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DOES ELASTIN CONTRIBUTE TO THE PERSISTENCE OF CORPORA ALBICANTIA IN THE OVARY OF THE COMMON DOLPHIN (*DELPHINUS DELPHIS*)

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ABSTRACT

The corpora albicantia (CAs) from the ovaries of 39 common dolphins (*Delphinus delphis*) caught in driving fisheries were used, and histologically and immunohistochemically examined. The area of each corpus albicans (CA) and the proportion of that area occupied by elastin were measured using NIH-image software. In all CAs, elastoid material (EM) was apparent although EM area varied in each CA. CAs increased in number with dolphin age. Smaller CAs contained a higher proportion of EM. EM was completely digested by elastase, but not by collagenase. Furthermore, EM was immunostained with anti-α-elastin antibody. These results demonstrated that EM was elastin. The present study is the first to describe the presence of elastin

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in cetacean CAs. The higher proportion of elastin in small-sized CAs of common dolphins is suggested as the likely cause of the persistence of CA.

Key words: corpora albicantia, ovary, elastin, histochemisrty, common dolphin, *Delphinus delphis*.

During the reproductive cycle of mammals, the corpus luteum (CL), which develops from an ovulated follicle, is a transient endocrine organ that provides progesterone to maintain pregnancy. The degeneration of the CL begins during the last part of pregnancy or during lactation, but sooner if fertilization does not occur. The degenerated CL is gradually reduced to small masses of connective tissue known as a corpus albicans (CA). In most mammals, the corpora albicantia (CAs) which contain relatively few collagen fibers, blend with the general ovarian stroma and disappear completely (Mossman and Duke 1973, Boyd *et al*. 1999, Fraser *et al.* 1999). In cetaceans, however, the number of CAs has been shown to increase with age or individual's body length, while their sizes gradually diminish, suggesting that CAs of cetaceans persist throughout the animal's life (Mackintosh and Wheeler 1929, Dempsey and Wislocki 1941, Miyazaki 1977, Marsh and Kasuya 1984, Akin *et al*. 1993, Boyd *et al*. 1999, Halldórsson and Víkingsson 2001). Thus, cetacean ovaries have been used as indices of relative age, sexual maturity, reproductive status, and records of reproductive life history. However, some researchers have reported that the CAs of some cetacean species may be eventually absorbed or regress to a size undetectable on gross examination (Harrison 1949, Sergeant 1962, Harrison *et al*. 1972, Harrison *et al.* 1981, Brook 2002). Brook (2002) found only three CAs in the ovaries of a captive bottlenose dolphin, *Tursiops aduncus*, for which three pregnancies and 18 ovulations had been recorded over 12 yr using ultrasound imaging and assessment of the serum progesterone level. The author has postulated that CA of pregnancy persists but CA of ovulation do not occur or do not remain in the ovaries, implying that ovulation rates cannot be determined by counting persisting ovarian corpora in this species.

In the ovaries of cattle and Japanese serow (*Capricornis crispus*), CAs of pregnancy probably persist throughout life, while CAs of infertile ovulations disappear at around 14 mo in cattle (Miyagi 1966) and around 5 mo in Japanese serow (Kita *et al*. 1983, Sugimura *et al*. 1984). Since the CA of pregnancy always contains more elastin fibers than that of ovulation (Miyagi 1966, Kita *et al*. 1983, Sugimura *et al*. 1984), it has been speculated that the accumulation of elastin in the CAs contributes to their persistence. To our knowledge, the presence of elastin in the CAs of cetaceans' ovaries has not yet been investigated. The purpose of the present study is to (1) determine if elastin is found in the ovaries of common dolphins (*Delphinus delphis*) and (2) relate its presence to the persistence of CA in the ovaries.

