FULL PAPER *Anatomy*

Immunohistochemical Localization of Steroidogenic Enzymes in the Corpus Luteum and the Placenta of the Ribbon Seal (*Phoca fasciata*) and Steller Sea Lion (*Eumetopias jubatus*)

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ABSTRACT. To study the luteal and placental function of pinnipeds, we analyzed the localization of steroidogenic enzymes (P450scc, 3 β HSD and P450arom) in the corpus luteum and the placenta of ribbon seals (*Phoca fasciata*) and Steller sea lions (*Eumetopias jubatus*) immunohistochemically. P450scc and 3 β HSD were present in all luteal cells of both species. Almost all of the luteal cells were immunostained for P450arom, while P450scc and 3 β HSD were negatively immunostained in placentae and P450arom was present in the syncytiotrophoblast of placentae. These findings suggest that 1) corpora lutea of both species synthesize pregnenolone, progesterone and estrogen during the entire pregnancy period, and 2) like other terrestrial carnivores in the suborder Caniformia, placentae of both species do not have the capability for synthesizing progesterone in the latter half of active pregnancy period.

KEY WORDS: corpus luteum, placenta, ribbon seal, Steller sea lion, steroidogenic enzyme.

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Both the ribbon seal (*Phoca fasciata*) and the Steller sea lion (*Eumetopias jubatus*) are highly adapted for an aquatic lifestyle, and they are classified in the order Carnivora [13, 20]. In the past, seals (family Phocidae), sea lions (family Otariidae) and walrus (family Odobenidae) were usually classified as a separate order, the Pinnipedia, or as a suborder of the order Carnivora. However, many morphological and molecular studies have recently revealed that affinities of the Pinnipedia lie within the suborder Caniformia of the order Carnivora, which includes Ursidae (bears) and Mustelidae (weasels, martens, etc.) [20].

From the viewpoint of reproduction, delayed implantation occurs in most pinnipeds as it does in bears and martens [1, 16, 17]. Although the total pregnancy periods of ribbon seals and Steller sea lions last almost one year, their post-implantation periods (active pregnancy periods) are shorter due to delayed implantations [14]. Timing of implantation in ribbon seals is assumed to occur in August [2], although details are unknown. The attachment of blastocyst in Steller sea lions occurs in late September and October [19]. The peak of pupping of ribbon seals and Steller sea lions occurs early in April and early in June, respectively [3, 19].

In some terrestrial carnivorous animals, the corpus luteum is the most important source of progesterone during the entire gestation period [6, 8, 18, 22, 23]. In seals, however, it is not known whether the corpus luteum can synthesize progesterone in late pregnancy, or if the placenta replaces the corpus luteum as a principal source of progesterone, as occurs in sheep and humans [18]. The aim of this study is to clarify whether the corpus luteum and the placenta of ribbon seals and Steller sea lions in the latter half of an active pregnancy period are capable of steroidogenesis.

MATERIALS AND METHODS

Ovaries and placentae were collected from 8 wild ribbon seals and 8 wild Steller sea lions. The animals were shot legally by hunters as part of nuisance control activities during winter in the Nemuro strait, Hokkaido, Japan. All animals were pregnant, and two ribbon seals had twins in their uteri. Each ovary of the mothers of twins had corpus luteum. In addition, an ovary containing corpus luteum was removed from the adult female Steller sea lion that died in captivity in July 1998. Further data on each individual are shown in Table 1. The age of each wild animal was determined by counting growth layers of dentinum and cementum annuli of the upper canine teeth [12].

Ovaries and placentae of two ribbon seals were fixed for about 12 hr in Bouin's solution, and the others were fixed and preserved in 10% formalin. After fixation, the specimens were dehydrated in an ethanol series and embedded in paraffin. Thin sections, 5 μm thick, were mounted on silan-coated glass slides (Matsunami, Tokyo, Japan). The sections were immunostained for steroidogenic enzymes by an avidin-biotin-peroxidase complex (ABC) method with a VECTASTAIN Elite ABC rabbit IgG kit (Vector Laboratories, Burlingame, CA, U.S.A.). The sections were deparaffinized with xylene and incubated with methanol that contained 0.3% H_2O_2 for 30 min to block endogenous peroxidase. The specimens were then washed in 0.01 M phosphate-buffered saline (PBS) for 5 min and treated with 1.5% normal goat serum in PBS for 40 min, before incubation

