Live-bearing without placenta: Physical estimation indicates the high oxygen-supplying ability of white shark uterus to the embryo

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One of the mysteries of shark aplanetal viviparity is the ability of the embryos to acquire oxygen from their mothers without a placental connection. It has been assumed that embryonic respiration in aplanetal viviparous shark depends on oxygen from the uterus wall, although this hypothesis has not been confirmed quantitatively. Morphological observations of the uterus wall of white shark (Carcharodon carcharias) provided the first quantitative evidence to support the ability of the uterus to supply ample oxygen to the embryos of viviparous elasmobranchs. The uterus surface of the white shark is characterized by (1) uterine lamellae that develop perpendicular to the uterus wall, (2) uterine lamellae folded in an accordion-like fashion, and (3) numerous micro-ridges on the lamellar surface. These modifications result in increased uterus surface area to up to 56 folds compared to the uterus with a smooth surface. Histological observations revealed that the diffusion barrier of the uterus wall is approximately 12 µm. By using these values, the oxygen-diffusion capacity of 1 cm² of the uterus wall of white shark was estimated to be 63.6 nmol·min⁻¹·torr⁻¹. This value is 250–400 times greater than that observed in other aplanetal viviparous sharks (Squalus spp.) and is comparable with that of fish gills.

Oxygen acquisition is essential for all living vertebrates, including the embryos developing in the maternal body. Therefore, viviparous (live-bearing) vertebrates have acquired various methods to supply oxygen to the embryo in utero. It has been assumed that some carcharhiniform sharks and mammals present convergent evolution of embryonic respiratory mechanism: The embryos/fetuses are connected to the uterus by a placenta and they acquire oxygen from the uterus through this connection1. However, the development of the placenta is restricted to few clades of vertebrates, and the mechanism of oxygen delivery to embryo in aplanetal vertebrates, including most viviparous elasmobranchs (sharks and batoids), is still largely unknown2.

It is widely believed that embryonic respiration in aplanetal elasmobranchs depends on the oxygen diffused from the uterus wall3–5. This hypothesis is supported by the presence of numerous surface projections (e.g., trophonemata and uterine villi) on the inner wall of the uterus, which increase the surface area for gas exchange. Recently, one of the authors (T.T.) and his colleagues examined this hypothesis through quantification of the oxygen-supplying ability of viviparous dogfishes (Squalus spp.)6. They used a physical model to estimate the rate of oxygen diffusion through the uterine villi and concluded that the oxygen supply from uterine villi cannot support the embryonic oxygen demand. Previous studies have showed that dogfish periodically exchange uterine fluid with external seawater through the cervix in late gestation5–7. Thus, it was suspected that the seawater introduced from the external environment is the main source of embryonic oxygen6. However, such studies have thus far been restricted to dogfish, and the large diversity in the uterus surface structure among elasmobranchs prevents direct generalization with other taxa.

The present study evaluates the oxygen-supplying ability of the white shark (Carcharodon carcharias), the largest piscivorous shark. To the best of our knowledge, this is the second aplanetal viviparous elasmbranch for which the oxygen-supplying ability has been estimated until date. This species produces 2 to 10 embryos through

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single gestation. The embryo grows to a total length of 1.6 m in utero by consuming lipid-rich uterine “milk” and unfertilized eggs. Lamniform sharks, including the white shark, are known to have numerous lamellae covering the entire inner surface of the uterus. This structure is hypothesised to ensure oxygenation of the uterine fluid, although this has never been tested quantitatively. Here, we describe the detailed morphology of the uterine surface structure of white shark for the first time and evaluate its ability to supply oxygen to the embryo.

