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The power of ultrasound: observation of nearly the entire gestation and embryonic developmental process of captive reef manta rays (*Mobula alfredi*)

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Abstract—The aim of this study was to make ultrasound observations of 2 captive reef manta rays (*Mobula alfredi*), thereby collecting the first known data regarding the embryonic development of reef manta rays throughout almost the entire gestation period. The gestation period and water temperature at parturition were ~1 year and ~27°C, both of which are consistent with observations of wild individuals. On the basis of embryonic features observed through ultrasound, 3 developmental stages were recognized: early (0–80 d after copulation), middle (80–150 d after copulation), and late (150–360 d after copulation). The middle stage was distinguished from the early stage by the presence of large, wing-shaped pectoral fins, cephalic lobes, and buccal pumping, whereas the late stage was distinguished from the middle stage by the overlapping of the right and left pectoral fins and cessation of motion of the entire body in utero. These criteria will be useful for determining the gestation stages of wild reef manta rays.

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Studies on the life history of large aquatic vertebrates, including the reef manta ray (*Mobula alfredi*) and the giant manta (*M. birostris*), are extremely difficult because such species move fast and have extensive ranges in vast marine environments and because technology for studying free-roaming aquatic animals does not exist (Castro, 2016). In the aquarium, observations are also difficult, because of the slow growth of animals, which can take decades to reach maturity, and because they are difficult to maintain for such long periods. Life history traits concerned with female reproduction, gestation cycle, gestation period, and fecundity are essential for studies on the conservation and management of large aquatic vertebrates.

Knowledge of the female reproductive biology of manta rays (Mobulidae) is still limited (Couturier et al., 2012). Marshall and Bennett (2010) observed the breeding behavior (e.g., courtship display, copulation, and parturition) of wild reef manta rays and estimated the gestation period for this species, on the basis of the duration between 2 consecutive parturition events for the same individual. However, their data were based on observations of females in the field, and no internal anatomical or embryonic developments were observed. The only documented observation of an embryo of a live reef manta ray can be found in Tomita et al. (2012), who conducted ultrasound examinations of a pregnant manta ray at the Okinawa Churaumi Aquarium, located in Okinawa, Japan, and described its embryonic respiratory behavior. However, this report provided only a "snapshot" of this species' embryonic development during late-term gestation, and this species' entire gestation process, from early to late term, has not been reported.

Long-term captivity and successful breeding of reef manta rays at the Okinawa Churaumi Aquarium have provided a unique opportunity to monitor the entire gestation process of this species. In 2007, a reef manta ray gave birth in the Kuroshio Tank (which has a volume of 7500 m^3). This event was the first captive birth of this species (Matsumoto and Uchida, 2008), and since then, 2 females have given birth to a total of 8 more offspring at the aquarium.

To the best of our knowledge, this report is the first to define almost the entire gestation process and embryonic development of the reef manta ray. This study would not have been possible without the recent development of underwater ultrasound (Tomita et al., 2019). Some previous studies have demonstrated that ultrasound is a powerful technique for observing the embryonic condition of captive, viviparous elasmobranchs (e.g., Carrier et al., 2003; Daly et al., 2007). However, the device used in this study is unique in that it can be used by scuba divers at water depths of 10 m or more, allowing the observation of pregnant females without handling the specimens and, therefore, minimizing potential stress.

Materials and methods

Experimental animals

The 2 female reef manta rays (specimens 1 and 2) examined were originally captured by using a set net off Okinawa Island, Japan. Specimen 1 was 205 cm in disc width (DW) when captured in 1998 and had grown to 387 cm DW by 2013, whereas specimen 2 was 227 cm DW when captured in 2008 and had grown to 368 cm DW by 2015. Specimen 1 gave birth 7 times from 2007 through 2013, and specimen 2 gave birth once in 2015.

Ultrasound examinations

Ultrasound examinations were conducted in the Kuroshio Tank at the Okinawa Churaumi Aquarium, by using a FAZONE M[1] ultrasound device (Fujifilm Global, Tokyo, Japan) and a transducer in a 3.525-MHz linear array and at depths up to 24 cm. The examinations were conducted by using either 1) water-surface ultrasound (Fig. 1) or 2) underwater ultrasound (Fig. 2). For water-surface ultrasound, the ultrasound probe was attached to the tip of a PVC pipe that was ~2 m long, and the observer approached the specimens from above during feeding. When the animals swam near the surface, the transducer was placed on the skin of the dorsal surface above the uterus. For underwater ultrasound, the device was housed in a water- and pressure-resistant case (Tomita et al., 2019), and scuba divers collected ultrasound data by placing the transducer on the skin of the dorsal surface above the uterus.