Table 1. General data of seal specimens used in this study

Species	Specimen Number	Date of death	Age (years)	Standard Length (cm)	Body Weight (kg)	Fetal Weight (kg)	Fixation	Special Remarks
Ribbon seal	K99004	Feb. 23, 1999.	N.D.[a)]	142	110	4.3	Bouin	
	K99013	Mar. 9, 1999	N.D.	135	95	5.65	Bouin	
	K98002	Feb. 19, 1998.	31	161	150	7.0, 7.4(twins)	10% formalin	
	K98003	Feb. 19, 1998.	8	140	105	6.2	10% formalin	
	K98004	Mar. 1, 1998.	13	150	100	6.5	10% formalin	
	K99020	Mar. 14, 1999.	N.D.	147	145	8.05	10% formalin	
	K98008	Mar. 19, 1998.	6	166	180	11.0	10% formalin	No placenta sample
	K98012	Mar. 29, 1998.	10	153	120	8.5, 7.5(twins)	10% fromalin	No placenta sample
Steller sea lion	99005	Jan. 23, 1999.	5	210	230	2.0	10% formalin	
	98012	Jan. 25, 1998.	13	220	230	N.D.	10% formalin	
	98013	Jan. 25, 1998.	4	214	280	N.D.	10% formalin	
	98017	Jan. 26, 1998.	11	235	335	N.D.	10% formalin	
	98019	Jan. 28, 1998.	3	202	210	N.D.	10% formalin	
	99013	Jan. 30, 1999.	8	215	245	2.55	10% formalin	
	98025	Feb. 2, 1998.	6	220	290	N.D.	10% formalin	
	98028	Feb. 3, 1998.	9	235	290	N.D.	10% formalin	
	980710AQ	July 10, 1998.	21+	N.D.	N.D.	–	10% formalin	No fetus was found, Died of Pneumonia

a) N.D.: No data

with the primary antiserum to reduce the background.

The sections were treated with the primary antiserum for 16–18 hr at 4°C. The following polyclonal antisera raised in rabbits were used: anti-cholesterol side-chain cleavage cytochrome P450 (P450scc) against rat adrenal P450scc (1:200; CHEMICON, Temecula, CA, U.S.A.) [21], anti-3 β-hydroxysteroid dehydrogenase (3 β HSD) against recombinant mouse type1 3 β HSD (1:1,000; prepared in the Royal Infirmary of Edinburgh NHS Trust) and anti-aromatase cytochrome P450 (P450arom) against human placental P450arom (1:200) [9]. After being washed in PBS for 30 min, the sections were incubated with biotinylated antibodies raised in goat against rabbit immunoglobulin (1:1,000; Vector) for 1 hr. They were then washed in PBS for 30 min followed by incubation with avidin-biotin-peroxidase complex solution (1:25; Vector) for 30 min at room temperature. After final washing in PBS for 30 min, sections were stained with 0.04% 3,3'-diaminobenzidine solution that contained 0.0004% H_2O_2. Control sections were treated with the same concentration of normal rabbit serum (Vector) instead of the primary antiserum. The sections were finally counter stained with Mayer's hematoxylin solution (Wako Pure Chemical, Osaka, Japan) and sealed under coverslips.

RESULTS

P450scc was present in all luteal cells of ribbon seals and Steller sea lions, irrespective of the fixatives or the dates of death (Fig. 1a). 3 β HSD was immunolocalized in all luteal cells of both species, similar to the way in which P450scc was localized (Fig. 1b,c), that is, the entire cytoplasm, except for the area of vacuoles, contained positively immunostained P450scc- and 3 β HSD- cells. Almost all luteal cells were immunostained for P450arom (Fig. 1d). Control sections were negatively stained except for the counterstaining of nuclei with hematoxylin (Fig. 1e). Luteal cells of the Steller sea lion that died in mid-July at an aquarium were obviously smaller and thinner than those of free-ranging sea lions that died in winter. However, immunolocalization of steroidogenic enzymes in luteal cells of this sea lion was similar to that of the other wild pregnant sea lions.