Materials and Methods

Animals and Tissue Preparations

Pairs of ovaries were collected from 39 common dolphins (*D. delphis*), which had been caught in the driving fishery off the coast of Wakayama Prefecture, Japan, in January 1982. The 78 ovaries were fixed in a 10% formalin solution. CLs and CAs were cut out transversally from the center of each ovulation scar found at the surface

of the ovary. After dissecting all CAs, the ovaries were sliced at 1.5 mm to check for corpora that were not detected by surface examination. A total of 267 ovarian corpora were obtained from 36 animals. The three remaining individuals were young dolphins without a single corpus. Twenty-one of the corpora were CLs of pregnancy, and four were very early regressing CLs or developing CLs. These 25 CLs were only used to study the relationship between the animal age and the number of CLs and CAs. Twenty-one luteinized unruptured follicles, so-called yellow body (corpora atretica *b*; Best 1967, Marsh and Kasuya 1984, Perrin and Donovan 1984) were excluded from the examination.

To age specimens, three teeth were collected from the middle of the lower mandible of each dolphin and fixed in 10% formalin solution.

Histological Observations

Four micrometer thick sections were cut from paraffin-embedded CA tissues. They were stained with hematoxylin–eosin and elastica van Gieson (sirius red was used instead of acid fuchsin), in which sirius red stained collagen to red and Weigert' resorchin-fuchsin stained elastin to dark purple. For the enzyme digestion experiment, four sections were cut at 3 μm and mounted on MAS coated slides (Matsunami, Japan). One section was incubated for 1 h at 37°C in 0.1 M boric acid–NaCl–borate buffer (pH 8.9) containing 40 units mL^{-1} elastase (EPC, USA). Another section was treated with 5 N KOH for 1 min at 60°C and then incubated in phosphate buffered saline (PBS) containing 2,000 units mL^{-1} collagenase (EPC) for 3 h at 37°C. Two remaining sections were used as controls, in which one was incubated in 0.1 M boric acid–NaCl–borate buffer without elastase for 1 h at 37°C, and the other was treated with 5 N KOH for 1 min at 60°C and incubated in PBS without collagenase for 3 h at 37°C. Following the incubation, all sections were stained with elastica van Gieson.

Immunohistochemical Observations

Sections were cut at 3 μm and mounted on poly-L-lysine coated slides. Immunostaining of α-elastin was performed by means of the avidin-biotinylated-enzyme complex (ABC) method (Hsu *et al*. 1981) using a VECTASTAIN kit (Vector Laboratories, U.S.A.). Wet heat-induced antigen retrieval of elastin was performed in an autoclave for 10 min at 121°C using DAKO retrieval solution (DAKO, Denmark). Afterward, the sections were cooled at room temperature, washed in PBS and treated with 3% hydrogen peroxide (H_2O_2) in methanol for 5 min to block the activity of endogenous peroxidase. After the treatment with 1.5% normal goat serum in PBS for 20 min, the sections were incubated with rabbit polyclonal antibody against bovine α-elastin (EPC) diluted 1:6,000 for 14 h at 4°C. After washing in PBS, the sections were incubated with biotinylated secondary antibody (goat anti-rabbit IgG) diluted 1:200 for 40 min at room temperature. The sections were washed again and incubated with the ABC-PO kit (Vector Laboratories) for 1 h. The reaction products were finally visualized by an addition of 3,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Japan) and counterstained with hematoxylin. The specificity for elastin was ascertained by a total loss of the staining when primary antibody was omitted.

Age Determination

Teeth sections were made according to Kasuya (1976). Ages were estimated by counting growth layer groups (GLGs; Gurevich *et al*. 1980, Perrin and Myrick 1980, Hohn and Fernandz 1999) under microscope (Olympus AX70, Olympus, Co., Japan). The age of three animals could not be estimated because the teeth were damaged during the preparation. They were only used for the analysis of the relationship between proportion of elastin and area of CA.

Areas Measurements of CAs and EM

Areas of CA and EM were measured by an image analysis method (NIH Image software) on digitized images from sections stained with elastica van Gieson. All images were obtained using a digital microscope camera (Cool SNAP color, resolution: 1.45 megapixels, Photometrics, Roper Scientific, U.S.A.) mounted on a stereoscopic microscope (Leica MZ12, Leica, Germany). The area of CA was estimated by a manually traced outline of CA and the area of EM within the CA was measured as the dark purple area stained with elastica van Gieson.

