遠洋水研報　第36号　17-25頁　平成11年3月
Bull. Nat. Res. Inst. Far Seas Fish., No. 36, 17-25, March 1999

Cytological Changes in Vaginal Smear and Epithelium Associated with the Reproductive Cycle in Northern Fur Seal, *Callorhinus ursinus*

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Numerical changes in vaginal smear cells are described in relation to serum steroid hormones in captive northern fur seals, *Callorhinus ursinus*. During anestrus, parabasal cells were the most common in vaginal smears. Intermediate cells increased in number at estrus accompanied with superficial and anucleated cells. Increase in intermediate cells was also observed in November when implantation was supposed to occur. The serum estrogen level peaked at estrus and implantation. The vaginal epithelium of pelagically sampled animals showed a difference related to the reproductive condition. The vaginal epithelium thickened at both estrus and implantation (100-200 μ m) in ovulated and pregnant females. Immature individuals did not show marked differences in thickness of the vaginal epithelium. Since vaginal smears show gradual cytological changes in relation to serum steroid levels, vaginal smears may provide a convenient tool for monitoring the reproductive cycles of captive and wild seals, but it appears to be unsuitable for detecting the exact timing of estrus and ovulation.

Key words: vaginal smear, estrogen, estrus, implantation, northern fur seal

Introduction

Vaginal smears are used as an easy and quick method to determine the estrus cycles in many mammals (Stenson, 1988; Wright, 1990). Pinnipeds have specialized annual reproductive cycles characterized by embryonic diapause and delayed implantation. If reproductive aspects of pinnipeds are easily checked by using vaginal smears, it may help husbandry of captive seals and monitoring the reproductive condition of wild animals. Bigg (1973) and Bigg *et al.* (1977, 1978) applied vaginal smears to two species of pinnipeds, harbor seal, *Phoca vitulina* and northern fur seal, *Callorhinus ursinus*, to determine estrus and ovulation. However, they showed only a qualitative description of changes in smear cells without mentioning the quantitative measurements in detail. In the present study, quantitative methods for counting vaginal smear cells were examined using captive northern fur seals. Numerical changes in vaginal smear cells are described in relation to serum steroid hormones. Histological characteristics of the vaginal epithelium associated with reproductive conditions were also investigated using tissue samples collected from wild animals.

Materials and Methods

Four female northern fur seals (designated as F-1, F-2, F-3 and F-4) were captured off northeastern Japan between 1982 and 1991 and brought to an aquarium (Izu-Mito Sea Paradise) in Numazu, Japan. During the experimental period from May 1993 to January 1995, they were held in an indoor facility. In summer season from April 7 to September 16, they were kept in two cement rooms separated by metal fences (2.6 × 3.0m compartment with a sea water pool measuring 1.9 × 1.5 × 1.2m; 2.6 × 3.9m compartment with a pool measuring 1.7 × 2.1 × 1.2m), each one containing two females. An adult male was added to each group during the breeding season. Females were kept together in the former compartment without males after the breeding season. Ages of the experimental individuals were estimated as 6-15 based on the body size and vibrissae color (Baba *et al.*, 1991).

Vaginal smear samples were collected either at five days' interval during the estrus period (May 27-August 30) and the implantation period (October 19-November 28) or at 10 days' interval during the other periods. Each female was kept in a dog cage and physically restrained during the sampling. Vaginal smears were taken using a cotton swab inserted to a depth of 10 cm using a vaginal speculum. Contents of the swab were expressed onto a slide glass, dried, and stained with Wright's stain. Vaginal cells were classified into four categories (parabasal, intermediate, superficial, anucleate) according to Wied (1958). Each type of epithelial cells and leucocytes was counted.

Blood samples were collected from the inter-digital vein of hind flippers into plain glass tubes when vaginal smears were taken. The samples were centrifuged at 3500 rpm for 15 minutes. The serum was stored at -20° C until steroid assay. Estradiol and progesterone were assayed using RIA system developed for human clinical use (Diagnostic Products Corporation, Los Angels). The assay was done by a commercial clinical company (SRL Corporation, Tokyo). Cross-reactions of estradiol antibody with ethinyl estradiol, estrone, 17 β-estradiol-3 β-D-glucuronide, and other steroids were

1999年1月26日受理　遠洋水産研究所業績　第375号
Accepted on January 26, 1999. Contribution No. 375 of the National Research Institute of Far Seas Fisheries.
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1.8%, 1.0%, 0.7%, and <0.5%, respectively. The progesterone antibody cross-reacted with 5 α -pregnan-3,20-dione (9.0%), 17 α -hydroxyprogesterone (3.4%), 5 β -pregnan-3,20-dione (3.2%), 11-deoxycorticosterone (2.2%) and other steroids (<0.5%). Sensitivity of each assay was 8 pg/ml for estradiol and 20 pg/ml for progesterone. The mean intra-assay coefficient of variation was 6.2% and 5.1% for estradiol and progesterone, respectively.