In the placentae, P450scc and 3 β HSD were negatively immunostained in both species (Fig. 2a,b). In contrast, P450arom was present in the syncytiotrophoblast of placentae of both species (Fig. 2c,d).

DISCUSSION

This study showed that the cytoplasm of all luteal cells of ribbon seals and Steller sea lions contained P450scc and 3 β HSD during the latter half of the active pregnancy period in each animal. P450scc converts cholesterol to pregnenolone in the mitochondria of luteal cells. Subsequently, 3 β HSD converts pregnenolone to progesterone. Therefore, our data suggest that the corpora lutea of both species synthesize progesterone from cholesterol using these two enzymes. In addition, corpora lutea appear to be capable of synthesizing estrogen, because the cytoplasm of almost all the luteal cells we studied contained P450arom. In the placenta, on the other hand, only P450arom was positively immunostained. Therefore, we conclude that the placentae of those two species are not capable of synthesizing progesterone in the latter half of an active pregnancy period, although they are capable of synthesizing estrogen.

Boyd [2] assumed that the placentae of grey seals (*Halichoerus grypus*) are capable of synthesizing progesterone in late pregnancy, based on the data of Hobson and Boyd [11]. However, they assayed concentrations of progesterone con-

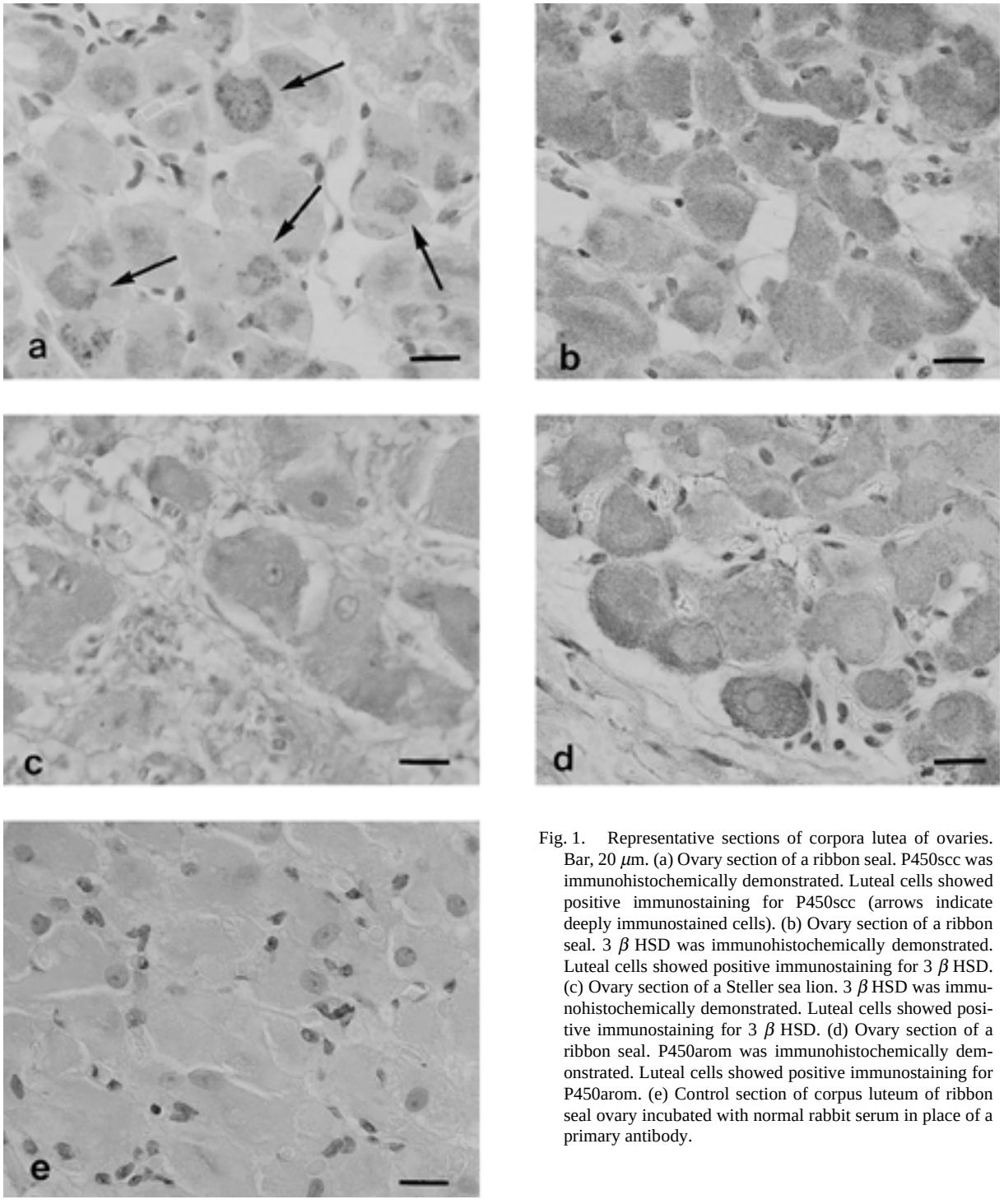