Results

Structure of uterine surface. Numerous uterine lamellae were observed on the entire surface of the uterine wall (Fig. 1). Each lamella was tightly folded in an accordion-like fashion (Fig. 2). Numerous micro-ridges ran parallel toward the free margin of the lamellae on both sides of the uterine lamellae (Fig. 3). Each micro-ridge
The mean face area of the white shark uterus is about 12 to 20 times greater than that of dogfish for the same tissue size. It has been suspected that the dogfish embryo uses uterine seawater from external environment as a major source of oxygen. However, this mechanism is unlikely to occur in white shark, because its cervix is quite narrow compared to the uterine size (maximum diameter of cervix was ca. 1.5 cm) and is tightly closed. This condition is also seen in another gravid white shark specimen (OCF-P 03018) that was incidentally caught by local fishermen in Okinawa Prefecture, Japan, in 2016. The uterine fluid (>130 L) of this specimen was retained in an intermediate, and posterior portion of the uterus (ANOVA, p > 0.05).

**Discussion**

The present study provides the first quantitative evidence to suggest the high oxygen-supplying ability of lamniform shark uterus. We found three morphological features, which greatly increase the surface area of the white shark uterus: (1) uterine lamellae developed perpendicular to the uterine wall, (2) uterine lamellae folded in an accordion-like fashion, and (3) numerous micro-ridges developed on the lamellar surface. These features result in the increase in uterine surface to 56 times compared to the uterus that has a smooth surface. Similar uterine accordion-like fashion, and (3) numerous micro-ridges developed on the lamellar surface. These features result in the increase in uterine surface to 56 times compared to the uterus that has a smooth surface. Similar uterine

![Figure 3](image-url) Scanning electron microscopy (SEM) images of the uterine wall. (a) Entire view of the uterine lamellae. (b) Close-up view of the white box in (a) showing the micro-ridges developed on the lamellar surface. (c) Cross section of the uterine lamellae showing a single blood vessel (bv) in each micro-ridge (mr).

**Oxygen-diffusing capacity of uterine lamellae.** Morphometric measurements for calculating the surface area and oxygen-diffusing capacity are listed in Table 1. The surface area of the whole uterus was estimated to be 21.6 ± 6.6 (SD) m²; the oxygen-diffusing capacity of the whole uterus was estimated to be 24.6 ± 8.8 (SD) × 10⁶ nmol·min⁻¹·torr⁻¹; and the oxygen-diffusing capacity of 1 cm² of the uterine wall was estimated to be 63.6 ± 22.7 (SD) nmol·min⁻¹·torr⁻¹. There was no significant difference in the oxygen-diffusing capacity among anterior, intermediate, and posterior portion of the uterus (ANOVA, p > 0.05).

| Specimen | Anterior | Intermediate | Posterior |
|----------|----------|--------------|-----------|
| OCF-P 03018 | 24.6 ± 8.8 (SD) | 25.2 ± 7.5 (SD) | 24.0 ± 6.3 (SD) |
| OCF-P 03019 | 23.9 ± 9.1 (SD) | 24.3 ± 8.7 (SD) | 23.8 ± 7.5 (SD) |

There was no significant difference in the oxygen-diffusing capacity among anterior, intermediate, and posterior portions of the uterus (ANOVA, p > 0.05).

The oxygen-diffusing capacity of white shark uterus is greater than that of viviparous dogfish. The surface area of the white shark uterus is about 12 to 20 times greater than that of dogfish for the same tissue size (mean ± SD = 1.97 ± 0.19 and 1.25 ± 0.42 cm² per 1-cm-squared uterine tissue in Squalus cf. mitsukuri and S. cubensis, respectively). Moreover, the gas-diffusing barrier of the uterine wall of white shark is about one-tenth of that of dogfish (mean ± SD = 0.11 ± 0.02 and 0.11 ± 0.01 mm in S.cf. mitsukuri and S. cubensis, respectively).