Vaginal tissues were excised from 28 female northern fur seals caught pelagically off northern Japan in 1976-1980. Tissues were fixed and preserved in 10%

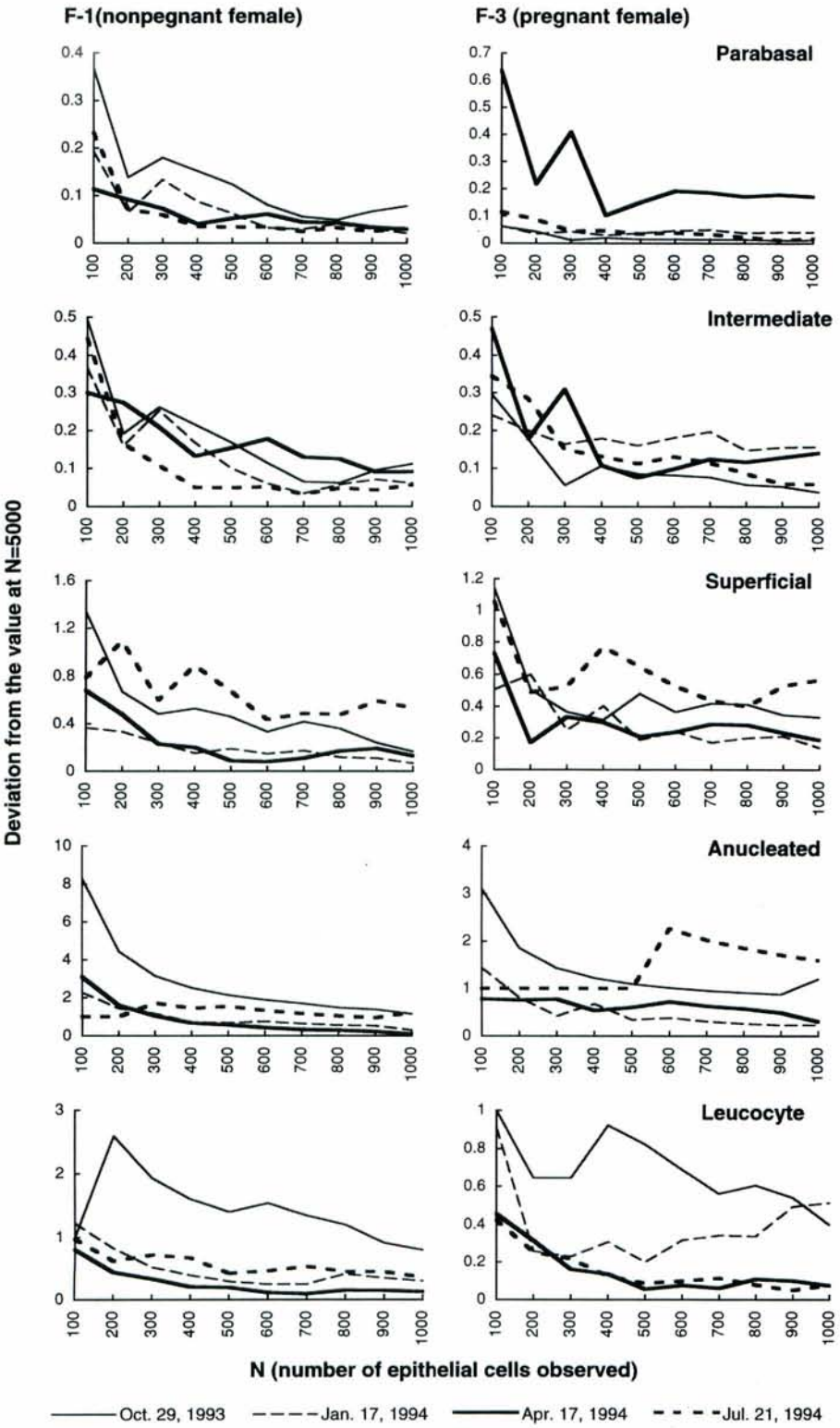

Fig. 1. Deviation of counts of vaginal smear cells related to sample size. Deviations from the value based on counts of 5000 epithelial cells were examined at ten different sample sizes. N epithelial cells were observed and each type of epithelial cells and leucocytes were counted. Eight vaginal smear samples from two different individuals in four seasons were used.

fromalin, dehydrated in graded concentrations of alcohol, embedded in paraffin, and sectioned at 5 μ m. The sections were stained according to the Hindenhain's modification of Mallory's method.

