## Materials and Methods

Both male and female Labrador Retrievers from a breeding colony for guide dogs for the blind were used in the present study. The animals were basically maintained by volunteers in their homes and were moved to the Hokkaido Guide Dog Association when the bitches exhibited signs of estrus. The embryo donors were 13 nulliparous animals that were 9-18 months of age and one multiparous animal that was 8 years of age (Table 1). The plasma progesterone concentrations of the bitches were measured daily by enzyme-linked fluorescent assay (SV-5010, Spotchem Vidas, Arkray, Kyoto, Japan) after the appearance of blood-tinged vaginal discharge. The day the plasma progesterone concentration exceeded 2 ng/ml was considered the day of the LH surge (day 0) [2]. A total of 14 bitches were inseminated by injecting freshly ejaculated semen into the vagina on day 4-6 after the estimated LH surge. The semen was collected from the dogs by digital manipulation. On day 9-15 after the LH surge (day 4-10 after insemination), the reproductive tracts of the bitches were excised after ovariohysterectomy at private clinics. The embryos were flushed from the oviduct, isthmus and uterus with phosphate buffered solution. The developmental stages of the collected embryos were determined under an inverted microscope. The number of ovulated oocytes was estimated by counting the number of corpora lutea present in both ovaries.

The animals used in this study were treated and cared for under the Guiding Principles for the Care and Use of Research Animals established by Obihiro University of Agriculture and Veterinary Medicine.

## Results and Discussion

To be able to utilize assisted reproductive technologies in research and clinical practice, it is essential to be able to determine the timing of ovulation. Although the onset of vulvar bleeding, vaginal smears and mating behavior have been used to assess

5 the timing of ovulation, their relative accuracy has proven to be highly variable. However, ovulation is much more closely related to the LH surge and progesterone concentration. There is little variation in the 2-day interval between the preovulatory LH surge and ovulation (36-50 h) [1]. The blood progesterone concentration at ovulation is also relatively stable and is thus extensively used to estimate ovulation time

10 [1]. Since measurement of the blood LH concentration is difficult to routinely carry out (i.e., it is time-consuming), we utilized measurement of the progesterone concentration to predict the LH surge in the present study.

Table 1 shows the developmental stages of the embryos from the Labrador Retrievers after artificial insemination with freshly collected semen. A total of 80

15 embryos were collected from 14 bitches that possessed a total of 101 corpus lutea (recovery rate: 79.2%). The recovery rate was 100% in 6 of the 14 animals (44%). The mean ovulation rate of the Labrador Retrievers used in the present study was 7.2 (n=14). The blastocyst stage of the embryos did not appear within 13 days after the LH surge. Beginning 14 days after the LH surge, blastosysts were recovered from the

20 uterus (Fig. 1). In a previous study using Beagles, blastocyst stage embryos were recovered on days 9 and 10 after the LH surge [6]. These results indicate that there is a considerable breed-related difference in the preimplantation development of embryos in canine species. In the present study, a majority of the expanded blastosysts were found to be shrunken at the time of collection or during washing of the embryos (Fig.

2-I). However, shrinkage of the blastocyst stage embryos was not observed during recovery.

Table 2 shows the localization of the embryos in the reproductive tract of the Labrador Retrievers after artificial insemination with freshly collected semen. Embryos that developed into morula migrated from the oviduct to the uterus beginning on day 11 after the LH surge. By day 13 after the LH surge, all of the embryos had moved to and were localized in the uterus. Although the embryonic stages of the collected embryos were synchronized in a majority of the bitches, the fact that the collected embryos from one particular bitch (KOH) exhibited a wide range of developmental stages, from the 4-cell stage to the morula stage, might indicate that ovulation can extend over a long period of time in some animals (Table 1). It is generally believed that ovulation from both ovaries is completed in a maximum of 24 to 36 h [1]. When freshly ejaculated semen was inseminated 4-6 days after the estimated LH surge, morula- and blastocyst-stage embryos (Fig. 2), which appear to be suitable for cryopreseravation and embryo transfer, might be collectable from the uterine horns of Labrador Retrievers on days 13 and 14, respectively (Fig. 1).