Fig. 1. Representative sections of corpora lutea of ovaries. Bar, 20 μm. (a) Ovary section of a ribbon seal. P450scc was immunohistochemically demonstrated. Luteal cells showed positive immunostaining for P450scc (arrows indicate deeply immunostained cells). (b) Ovary section of a ribbon seal. 3 β HSD was immunohistochemically demonstrated. Luteal cells showed positive immunostaining for 3 β HSD. (c) Ovary section of a Steller sea lion. 3 β HSD was immunohistochemically demonstrated. Luteal cells showed positive immunostaining for 3 β HSD. (d) Ovary section of a ribbon seal. P450arom was immunohistochemically demonstrated. Luteal cells showed positive immunostaining for P450arom. (e) Control section of corpus luteum of ribbon seal ovary incubated with normal rabbit serum in place of a primary antibody.

tained in the homogenized placentae, thus they were unable to detect the source of the progesterone with their methods. Progesterone is presumably produced not in placentae but in maternal ovaries in grey seals.

In other carnivores, such as mink (*Mustela vision*), there is a strong evidence that the placenta does not secrete progesterone, because no mRNA encoding 3 β HSD was found in the placenta [8]. Using immunohistochemical methods, Tsubota *et al*. [22, personal communications] also found that the placenta does not synthesize progesterone but that it does synthesize estrogen in Japanese black bears (*Ursus thibetanus japonicus*) and northern fur seals (*Cal-*