Results

Evaluation of Methods for Counting Vaginal Smear Cells

Quantitative methods to measure the relative frequency of each type of cells in vaginal smears were examined. A certain number (N) of epithelial cells were counted, and the frequency of each type as well as the number of leucocytes observed while counting epithelial cells was recorded using eight different smear samples collected from two different females in four different seasons. N increased at ten levels (i.e., 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000), and counts were repeated five times for each level. Mean deviations of counts at each N relative to the counts of 5000 epithelial cells are plotted in Fig. 1, where

$$\text{Mean deviation} = \frac{\Sigma \mid C_N - C_{5000} \mid}{(5 \times C_{5000})}$$

C_N=counts of each cell type while counting N epithelial cells

C_{5000}=counts of each cell type while counting 5000 epithelial cells

In parabasal and intermediate cells which occurred abundantly in vaginal smears, mean deviations decreased as N increased. Anucleated cells and leucocytes sometimes showed high deviation when their occurrence was extremely low (<1%). In most

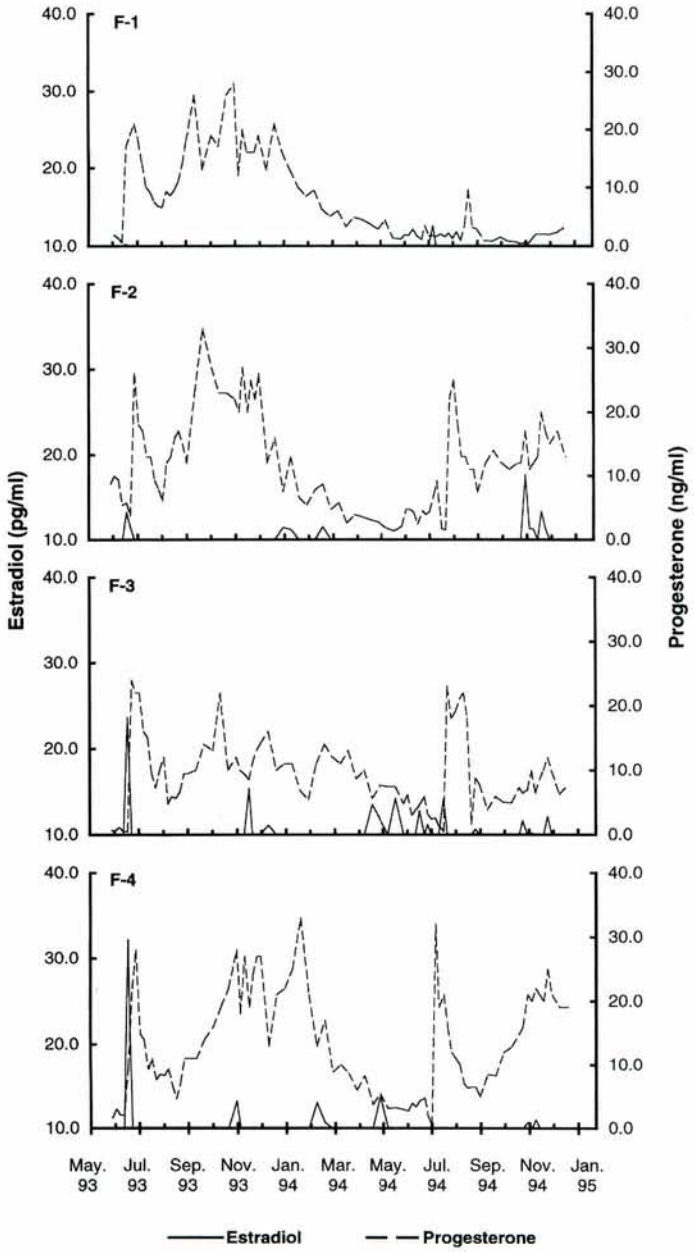

Fig. 2. Changes in serum estradiol and progesterone levels in four captive northern fur seals.