We conducted ultrasound experiments for 93 d, about once every month. The beginning of the ultrasound observations was timed differently for each pregnancy, starting from 12 h to 31 d after mating.

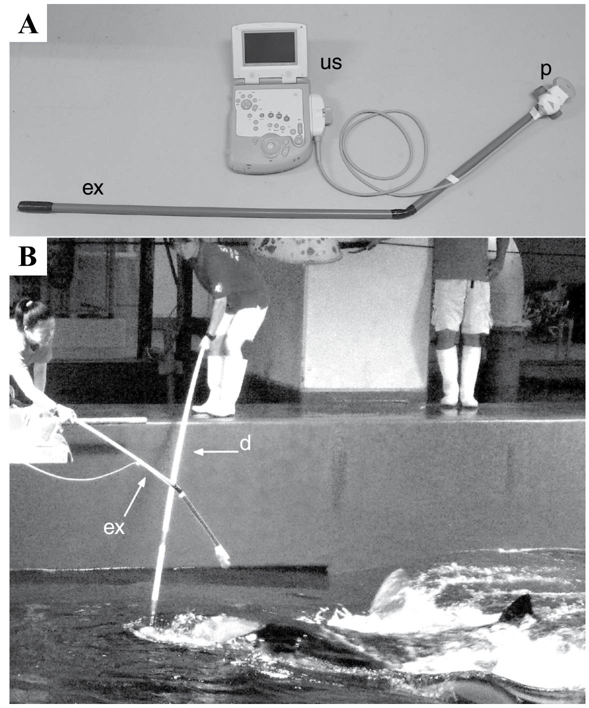

Figure 1

Photographs of the water-surface ultrasound device and technique used to make observations of the gestation and embryonic developmental process of captive reef manta rays (*Mobula alfredi*) during 2007–2015 at the Okinawa Churaumi Aquarium in Japan. (**A**) The probe (p) of the ultrasound device (us) was extended with a PVC pipe (ex). (**B**) An aquarium staff member persuades the reef manta ray to swim to the surface by serving krill with a long-handled dipper (d), while another staff member obtains ultrasound footage from above.

Analysis of ultrasound footage was conducted by using software designed to edit images in the DICOM file format (OsiriX, vers. 3.9.2–3.9.4, Pixmeo SARL, Geneva, Switzerland). Through analysis of footage, we confirmed the developmental condition of the embryo, such as the presence or absence of yolk sac and cephalic lobes and the size of the pectoral fins. In addition, we observed the kinematics of the embryo, confirming the presence or absence of buccal movements.

Results

We tracked a total of 7 pregnancies in 2 female reef manta rays, and 6 of these pregnancies resulted in successful births. Mating events were observed for all pregnancies. The gestation periods, calculated from the 6 pregnancy events of the 2 captive females from 2007 through 2015 with the assumption that fertilization occurred on the day

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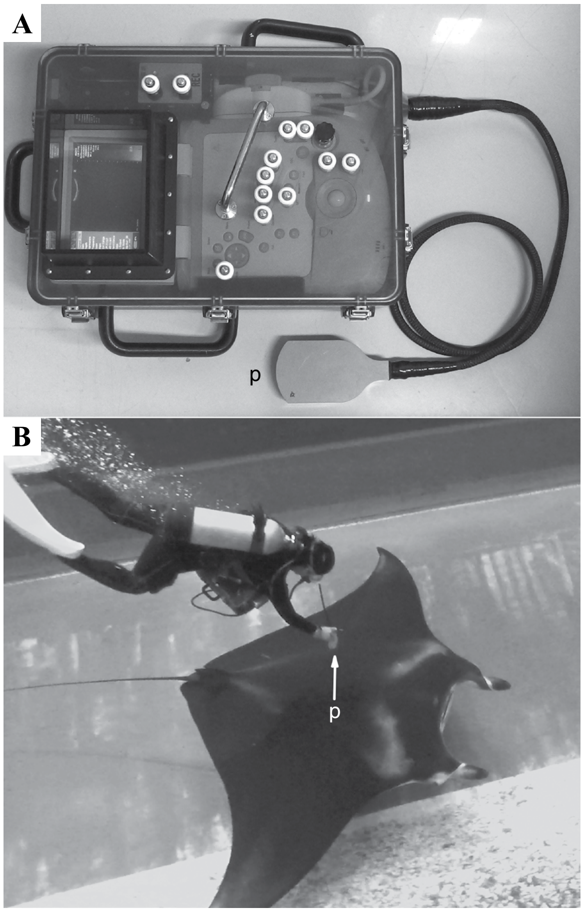