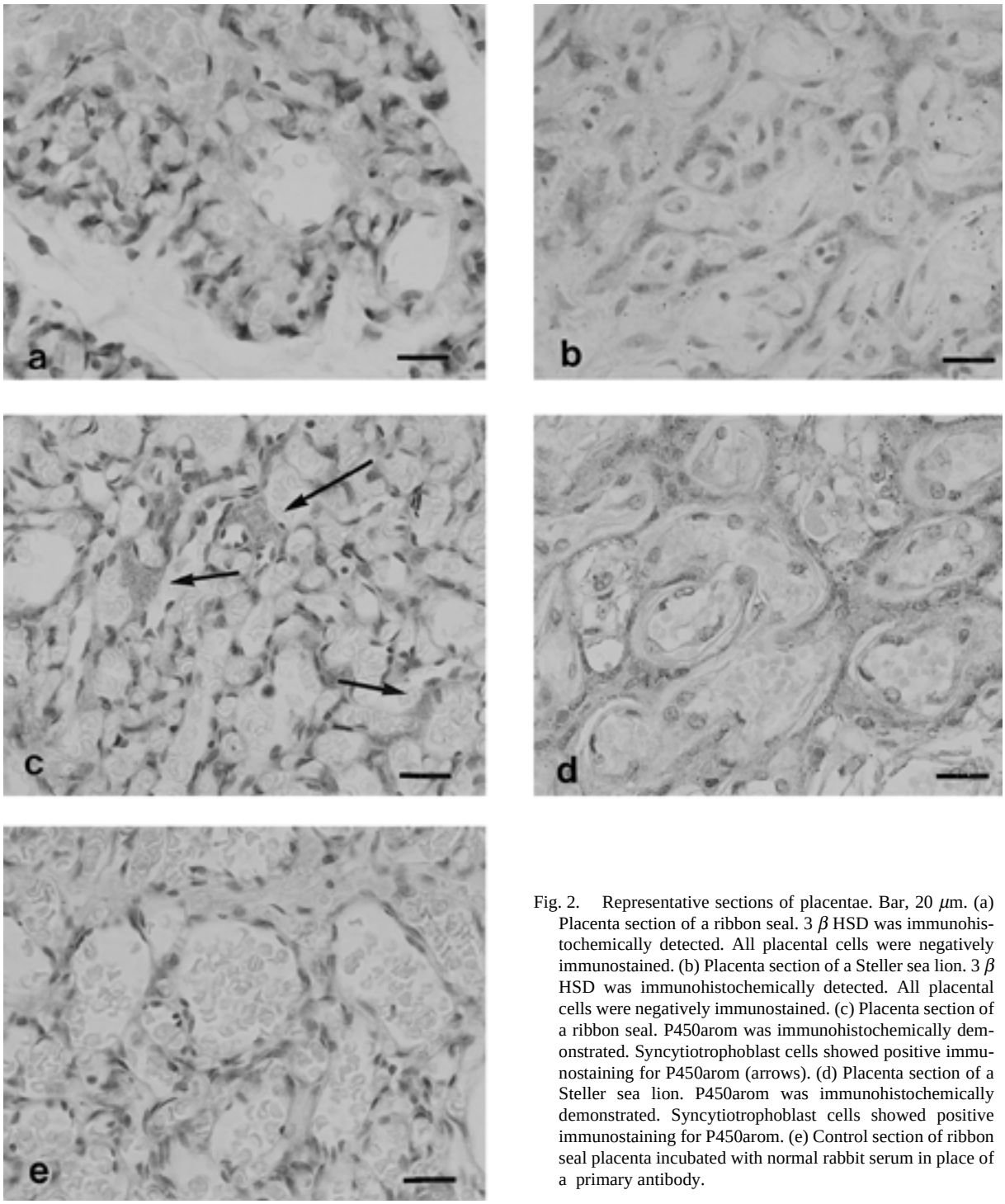

Fig. 2. Representative sections of placentae. Bar, 20 μm. (a) Placenta section of a ribbon seal. 3 β HSD was immunohistochemically detected. All placental cells were negatively immunostained. (b) Placenta section of a Steller sea lion. 3 β HSD was immunohistochemically detected. All placental cells were negatively immunostained. (c) Placenta section of a ribbon seal. P450arom was immunohistochemically demonstrated. Syncytiotrophoblast cells showed positive immunostaining for P450arom (arrows). (d) Placenta section of a Steller sea lion. P450arom was immunohistochemically demonstrated. Syncytiotrophoblast cells showed positive immunostaining for P450arom. (e) Control section of ribbon seal placenta incubated with normal rabbit serum in place of a primary antibody.

lorhinus ursinus). Our immunohistochemical study strongly suggests that the corpora lutea of ribbon seals and Steller sea lions are a principal source of progesterone during the entire pregnancy period. This endocrinological characteristic may be universal to carnivores, and supports the recent classification of the Phocidae and the Otariidae in the order Carnivora.

From the viewpoint of wildlife conservation, the declining birth rates of these pinnipeds is a serious matter. Resorption, abortion, and premature birth during pregnancies have resulted in declining birth rates [4, 7, 10, 19]. For example, in the Gulf of Alaska, nutritional stress which

were responsible for walleye pollock (*Theragra chalcogramma*) fisheries was considered to be the cause of abortion in the Steller sea lions population [15]. However, the direct physiological trigger of abortion is unknown. Although we expected that irregular replacement of the source of progesterone secretion from the corpus luteum to the placenta is one factor causing abortion, this study suggests that the placentae of seals and sea lions do not synthesize progesterone during late pregnancy. Douglas *et al.* [8] reported that prolactin and luteinizing hormone (LH) are necessary for maintenance of the corpus luteum during postimplantation gestation in mink. Normal luteal function in dogs also requires both prolactin and LH [5, 6]. Further studies are required to clarify the immediate trigger of abortion during the active pregnancy period in pinnipeds.

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