Statistics Used

Simple regression was used to highlight trends between number of CAs and animal age. Statistical tests were performed using Statview (version 4.57, Abacus Concepts Inc. 1996) following Sokal and Rohlf (1969). Differences in EM proportion between CA sizes were assessed by a non-parametric Steel-Dwass test following the Kruskal-Wallis test. $P < 0.05$ was considered statistically significant.

RESULTS

Number of Corpora and Age

The age of the common dolphins ranged from 4 to 27 yr old. The total number of CLs and CAs in both ovaries per animal ranged from 0 to 13. Three young dolphins (4–6 yr old) did not have a single corpus. The number of corpora (CLs plus CAs) in the ovaries was significantly and linearly related to the age of the dolphins ($y = 0.48\,x - 2.00$, $r^2 = 0.53$, Fig. 1).

Histological Changes of CA

The size of CAs ranged from 1.8 to 95.8 mm^2. The CAs were relatively acellular, and consisted of collagen fibers and elastin-like material (EM) estimated by elastica van Gieson staining. The EM was observed in all CAs, and was found near the central part of each CA and at the walls of blood vessels and the perivascular connective tissue. The proportion of EM in CAs varied depending on the sizes of CAs. Overall, the proportion of EM tended to increase with decreasing CA sizes (Fig. 2).

Large-sized CAs ($\geq$50 mm^2) were highly collagenous (Fig. 3a) and contained many small arteries, but few capillaries (Fig. 3b). EM appeared sparsely at the septum and the perivascular connective tissue, and was distinguished from collagen fibers (Fig. 3b). EM regions contained more cells than other collagenous regions, and accounted for less than 14% of the CA area (Fig. 2).

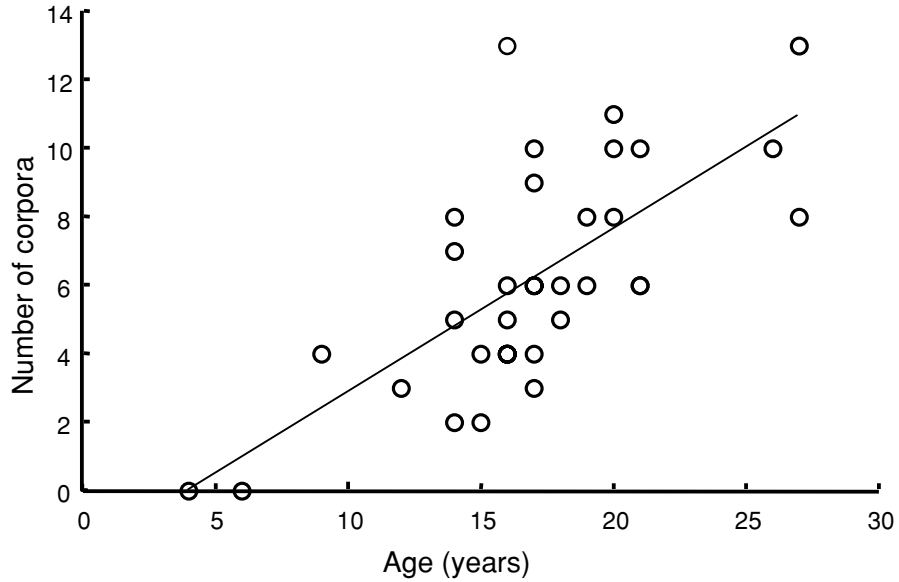

Figure 1. Relationship between number of corpora (CAs plus CLs) and age of common dolphins.