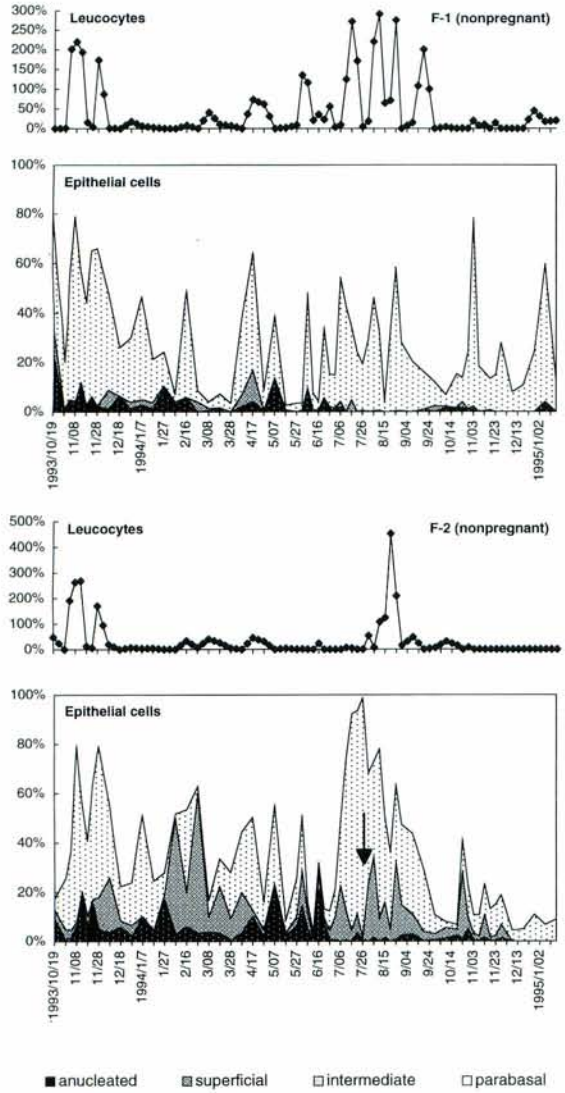

Fig. 3A. Counts of leucocytes and epithelial cells in vaginal smears of captive northern fur seals (F-1 and F-2). Percentage of each cell type per 500 epithelial cells is shown. Arrows indicate ovulation determined from sudden rise of serum progesterone.

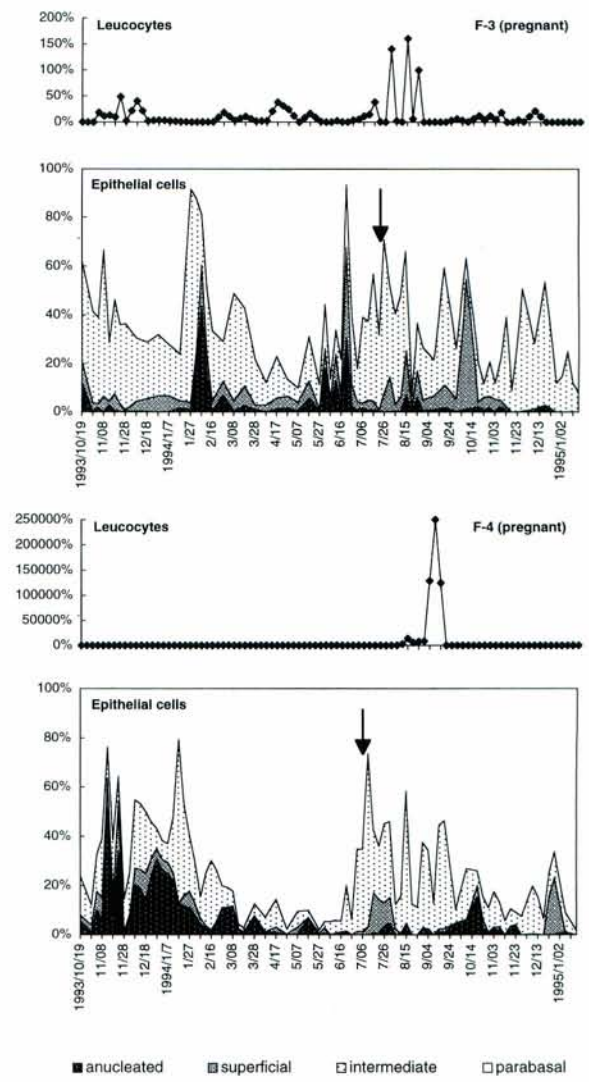

Fig. 3B. Counts of leucocytes and epithelial cells in vaginal smears of captive northern fur seals (F-3 and F-4). Percentage of each cell type per 500 epithelial cells is shown. Arrows indicate ovulation determined from sudden rise of serum progesterone.

cases, deviation of common cells were smaller than 0.1 when $N \geqq 500$. Thus, counting each type of epithelial cells and leucocytes while observing 500 epithelial cells per one slide glass seems a practical method for routine inspection of vaginal smears.