Using these values, the oxygen-diffusing capacity of white shark is estimated to be about 250–400 times greater than that of dogfish for the same tissue size. Narrow and tightly closed cervix was also reported in lamnoid Cetorhinus maximum (Matthews, 1949). In addition, little or no water input from the external environment was supported by the chemical composition of uterine fluid of the white shark (OCF-P...
**Figure 4.** Histological thin section of the uterine lamellae. (a) Cross section of the uterine lamellae cut through the mid-half of lamellar height. (b) Close-up view of the uterine lamellae. (c) Close-up view of a part of a single lamella showing a blood vessel (bv) running through each micro-ridge (mr). (d) PAS-stained uterine surface. (e) Schematic representation of the cross section of uterine lamellae showing the locations of morphometric measurements. Abbreviations: bm, basement membrane; vd, blood vessel diameter; rc, red blood cell; CD, curved distance along the lamellar surface between two points (p1 and p2; linear distance between p1 and p2 = 200 µm); X, thickness of the diffusion barrier. Scale bars = 1 mm in (a) and 100 µm in (b), (c) and (d).

| Sampling locations | Projected area of lamellae S (cm²) | Surface increase because of the presence of microridges α | Thickness of the diffusion barrier X (µm) | Oxygen-diffusion capacity D (nmol × min⁻¹ × torr⁻¹)²² |
|--------------------|---------------------------------|---------------------------------------------------------|-----------------------------------------|-----------------------------------------------|
| Anterior (s1)      | 16.1 (±0.4)                     | 1.96 (±0.50)                                           | 11.9 (±1.9)                             | 69.5 (±21.0)                                  |
| Intermediate (s2)  | 12.1 (±2.5)                     | 2.17 (±0.38)                                           | 11.8 (±2.0)                             | 58.3 (±18.6)                                  |
| Posterior (s3)     | 11.1 (±1.8)                     | 2.25 (±0.49)                                           | 10.8 (±2.3)                             | 60.6 (±20.9)                                  |
| Total              | 13.1 (±2.8)                     | 2.13 (±0.47)                                           | 11.5 (±2.1)                             | 63.6 (±22.7)                                  |

Table 1. Morphometric measurements (±SD) of the uterine lamellae, and estimated oxygen-diffusion capacity of white shark uterus. ¹¹Values for 1 cm² of the uterine wall ²²D = K × 2 × α × S/X, K = 1.31 × 10⁻³ nmol × cm⁻¹ × min⁻¹ × torr⁻¹ (see text for details).
By using DRI-CHEM 7000 V (Fujifilm Co., Tokyo, Japan), Na⁺, K⁺, and Cl⁻ concentration of the uterine fluid was measured to be 402, 4.5, and 366 mEq/L, respectively, and all of these values were significantly lower than those of seawater. From these observations, we can hypothesize that the amount of water input through cervix, if any, is quite limited in white shark, and the embryonic oxygen is mainly supplied from the uterine wall.

The present study also revealed that the oxygen-diffusing capacity of white shark is comparable to that of fish gills. Oxygen-diffusing capacities of the gills vary among species reflecting their locomotor lifestyles: Demersal fishes generally have lower oxygen-diffusing capacity compared to fast-swimming pelagic fishes. Based on our calculations, the oxygen-diffusing capacity of 1 cm² gill tissue of benthic Scyliorhinus stellaris was 6.7 nmol-min⁻¹-torr⁻¹, whereas it was 82.3 nmol-min⁻¹-torr⁻¹ in pelagic Isurus oxyrinchus. This indicates that the oxygen-diffusing capacity of white shark uterus (63.6 nmol-min⁻¹-torr⁻¹) is nearly comparable to the value of the gills of I. oxyrinchus.

The uterine wall of white shark probably exchanges many kinds of molecules and materials with the uterine fluid but their contents and proportions may be drastically changed during gestation. Previous study showed that the uterine wall in early gestation includes many secretory cells that produce lipid-rich "milk". Our study showed that such milk secretion probably ceased in late gestation, because PAS-staining reaction to the uterine surface in late gestation is much weaker than that in early gestation (Fig. 4 in ref.15). On the other hand, micro-ridges of the uterine lamellae become more prominent, and the epithelium of uterine lamella is changed from double- to single-cell layered from early to late gestation (Fig. 4 in ref.11). These modifications result in the increase in surface area and decrease in the thickness of the diffusion barrier, both leading to increase in oxygen-diffusion capacity. Based on these observations, it is likely that major functions of the uterine wall involve both milk secretion and respiration in early gestation but are more specialized to respiration in late gestation. This phenomenon may be reflected by the abrupt increase in oxygen demand in large developing embryos, which was observed in oviparous species21-23.