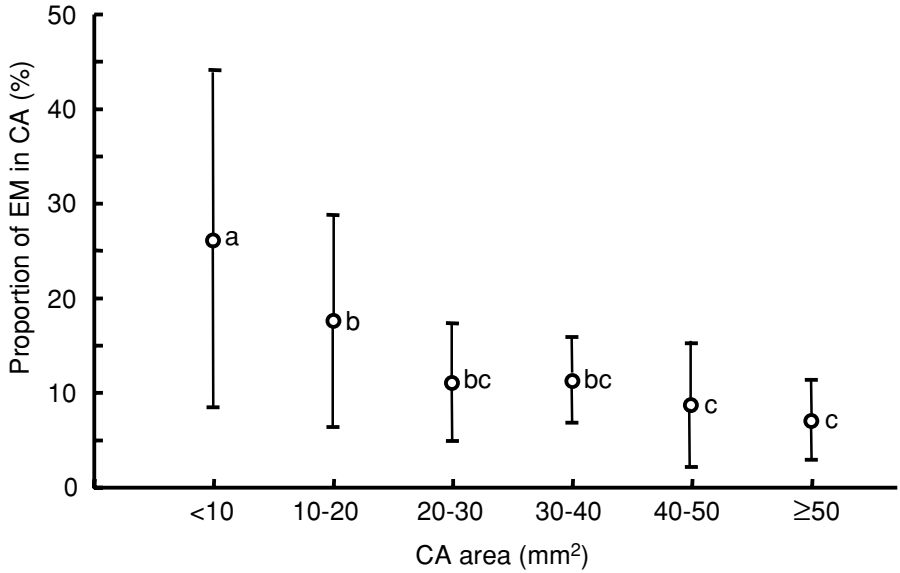

Figure 2. Relationship between EM proportion in CA and size of CA. Bars represent mean ± SD ($n = 78$ in <10, $n = 79$ in 10–20, $n = 32$ in 20–30, $n = 10$ in 30–40, $n = 10$ in 40–50, $n = 12$ in ≥50). Values with different letters (a, b, c) are significantly different ($P < 0.05$).