Changes in Vaginal Smear and Serum Steroids

Fig. 2 shows the levels of serum steroids in four female northern fur seals during the experiment. Sudden rise of progesterone in July indicates ovulation and subsequent formation of corpus luteum in all the four females in 1993 and three females (F-2, F-3, F-4) in 1994. Remarkable peaks of estradiol were observed only for two females (F-3 and F-4) in June 1996, indicating that blood sampling at 5 days' interval was insufficient for detecting the estrogen surge at ovulation. These two females became pregnant in 1993 and delivered pups on July 16 and June 29, 1994, respectively. Progesterone level decreased in August associated with embryonic diapause. Secretion of estradiol was also detected at the time of implantation in October-November and during late pregnancy (February-June).

The relative frequency of epithelial cells and leucocytes in vaginal smears is shown in Fig. 3. Parabasal cells were the most common cell type during the anestrus period (Fig. 4). Intermediate cells increased in number in the estrus period accompanied by superficial and anucleated cells. The estrus pattern of vaginal epithelial cells was the most conspicuous in F-2, moderate in F-3 and F-4, and obscure in F-1. Judging from the serum steroid profile, the female F-1 did not have estrus in 1994. Increase in intermediate cells was also observed in November, possibly related with the estrogen secretion at implantation. Changes in abundance of leucocytes were more variable than those of epithelial cells. All four females showed a higher number of leucocytes in August. In some individuals, leucocytes also increased in November. The number of leucocytes relative to epithelial cells differed among

individuals. F-4 had a higher number of leucocytes which might be related to factors other than the reproductive condition (e.g., infection). In conclusion, vaginal smears showed gradual cytological changes related to the reproductive cycle.

Differences in Vaginal Epithelium Associated with Reproductive Status

Fig. 5 shows sections of the vagina at different reproductive stages. The vaginal mucosa of estrus females were folded, and the surface depressions were filled with squamous cells and superficial cells. At embryonic diapause in August, the vaginal epithelium was thinner and consisted of 4-5 layers of undifferentiated cells. The vaginal epithelium thickened in November during the implantation period, but its thickness decreased again in the early gestation period (December-January) in pregnant females. Fig. 6 compares the differences in thickness and number of cell layers of the vaginal epithelium between different reproductive stages. The vaginal epithelium of mature females was thicker (100-200 μ m) at estrus than at embryonic diapause and anestrus. Pregnant females also showed thickening of the vaginal epithelium during implantation period. Number of cell layers showed a similar increase at estrus and implantation in mature females. On the contrary, thickening of the vaginal epithelium was not conspicuous in immature females.

Discussion

This study demonstrated the changes in vaginal smear cells in relation to the serum estrogen level which fluctuated according to reproductive cycles in captive female northern fur seals. The increased abundance of intermediate cells and the presence of superficial and anucleated cells during the estrus period must be caused by the elevated estrogen level. In rats *(Rattus rattus)*, estrogen is known to induce proliferation and cornification of vaginal epithelium which increases the exfoliation of epithelial cells into vaginal smear (Montes and Luque, 1988). In northern fur seals, the cytological change should be adopted to