**Materials and Methods**

**White shark uterus.** The uterine specimen was obtained from a single female white shark OCF-P 03062 (5.05 m in total length, TL), which was the bycatch of the set net in 2016 by local fishermen in Okinawa Prefecture, Japan (Fig. 1). The left uterus of the specimen included four late-term embryos (c.a. 100 cm TL), and the right uterus was empty. The description of the embryos was provided previously11. We took the samples of the uterine walls (c.a. 5 cm × 5 cm) from the anterior, intermediate, and posterior portions of the left uterus (sites s1–s3 in Fig. 1c) and fixed it in Bouin’s solution for histological study (see below). The rest of the uterine specimen was fixed in 10% formalin for further studies. All the specimens used in this study were preserved at Okinawa Churashima Research Center (Okinawa Churashima Foundation, Okinawa, Japan). The small sample size is a consequence of the scarcity in the availability of pregnant white shark specimens.

**Histological thin section.** The tissue samples were sectioned at 7 µm almost parallel to the wall and were stained with Delafield’s hematoxylin and eosin (Fig. 4a–c). Some sections were stained with PAS staining kit (1.01646.0001; Merck KGaA) and counterstained with hematoxylin to confirm the presence/absence of milk secretion.

**Oxygen-diffusing capacity.** We evaluated the oxygen-supplying ability of the uterine wall by using oxygen-diffusing capacity (D, nmol-min⁻¹-torr⁻¹). This index is widely used to quantify the efficiency of passage of gas molecules through the biological membrane, such as lung and fish gills. Oxygen-diffusing capacity is defined as follows:

\[
D = K \times \frac{A}{X}
\]

(1)

where K is the Krogh’s diffusion coefficient, A (cm²) is the surface area of the uterine surface, and X (cm) is the thickness of the diffusion barrier. The value of K varies among tissues, but its value in a biological membrane is typically one-third the value in pure water. Following a prior study of oxygen diffusion in skate external gill filaments, we used the value of 1.31 × 10⁻⁹ nmol-cm⁻¹-min⁻¹-torr⁻¹ for K²².

**Surface area and thickness of the diffusion barrier of the uterus.** The tissue samples of the uterine wall (1 cm × 1 cm) were obtained from the anterior, intermediate, and posterior portion of the left uterus (sites s1–s3 in Fig. 1c). We collected five samples from each site. Then, we used a scapula to isolate all the uterine lamellae for each tissue and mounted them on slide glasses with Aqueous Mount-Quick (Daido Sangyo Co., Tokyo, Japan) (Fig. S1). All these lamellae were photographed using a digital microscope (BX53; Olympus Co., Tokyo, Japan) at Okinawa Churashima Research Center, and the projected areas of the lamellae were measured using ImageJ software (US National Institutes of Health, Bethesda, MD, USA).

The effect of the increase in surface caused by the micro-ridges was evaluated in the following sequence. First, the histological sections were photographed using a digital microscope (BX53; Olympus Co., Tokyo, Japan). Second, a pair of points located on the lamellar surface was set for these photographs, so that the linear distance between these points was 200 µm (p1 and p2 in Fig. 4d). Third, the curved distance (CD, µm) along the surface of the uterine wall between two points was measured using ImageJ. Fourth, CD/200 (µm/µm) was calculated to determine the effect of the increase in surface caused by the micro-ridges.