In conclusion, as shown in Fig. 1, the present study demonstrates that embryos migrate from the oviduct to the uterus beginning on day 11 after the LH surge in the Labrador Retrievers. This transport might be completed within 24 h. The embryos developed to the morula and to blastocyst stages by 11-13 and 14 days after the LH surge, respectively. Fundamental research is still required to understand the mechanisms of fertilization and early embryonic development in order to better control and mimic them in vitro in canines.

**Acknowledgement**

Figure legend

Fig. 1: Preimplantation development and localization of embryos in the Labrador
Retrievers.    The day the plasma concentration of progesterone exceeded 2 ng/ml was
considered to be the day of the LH surge (day 0).    The embryos developed to the
morula and blastocyst stages by 11-13 and 14 days after the LH surge, respectively.
The embryos began to migrate from the oviduct to the uterus on day 11 after the LH
surge.    By day 13 after the LH surge, all of the embryos had moved and were localized
in the uterus.

Fig. 2: Preimplantation stages of embryos recovered from the female reproductive tract
after artificial insemination in the Labrador Retriever.    A-B: 8-cell stage. C-D: 16-cell
stage. E-F: Morula. G: Expanded blastocyst. H: Large expanded blastocyst. I: Shrunken
blastocyst.    Bars indicate 100 um.

# References

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3) **Concannon PW, Lein DH.** Hormonal and clinical correlates of ovarian cycles, ovulation, pseudopregnancy and pregnancy in dogs. *In:* Kirk R (ed.) Curr Vet Ther Small Anim Pract, vol. 10. Philadelphia: WB Saunders Company; 1989: 1269-1282.

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6) **Tsutsui T, Hori T, Okazaki H, Tanaka A, Shiono M, Yokosuka M, Kawakami E.** Transfer of canine embryos at various developmental stages recovered by hysterectomy or surgical uterine flushing. *J Vet Med Sci* 2001; 63: 401-405.

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Table 1. Developmental stages of embryos in Labrador Retrievers after artificial insemination