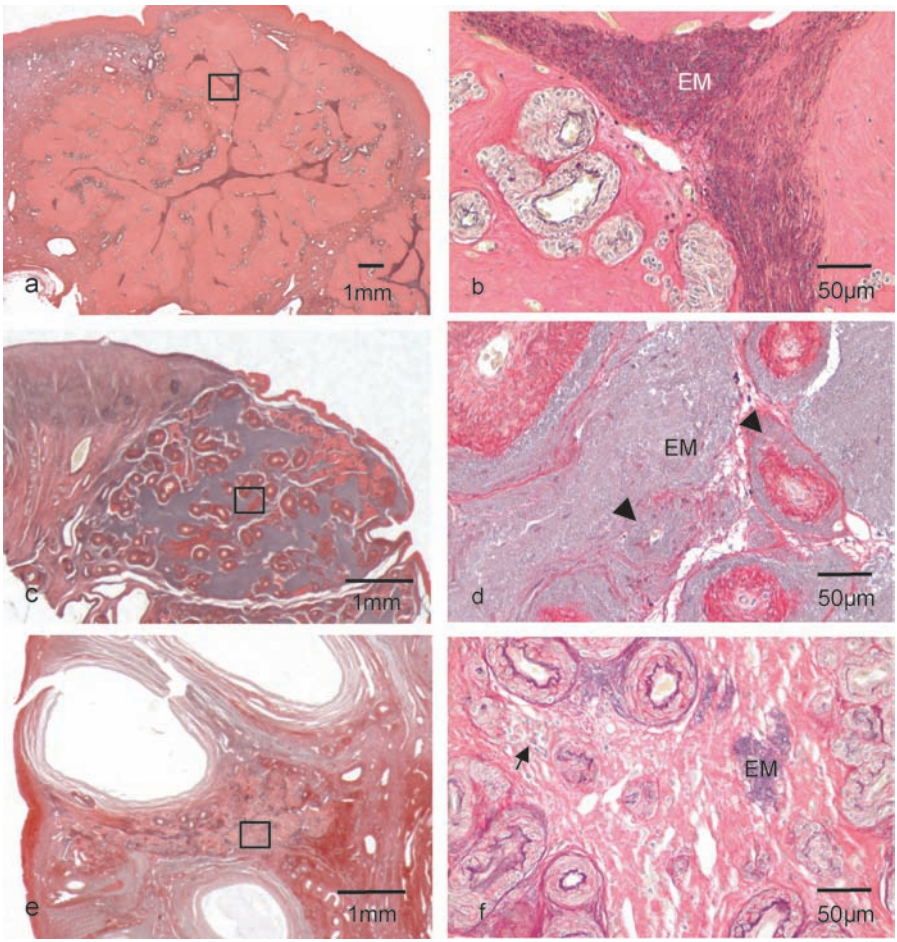

Figure 3. Elastica van Gieson staining of CAs. Collagen and EM are stained red and dark purple, respectively. The area enclosed by rectangles in a, c, and e is magnified in b, d, and f, respectively. In a large-sized CA (78.39 mm^2; a, b), EM distributes sparsely on the septa. In a small-sized CA (9.27 mm^2) of high proportion of EM (c, d), EM and hypertrophied blood vessels are predominant, and the walls of blood vessels are covered with EM (d: arrow head). In a small-sized CA (3.83 mm^2) of low proportion of EM (e, f), loose connective tissue contains degenerating luteal cells. Arrow (f) indicates cluster of degenerating luteal cells.

In middle-sized CAs (20–40 mm^2), EM extended to the surrounding region and accounted for up to 35% of the CA area, but most of CAs were relatively collagenous. EM region was acellular connective tissue and mainly consisted of loosely structured EM (loose EM), in which a few collagen fiber and cells were contained. The walls of some blood vessels thickened and EM was relatively abundant in the perivascular connective tissue.

In the small CAs (<10 mm^2), the proportion of EM varied from 2.7% to 87.0% of the CA area. The mean proportion of EM in CAs under 10 mm^2 was significantly

larger than that of large and middle-sized CAs (Fig. 2). Over one-third of small CAs contained greater than 30% EM. The CAs consisted of loose EM and blood vessels with a small amount of collagenous tissue distributed around EM. In the EM region, collagen fibers and cells were rarely detected (Fig. 3c, d). The walls of the blood vessels were hypertrophied. EM prominently accumulated and replaced the collagen fibers in the walls of the blood vessels (Fig. 3d). Collagenous tissue around EM region was sparse and appeared as a "meshwork" that contained leukocyte-like cells. Under one-third of small-sized CAs contained less than 15% EM. About 70% of them mainly consisted of loose collagenous tissue and contained degenerating luteal cells and/or their clusters, and relatively many leukocyte-like cells (Fig. 3e, f). The walls of the blood vessels were slightly hypertrophied and EM was sporadically distributed around them (Fig. 3f).

Identification of EM as Elastin

Elastin digestion techniques demonstrated that EM was completely removed by elastase treatment, whereas collagenous tissue was unaffected (Fig. 4a, b). In contrast, collagenase treatment led to a loss of collagenous tissues, but EM was unaffected (Fig. 4c, d). Futhermore, the immunostaining with bovine α-elastin antibody positively reacted in the area where EM was observed (Fig. 5a, b). These findings confirmed the identity of EM as elastin.

