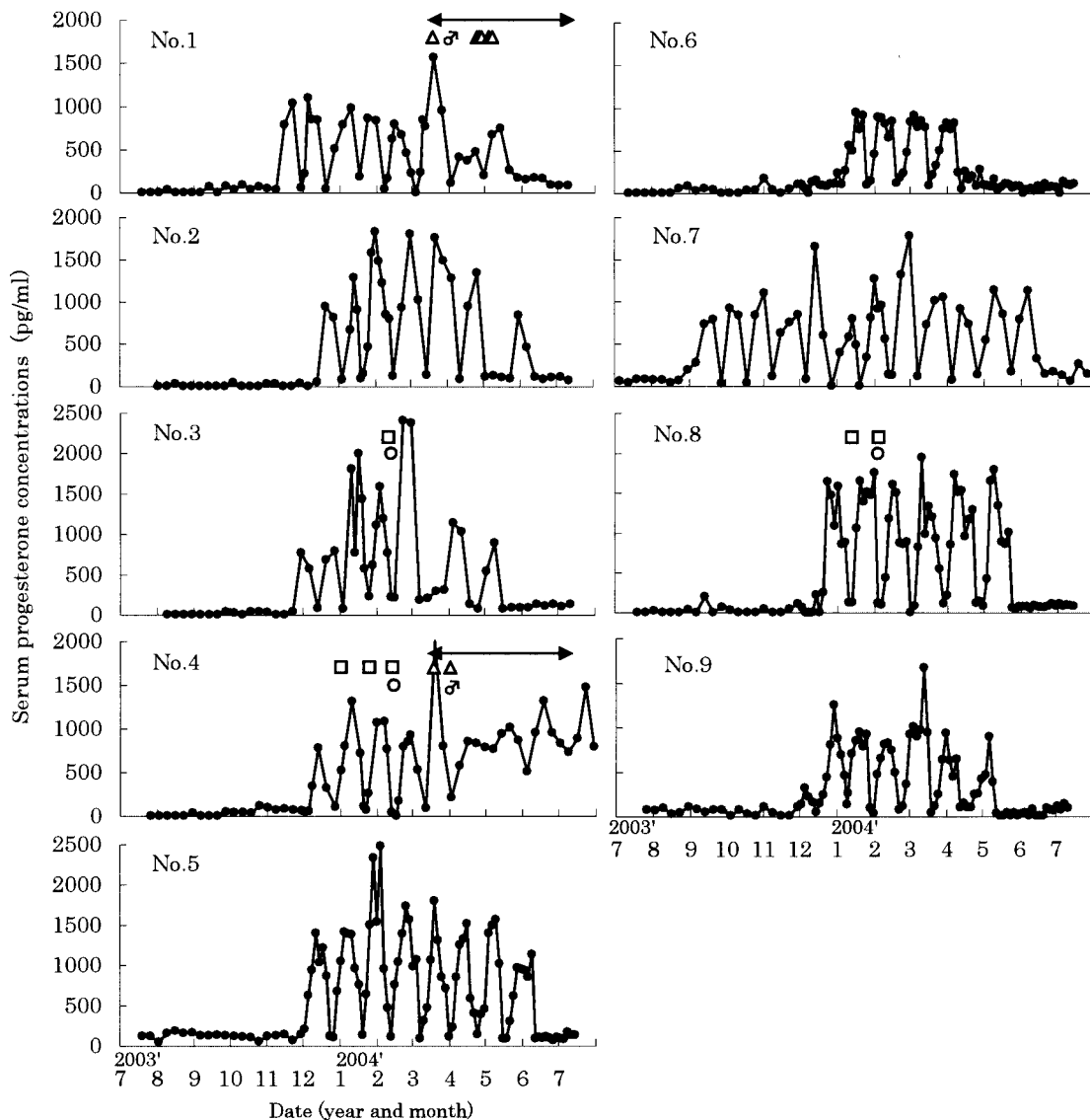


Fig. 1. Seasonal changes in serum progesterone concentrations of the female bharals. □: Chasing behavior to other females. ○: Mucous discharge from the vulva. △: Received mounting and/or chasing behaviors by males. ♂: Copulation. ↔: Periods housed together with males.

deliveries from the eight females, and two of the deliveries were twins. This produced a total number of offspring of 39 animals (22 females and 17 males). Of the 37 deliveries, 3 were in April, 12 were in May, 13 were in June, 4 were in July, 3 were in August, and 2 were in September. There were no deliveries in other months (Fig. 2). About 70% (25 deliveries) of all deliveries were concentrated in May and June. Moreover, the conception month determined on the day of birth was between October and April for all animals, and 54% (20 conceptions) of conceptions occurred in December (Fig. 2). The age at first delivery for the eight females was 2.0–3.1 years old (Table 2).