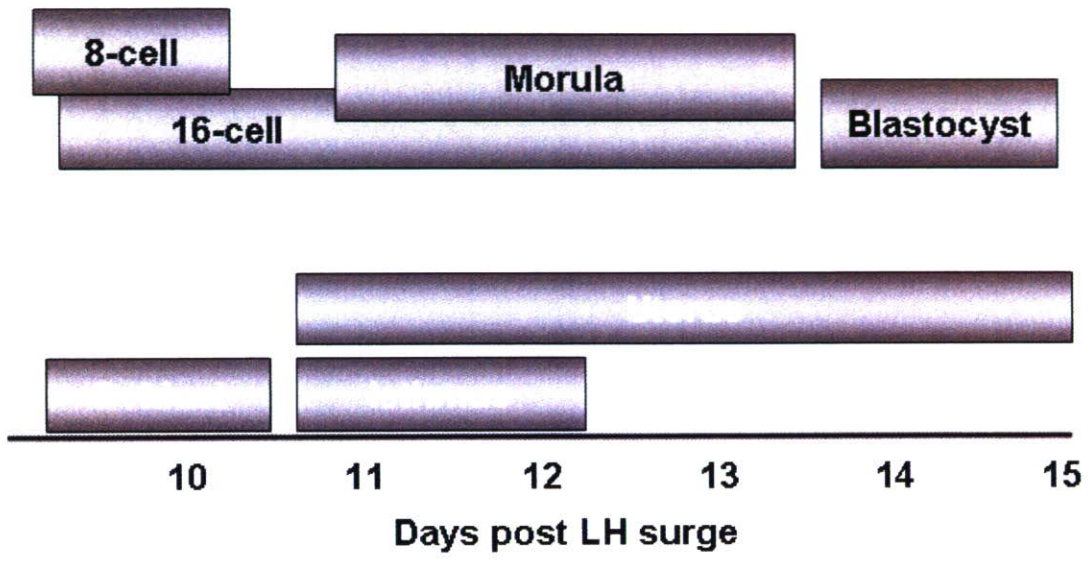| Bitch | Age (M) | Multipara or nullipara | Days post LH surge | Days at AI post LH surge | Days after AI | No. (%) embryos collected / no. CL | Developmental stage | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | UF | 4C | 8C | 16C | M | ExB | LEB | F |
| WIN | 16 | N | 9 | 5 | 4 | 5/8 (63) | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 |
| ROS | 18 | N | 10 | 6 | 4 | 7/7 (100) | 1 | 0 | 6 | 0 | 0 | 0 | 0 | 0 |
| KOK | 16 | N | 10 | 4 | 6 | 5/8 (63) | 1 | 0 | 0 | 4 | 0 | 0 | 0 | 0 |
| KOH | 17 | N | 11 | 5 | 6 | 7/7 (100) | 0 | 3 | 1 | 0 | 1 | 0 | 0 | 2 |
| HIN | 14 | N | 11 | 4 | 7 | 4/5 (80) | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 |
| HAR | 10 | N | 11 | 4 | 7 | 7/8 (88) | 0 | 0 | 0 | 6 | 1 | 0 | 0 | 0 |
| ELU | 10 | N | 12 | 6 | 6 | 9/9 (100) | 0 | 0 | 0 | 8 | 1 | 0 | 0 | 0 |
| EMI | 9 | N | 12 | 5 | 7 | 4/7 (57) | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 |
| PET | 13 | N | 13 | 4 | 9 | 4/5 (80) | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 |
| PEZ | 11 | N | 14 | 6 | 8 | 8/8 (100) | 0 | 0 | 0 | 3 | 1 | 0 | 0 | 4 |
| DYI | 10 | N | 14 | 5 | 9 | 1/7 (14) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| BET | 107 | M | 14 | 4 | 10 | 5/5 (100) | 0 | 0 | 0 | 0 | 0 | 5 | 0 | 0 |
| JIN | 15 | N | 14 | 4 | 10 | 7/7 (100) | 0 | 0 | 0 | 0 | 0 | 0 | 7 | 0 |
| BIA | 9 | N. | 15 | 5 | 10 | 7/10 (70) | 0 | 0 | 0 | 0 | 0 | 0 | 7 | 0 |
| Total | | | - | - | - | 80/101 (79) | 2 | 3 | 12 | 29 | 8 | 5 | 14 | 7 |

*Embryos were collected surgically after non-surgical flushing of the reproductive tract.

UF: Unfertilized egg. 4C: 4-cell stage. 8C: 8-cell stage. 16C: 16-cell stage. M: Morula. ExB: Expanded blastocyst. LEB: Large expanded blastocyst. F: Fragmented.