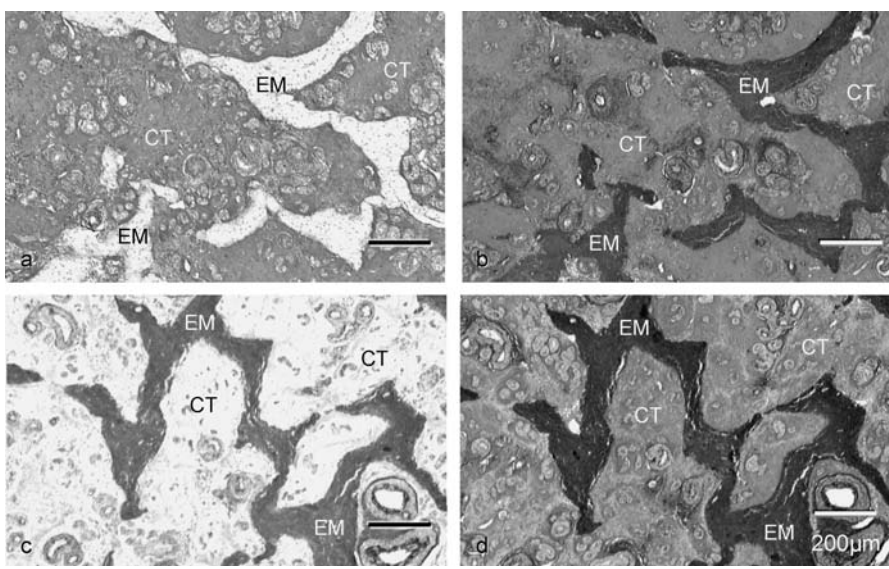

Figure 4. Elastase digestion of EM. EM is not detected by elastase digestion (a) and remained by collagenase digestion (c). Sections were stained with elastica van Gieson after enzyme digestion. Sections a and b, and c and d are serial sections. a: elastase treatment, b: control, c: collagenase treatment, d: control. CT: collagenous tissue.

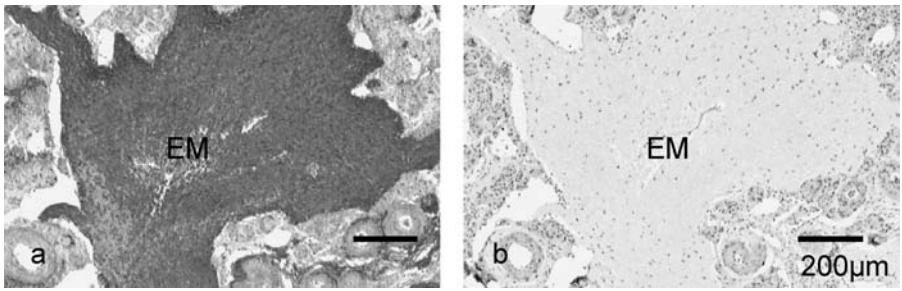

Figure 5. Immunstaining of α-elastin. Positive reaction for bovine α-elastin is detected in EM area (a) but not in control section without the primary antibody (b).

Discussion

The EM in CAs under 10 mm^2 occupied a significantly larger proportion than that of large and middle-sized CAs, and was identified as elastin by elastase digestion. These results for common dolphin are, to our knowledge, the first confirmation of the presence of elastin in the CAs of ovaries of a cetacean species. Previous studies examining the histological composition of cetacean ovaries have reported that CAs consist of acellular connective tissue (*e.g.,* Laws 1961, Best 1967, Fisher and Harrison 1970, Hirose *et al*. 1970, Harrison and McBreaty 1977, Marsh and Kasuya 1984), which was largely composed of unpigmented collagen (Laws 1961), or acellular fibrioid material that contains patches of PAS reaction-positive material (Fisher and Harrison 1970), or collagenous fibers and amorphous material containing granules (Harrison *et al*. 1972). However, these authors did not mention the presence of elastin in the CAs.

Histological characteristics of CAs in odontocetes have been described in detail by Harrison and his coworkers (Harrison *et al*. 1969, Fisher and Harrison 1970, Harrison *et al*. 1972, Harrison and McBrearty 1977, Collet and Harrison 1981, Harrison *et al*. 1981). In their descriptions, the fully developed CL of pregnancy is the source of a persistent, firmly formed, fibrotic and hyalinized CA. Infertile ovulations do not develop into well-organized corpora, in which the CAs are eventually absorbed (Fisher and Harrison 1970, Harrison *et al*. 1972). Harrison and colleagues have further classified the CAs of Pacific white-sided dolphins (*Lagenorhychus obliquidens*), harbor porpoises (*Phocoena phocoena*), bottlenose dolphins (*Tursiops truncatus*), and common dolphins into two types (Harrison *et al*. 1969, Fisher and Harrison 1970, Harrison *et al*. 1972, Collet and Harrison 1981). In common dolphins, one type of CAs is largely formed by acellular and hyaline materials arranged in lobules. The other type is little more than coils of blood vessels and sparse hyaline material. The former derives from CLs of pregnancy, while the latter derives from CLs of infertile ovulation or of a luteinized follicle, and subsequently disappears. In these studies, Collet and Harrison (1981) have attributed the persistence of CA to the presence of the hyaline material. Hyalinization generally proceeds from the degeneration of collagen tissue (Jaworski and Arvanitis 1999). Similarly, in the present study, hyalinization of the small CAs was observed in the collagenous tissue but did not occur in the elastin tissues. These observations suggest that the hyaline material is degenerated collagen tissue. Because the proportion of collagen tissue decreased, while the proportion of elastin increased with decreasing CA size, the shrinking of the CAs may

result from the progressive loss of collagen. The likely disappearance of collagen observed in the present study contradicts with the results of Collet and Harrison (1981).