DISCUSSION

It is known that physiological phenomena, such as reproductive hormone concentrations, gonadal morphology, and histology, usually change seasonally in male and female wild mammals living in seasonal natural habitats, such as temperate zones [1, 11]. Serum testosterone and testis size in male bharals also changes seasonally (S. Kusuda, unpublished data). Although there are endocrinological reports on the breeding season and puberty in domestic and wild species of the related genera *Ovis* and *Capra*, this may be the first report on the reproductive endocrinological characteristics of genus *Pseudois*.

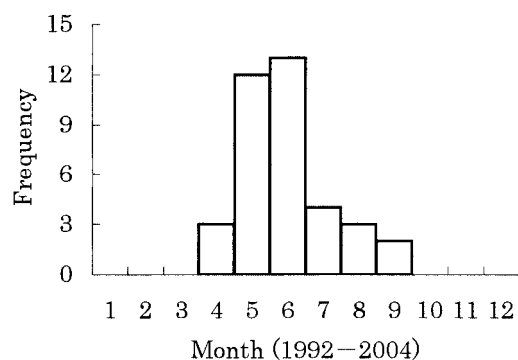


Fig. 2. Delivery month of the bharals at Himeji Central Park over the course of 12 years (April 1992–September 2004).

Table 2. Age at first parturition of bharals kept at Himeji Central Park

Name (Individual number*)	Age at first parturition (year)
Kuer	2.0
Kei (No. 4)	2.0
Celong (No. 7)	2.0
Sirong (No. 2)	2.1
Puer (No. 3)	2.1
Syarong	2.9
Wurong (No. 1)	3.0
Kate	3.1

* Bharals included in this study (Table 1).

The breeding season of female bharals was clarified endocrinologically in our study. The results indicated obvious seasonal P_4 changes, and the breeding season based on their profile was from early winter to late spring (November–June). Chasing insistently of other females and discharge of mucus from the vulva were observed around the time when the serum P_4 concentrations began to increase, that is, at estrus. Therefore, we considered the chasing behavior and discharge of mucus to be external indicators of estrus in female bharals.

The mating season of bharals living in a natural habitat is October–January and the parturition season is from May to early July [6]. That is, bharals are seasonal breeding animals. The parturition season at Himeji Central Park is mainly May–June, and the conception period determined from the birth date was October–April (December was the peak). This timing almost matches the serum P_4 profiles in our results, and a report on the breeding season in the wild population. Thus, bharals have a very limited breeding season. The peak of the birth season corresponds to the period of spring to early summer when food resources are abundant in natural habitats. This might be very suitable for the extreme climatic and nutritional environment of mountainous zones.

The length of the estrous cycles in domestic sheep and goats varies according to breed and averages 20–24 days for the Rambouillets breed of sheep, 16–19 days for the Colum-

bia breed of sheep, and 19–21 days for goats [5]. There are reports concerning the estrous cycle in wild species of genuses, *Ovis* and *Capra*. It has been reported that the average length of the estrous cycle in Dall's sheep (*O. dalli dalli*) is 18.2 days based on the changes in pregnanediol-3-glucuronide concentrations in urine [3]. The average length of the estrous cycle was reported to be 17.2 days and 19.4 days in mouflon [9] and Spanish ibex [8], respectively, based on plasma P_4 concentrations. The mean length of estrous cycles in the bharals was 24.9 days based on our results, and this was slightly longer compared with the wild species of genuses, *Ovis* and *Capra*.

The number of estrous cycles during one breeding season varied and ranged between 4–12 cycles according to individual. It has been reported that the breeding season in the mouflon begins in the middle of October and terminates randomly within the period of February–May based on the plasma P_4 concentrations [9]. It had been also suggested that the length of the breeding season in young mouflons (two years old or less) is shorter compared with individuals that are 3–6 years old. However, there were no relationships between the number of estrous cycles or length of the breeding season and age in our results.

Generally, it is known that the bharal reaches sexual maturation at 18 months of age [6]. Serum P_4 concentrations in the two youngest females (Fig. 1, Nos. 8 and 9) used in our study began to change at the age of 20 months, and there was no difference when compared with the P_4 profiles of females that were two years old or more. In addition, the age at first parturition was 2.0–3.1 years for eight of the females at Himeji Central Park. The age at first conception determined from the first parturition date was 1.6–2.7 years (19–33 months). Puberty in the domestic Shiba goat occurs at the age of 27.0 ± 0.9 weeks ($n=11$ out of 12 goats) based on change in plasma P_4 concentrations [7]. In at least 5 of these goats, the first estrous behavior was observed at the second ovulation. A female mouflon that was born at the beginning of the birth season was reported to be in puberty at 8 months of age during the breeding season this year [9]. Consequently, puberty in the female bharal is probably later than that of the mouflon and domestic goat. Female bharals that are born in spring to early summer reach sexual maturation during the breeding season (the second winter) of the following year and could become pregnant at the beginning of the breeding season.

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