Figure 2

Photographs of the underwater ultrasound device and technique used to make observations of the gestation and embryonic developmental process of captive reef manta rays (*Mobula alfredi*) during 2007–2015 at the Okinawa Churaumi Aquarium in Japan. (**A**) The ultrasound device was housed in a pressure- and water-proof case. (**B**) A scuba diver positions the ultrasound probe (p) on the dorsal surface of a reef manta ray.

of mating, ranged from 363 to 379 d (mean: 370.5 d [standard deviation (SD) 5.7]) (Table 1). All the parturition events occurred in May and June, and the water temperature during these events ranged from 24.9°C to 27.5°C (mean: 27.0°C [SD 0.8]) (Table 1). All pregnant females observed in this study bore a single embryo. The birth size of the manta ray offspring ranged from 153.5 to 192.0 cm DW (mean: 180.3 cm [SD 14.1]) (Table 1).

The gestation process of the reef manta rays was divided into 3 stages: early (0–80 d after copulation; Fig. 3), middle (80–150 d after copulation; Fig. 4), and late (150–360 d after copulation; Fig. 5). These 3 stages were recognized in 3 pregnancies, after the underwater ultrasound was introduced in this study in 2010.

At the beginning of the early stage (0–20 d after copulation), the amount of uterine fluid was relatively small, as indicated by the diameter of the internal uterine cavity (maximum of ~10 cm; Fig. 3A). The embryo was not yet visible, and yolk could be observed. Later (~50 d after copulation), the motionless embryo could be observed in utero (Fig. 3B).

The amount of uterine fluid was greater during the middle stage than during the early stage, as indicated by the diameter of the internal uterine cavity (maximum of 30 cm). In addition, the pectoral and dorsal fins and cephalic lobes were well developed (Fig. 4B), and the yolk sac was slender, cylindrical, and easily identifiable in ultrasound images (diameter of ~2 cm) (Fig. 4C). Furthermore, the tips of the right and left pectoral fins were bent dorsally or ventrally along the curve of the uterine wall but never overlapped, and the embryo rotated its body inside the uterine cavity and exhibited buccal pumping (Fig. 4D).

During the late stage, the relative amount of uterine fluid decreased, as the size of the embryonic body increased, and a conspicuous bulge was observed on the dorsal surface of the maternal body. The uterus was greatly expanded; therefore, the entire uterine image could not be acquired by ultrasound, and uterine diameter was no longer available. The yolk sac was still present but was rarely observed in ultrasound images, possibly because of its small size, and the pectoral fins were folded dorsally, with the right and left pectoral fins largely overlapping (Fig. 5A). The embryo did not show complete body movements, such as body rotation, in utero. Internal organs (e.g., spiral intestine) were visible (Fig. 5B). However, during the last part of this stage, the embryonic head was rarely observed because the depth of the embryonic head from the maternal skin surface was >24 cm, a depth that exceeds the limit of the ultrasound used. Therefore, buccal pumping was not observed.

Discussion

This study produced the first data of the entire gestation period of a manta ray. The gestation period of 370.5 d (SD 5.7), estimated from the 6 pregnancy events of the 2 captive females, is consistent with the 1-year gestation period that has been reported for wild reef manta rays (Marshall and Bennett, 2010). It also is significantly longer than the gestation period reported for other stingrays, for example, periods of 2, 3, and 4 months in the pelagic stingray (*Pteroplatytrygon violacea*), the round stingray (*Urobatis halleri*), and the bull ray (*Aetomylaeus bovinus*), respectively (Ranzi, 1934; Babel, 1967). The results of this study also indicate that parturition occurred in the summer, at water temperatures of ~27°C, as previously reported for wild reef manta rays (Marshall and Bennett, 2010).