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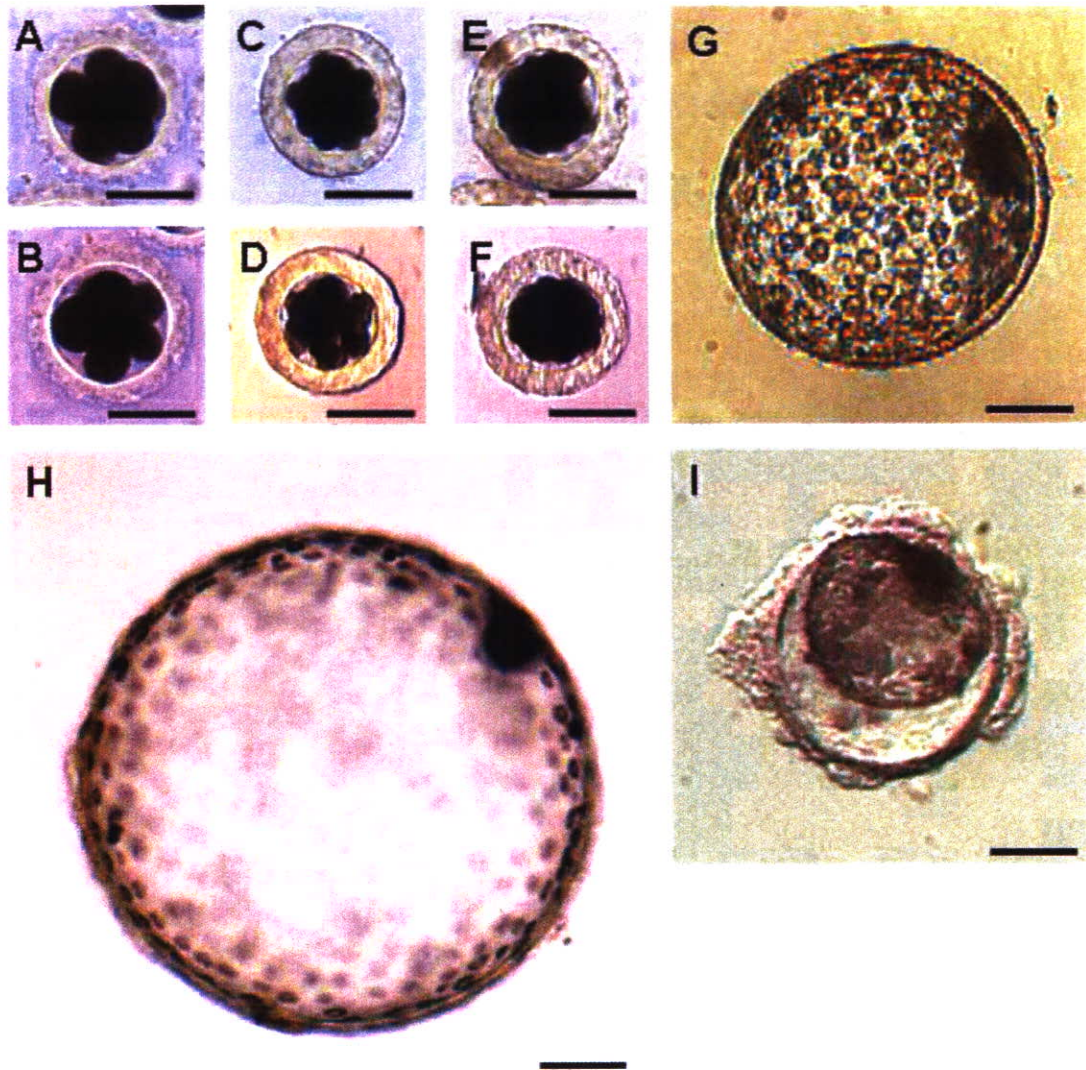Table 2. Localization of embryos in the reproductive tract of Labrador Retrievers after artificial insemination

| Bitch | Days post LH surge | Days after AI | No. embryos collected | Localization of embryo | | |
|-------|------------------|---------------|----------------------|---------|---------|--------|
| | | | | Oviduct | Isthmus | Uterus |
| WIN | 9 | 4 | 5 | 5 | 0 | 0 |
| ROS | 10 | 4 | 7 | 7 | 0 | 0 |
| KOK | 10 | 6 | 5 | 5 | 0 | 0 |
| KOH | 11 | 6 | 7 | 0 | 7 | 0 |
| HIN | 11 | 7 | 4 | 0 | 3 | 1 |
| HAR | 11 | 7 | 7 | 2 | 1 | 4 |
| ELU | 12 | 6 | 9 | 0 | 0 | 9 |
| EMI | 12 | 7 | 4` | 0 | 0 | 4 |
| PET | 13 | 9 | 4 | 0 | 0 | 4 |
| PEZ | 14 | 8 | 8 | 0 | 0 | 8 |
| DYI | 14 | 9 | 1 | 0 | 0 | 1 |
| BET | 14 | 10 | 5 | 0 | 0 | 5 |
| JIN | 14 | 10 | 7 | 0 | 0 | 7 |
| BIA | 15 | 10 | 7 | 0 | 0 | 7 |
| Total | - | - | 80 | 19 | 11 | 50 |

*Embryos were collected surgically after non-surgical flushing of the reproductive tract.

Fig. 1

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Fig. 2