Assuming the cylindrical morphology of the uterus, we calculated the surface area of the whole uterus as follows:

\[
A = 2 \times \pi \times S \times UD \times UC
\]

(2)
where A (cm²) is the surface area of the uterine wall, S (cm²) is total projected area of lamellae within 1-cm-squared tissue sample, α is the effect of the increase in surface caused by the micro-ridge on the lamellar surface, UD (cm) is the antero-posterior length of the uterus, and UC (cm) is the circumference of the uterus at the middle half of the uterus. Using a measuring tape, we measured UD and UC, which were 90 and 43 cm, respectively.

The thickness of the diffusion barrier (X) was measured from histological thin sections using the digital microscope (BX53; Olympus Co., Tokyo, Japan) (Fig. 4d). Thirty measurements were taken from each sampling site (sites s1–s3 in Fig. 1c), and the average was calculated for each site.

Notably, the isthmus region was not included in our physical calculation. Isthmus region, which is the anterior-most small region of the uterus, is characterized by the development of large ridges on the surface (Fig. S2a). This feature is presumed to allow enhanced oxygen supply in this region12,13. However, this notion is not supported by the following two lines of evidence. (1) Contribution of large ledges to increase in surface-area is relatively small; it is approximately four times over that of the flat surface (=1.4 cm [number of large ridges per cm of the isthmus region] × 1.4 cm [approximate height of the large ridges] × 2 [the number of sides of each large ridge]). (2) Microstructure suggests that the ability of oxygen supply of the isthmus region is lower than that of the other regions. The height of uterine lamellae is approximately one-tenth of the other regions, and the diffusion barrier is approximately 2.3 times thicker than that of the other regions (height of uterine lamellae = 0.54 ± 0.07 [SD] mm; thickness of diffusion barrier = 24.9 ± 3.6 [SD] μm; Fig. S2b–d). Based on these observations, the amount of oxygen supply in this region is probably smaller than that in other regions, suggesting that the exclusion of the isthmus region may have little effect on our conclusions.

Oxygen-diffusing capacity of fish gills. The oxygen-diffusing capacity of gills was calculated for two elasmobranch species, Isurus oxyrinchus and Scyliorhinus stellaris. Based on the geometrical model for fish gills in ref. 3, the oxygen-diffusing capacity of single gill filament (Dgill) was calculated as follows:

\[ D_{\text{gill}} = K \times (2nLW \times LW) / X_{\text{gill}} \]  

where K is Krogh’s diffusion coefficient, n is average number of half-side of secondary lamella of a single filament, LW (cm) is the average width of secondary lamella, LH (cm) is the average height of secondary lamella, and Xgill (cm) is the thickness of the diffusion barrier of the gills. Dgill was divided by the average projected area of a single gill filament (=average length of gill filament [cm] × average width of gill filament [cm]) for estimating the oxygen-diffusing capacity of 1 cm² gill tissues. All morphometric data of the gills used for this analysis were obtained from previous publications1,2,5,9,10. The value of 1.31 × 10⁻³ mmol·cm⁻¹·min⁻¹·torr⁻¹ was used for K.

Scanning electron microscopy. Scanning electron microscope (SEM) images were obtained for five tissue samples of the uterine wall (approximately 3 mm × 3 mm) using a VE-8800 (Keyence Co., Osaka, Japan) at 1–3 kV at the Okinawa Churaumi Aquarium (Okinawa, Japan) from each of the three sites. Before SEM imaging, the tissue samples were dehydrated through a graded series of ethanol (70, 80, 90, 95, and 99.5%) and tert-butyl alcohol and were freeze-dried using EYELA FDU-1200 (Tokyo Rikakikai Co., Tokyo, Japan) at Okinawa Churaumi Aquarium.

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Author Contributions
T.T. wrote the main body of the manuscript. T.T., R.N., M.N., S.M., K.M., and K.S. contributed to the collection of data reported in this study and reviewed the manuscript. M.N. prepared the histological thin sections. T.T. and K.M. prepared the figures.

Additional Information
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