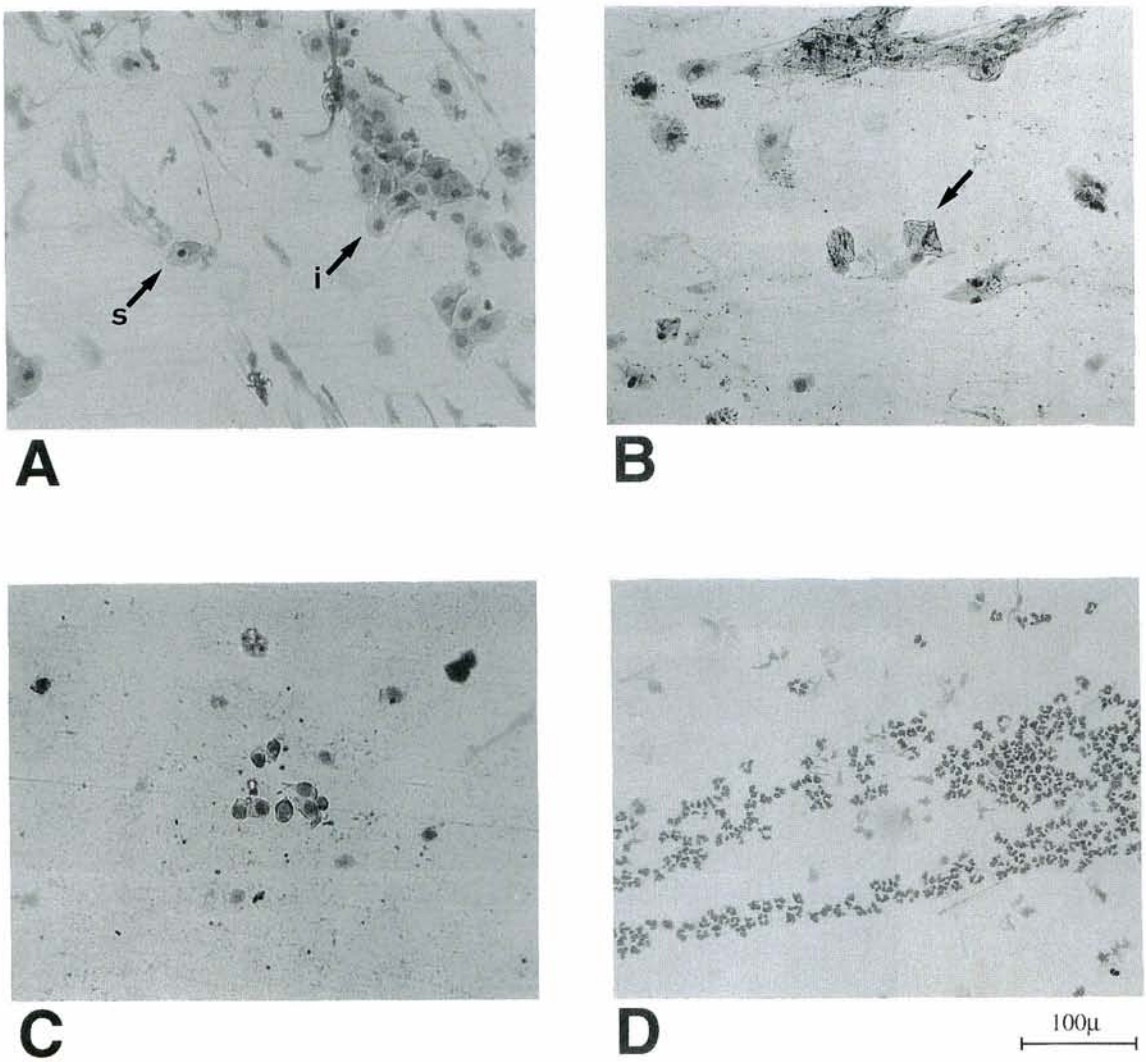

Fig. 4. Photomicrographs of vaginal smears. A: vaginal smear showing intermediate cells (i) and a superficial cell (s) with pyknotic nucleus at estrus (F-4, July 11, 1994), B: Anucleated cells (arrow) at anestrus (F-2, May 17, 1994), C: Parabasal cells at anestrus (F-2, April 17, 1994), D: Leucocytes at embryonic diapause (F-4, September 9, 1994).