The present study showed that the hypertrophied arteries existed in small CAs where connective tissue was replaced with elastin and some blood vessels were almost completely covered with elastin. Marsh and Kasuya (1984) have reported that CAs of short-finned pilot whales (*Globicephala macrorhynchus*) are formed predominantly by coiled blood vessels and that they are persistent, because the connective tissue elements in the walls of the arteries of CA become particularly resistant to degradation. In CAs, the elastin in the walls of blood vessels is prominent in bottlenose dolphins (Harrison *et al*. 1972), pigs (Yamashita 1960), cows (Miyagi 1966), and Japanese serows (Sugimura *et al*. 1984). Elastic fibers increase in the tunica media of arterioles and especially veins around the fibrous capsule of CAs as CA decrease in size in Japanese serows (Sugimura *et al*. 1984). Furthermore, Bauer *et al*. (2003) have reported a formation of elastin fibers in arterial vessels of regressing bovine CLs. These results lead us to consider that the increased elastin in the walls of blood vessels during the CL regression phase gradually covers the totality of the blood vessels during the shrinkage of persistent CA.

In the present study, the possibility that CAs persist for a long time was indicated by the relationship between the total number of CLs and CAs, and the age of common dolphins. The proportion of elastin area in CAs showed a tendency to increase with decreasing size of CAs with age. This shows that collagen disappears and elastin remains in the CA. In CAs smaller than 10 mm^2, over one-third of CAs contained EM proportions over 30% in the cross-sectional area. This indicates that elastin accumulation in the CAs of common dolphins seems to cause their persistence for long periods of time. Brook (2002) has revealed that the number of CAs corresponds with the times of pregnancies in the ovaries of a captive bottlenose dolphin, *Tursiops aduncus*, indicating that CA of pregnancy persists for life. From these findings, it is strongly suggested that small CAs (under 10 mm^2) with a high percentage of elastin may persist for long periods of time as a record of pregnancy.

On the other hand, under one-third of small CAs (under 10 mm^2) contained a low proportion of elastin under 15% and also a substantial amount of collagen. Most of these CAs contained degenerating luteal cells, so that they were considered young corpora derived from luteinized unruptured follicles. On CAs of infertile ovulation, Brook (2002) has reported that they do not occur or not remain in the ovaries. CLs without pregnancy are recognized in only 5.4% of females with CL in *Stenella attenuata* (Perrin *et at*. 1976) and in only 2.2% of mature females of *S. longirostris* (Perrin *et al*. 1977). These findings suggest that CAs from infertile ovulations are rare in wild dolphins. Therefore, the remnants of small CA containing elastin under 15% in common dolphin may derive from infertile ovulation CLs.

Elastin has a long half-life of 40–70 yr (Prediman 1997) and is relative resistant to proteolysis by all but a limited number of proteinases (Mecham *et al*. 1997). The persistent CAs of pregnancy in cattle and Japanese serow contain elastin (Miyagi 1966, Sugimura *et al*. 1984). Iwasa and Atkinson (1996) have proposed that CAs of ovaries in Hawaiian monk seals (*Monachus schauinslandi*) are not persistent because the collagenous fibrous tissues in regressing CLs are not replaced by elastic fibrous tissues which last longer than collagenous fibrous tissues. Taken together, these findings strongly suggest that the persistence of CAs depends on their elastin content.

We suggest that the presence of elastin in the CAs of common dolphins is closely associated with the persistence of the CAs for long periods of time. The counting of

persistent CAs has become a highly useful tool to estimate the lifetime reproductive history. Therefore, further investigations are needed to confirm whether CAs containing a higher EM proportion persist throughout the life of common dolphins.

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