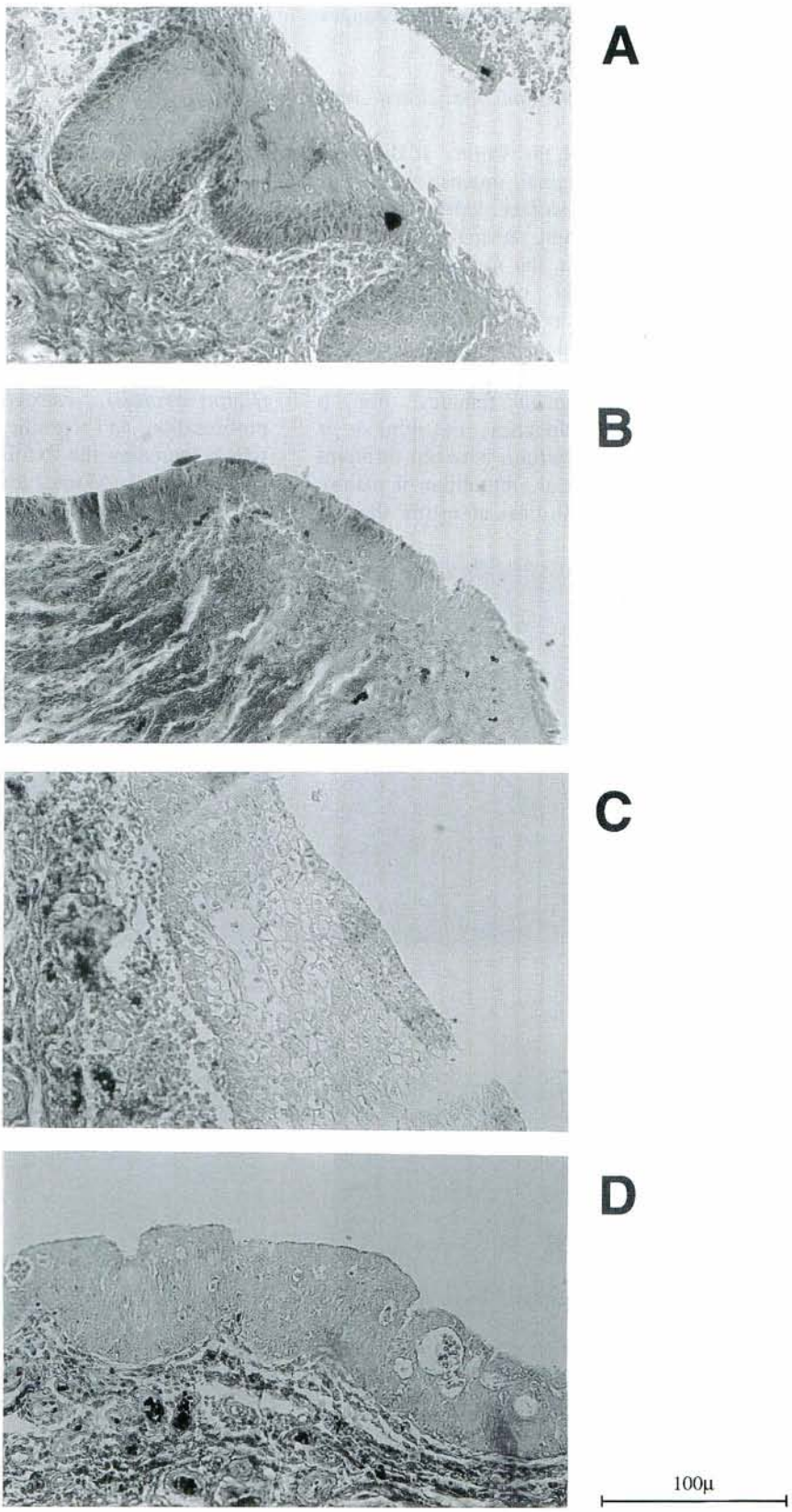

Fig. 5. Photomicrographs of the vaginal epithelium of four mature female northern fur seals of different reproductive stages. A: estrus, B: embryonic diapause, C: implantation, D: early pregnancy.

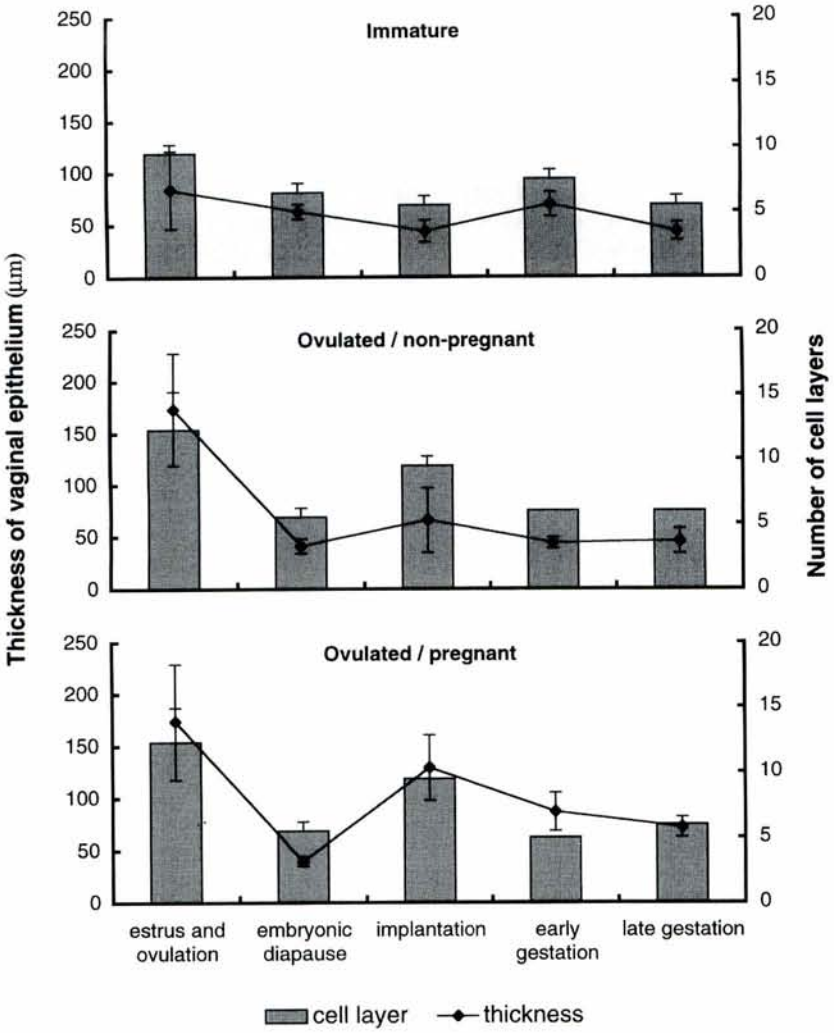

Fig. 6. Differences in thickness and number of cell layers in the vaginal epithelium of northern fur seals according to reproductive conditions. Vertical bars indicate standard deviations.

copulation. Similar thickening of the vaginal epithelium was observed at implantation as well. However, it does not necessarily imply the possibility of the second estrus in late autumn because no copulatory behavior was observed then. The phenomenon seems to be related to the physiological changes associated with delayed implantation. Daniel (1974) found a peak of estrogen synchronized with termination of embryonic diapause in northern fur seals. Similar peaks were detected in some individuals in this study. Boyd (1991) proposed a model of pinniped reproduction in which active and inactive phases alternated to accomplish annual reproductive cycle. The thickening of the vaginal epithelium at implantation seems to be a by-product of activation of the reproductive system from inactive diapause stage to gestation stage, and the epithelial change itself does not seem to have an adaptive value. Immature females did not show remarkable changes in vaginal cells probably because their reproductive system had not switched-on yet.

Serum estrogen is secreted in peaked pattern and is hardly detected by infrequent blood sampling. Vaginal cytology reflects the physiological action of estrogen and can be used as a good index of the reproductive condition. Because vaginal smears show relatively slow changes in which a similar condition continues for 10-20 days or more, the occurrence of estrus and ovulation will be detected by less frequent sampling compared to serum estrogen. The method provides an easy tool for monitoring the reproductive conditions of captive and wild seals. However, on the contrary, it is not suitable for examining the exact timing of estrus and ovulation. For the latter purpose, frequent sampling of sexual steroids may have advantage. Sampling of steroids without restraining animals, using urinary or fecal materials for example, may be helpful for that kind of study.

Acknowledgements

We thank the staff of Izu-Mito Sea Paradise for taking care of the captive animals and for helping the sampling. Thanks also go to Norihisa Baba of the National Research Institute of Far Seas Fisheries, Shimizu, for his advice and encouragement during this study.

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キタオットセイの繁殖周期に関連した膣粘液塗抹と上皮細胞の変化

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摘　要

飼育下のキタオットセイの膣粘液塗抹中の細胞の定量的変化と血清中ステロイドホルモンの関係を調べ，繁殖周期の簡便な判定法としての膣粘液塗抹の有効性を評価した．非発情期には膣粘液塗抹中には基底細胞が最も多かったが，発情排卵期には中間細胞の個数が増加し表面細胞と無核細胞が出現した．中間細胞の増加は11月の着床時期にも観察された．血清中発情ホルモンのピークは発情期と着床期に検出された．海上で捕殺した妊娠獣の膣上皮は，発情期と着床期に肥厚する傾向を示したが，未成熟個体の膣上皮細胞は明瞭な変化を示さなかった．膣粘液塗抹は血清中ステロイドホルモンの変動に応じて緩やかな細胞学的変化を示すため，発情排卵の正確な時期を知るのには適していないが，飼育個体や野生個体の繁殖周期のモニタリングには役立つものと思われる．

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