The results of this study reveal that buccal pumping is a good indicator for distinguishing between the early and middle developmental stages of the reef manta ray. This observation is similar to the observations of the oviparous catshark species, the small-spotted catshark (*Scyliorhinus canicula*), and the cloudy catshark (*Scyliorhinus torazame*),

Table 1

Summary information for 7 captive births of reef manta rays (*Mobula alfredi*) at Okinawa Churaumi Aquarium during 2007–2015. Averages, calculated by excluding the stillborn event on 13 May 2012, are given with standard deviations (SDs) in parentheses. Two females accounted for these birth events. Specimen 1 gave birth 6 times before dying during pregnancy on 31 May 2013. Specimen 2 gave birth once during the study period, on 1 June 2015. N/A=not available.

		Birth size				
Birth events	Sex of newborns	Disc width (cm)	Body weight (kg)	Water temperature (°C)	Gestation duration (days)	Birth status
16 June 2007	Female	192.0	68.5	26.0	374	Normal
17 June 2008	Male	182.0	N/A	27.5	368	Normal
24 June 2009	Female	192.0	70.0	27.0	372	Normal
26 June 2010	Female	182.0	66.0	27.5	367	Normal
24 June 2011	Female	180.0	60.5	27.8	363	Normal
13 May 2012	Male	158.4	49.0	24.9	329	Stillborn
1 June 2015	Female	153.5	31.5	26.0	379	Normal
Average (SD)		180.3 (14.1)	59.3 (16.0)	27.0 (0.8)	370.5 (5.7)	

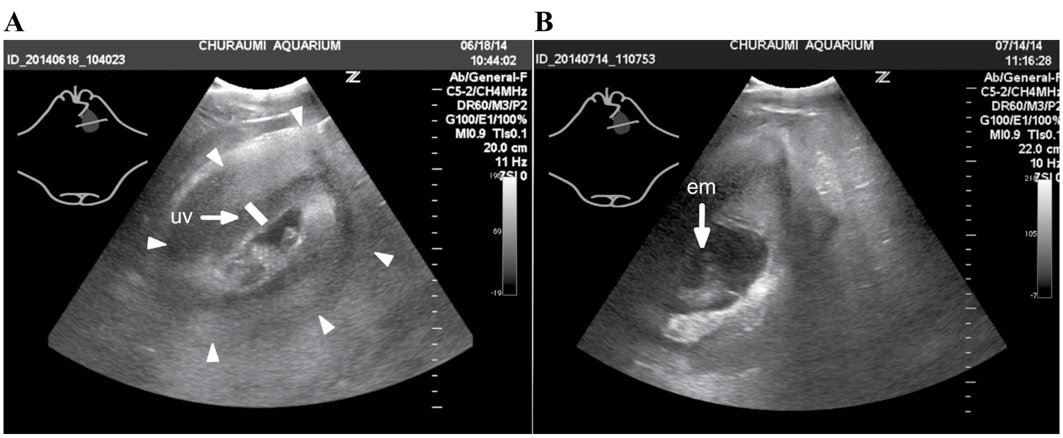

Figure 3

Ultrasound images of embryos in the early developmental stage (0–80 d after copulation) in a captive reef manta ray (*Mobula alfredi*) at the Okinawa Churaumi Aquarium in Japan: (**A**) embryo at 20 d after copulation, from ultrasound of specimen 2 on 18 June 2014, and (**B**) embryo (em) at 46 d after copulation, from ultrasound of specimen 2 on 14 July 2014. In panel A, the white arrows indicate the uterine chamber surface and the white line indicates the thick layer of uterine villi (uv). In panel B, the embryo is visible inside the uterine chamber.

in which embryos first exhibited buccal pumping during the middle gestation period, after the jaw and hyoid structure are fully developed (Diez and Davenport, 1987; Thomason et al., 1996; Tomita et al., 2014). Interestingly, in this study, we failed to observe any evidence of tail-waving movement, which is typically observed in oviparous batoids (Diez and Davenport, 1987; Thomason et al., 1996). The tail motion of oviparous batoid embryos is thought to create water flow inside the egg case and to promote the exchange of water between the inside and outside of the egg case. However, the lack of embryonic tail motion in the reef manta ray probably reflects the nonnecessity of such water replacement during viviparous reproduction.

The results of this study also indicate that the overlapping of the pectoral fins is a good indicator of the late developmental stage of the reef manta ray. According to our observations, the right and left pectoral fins are tightly folded posteriorly as the embryo grows and the tips of the right and left pectoral fins begin to overlap at 150 d after copulation. Because this change almost coincides with the

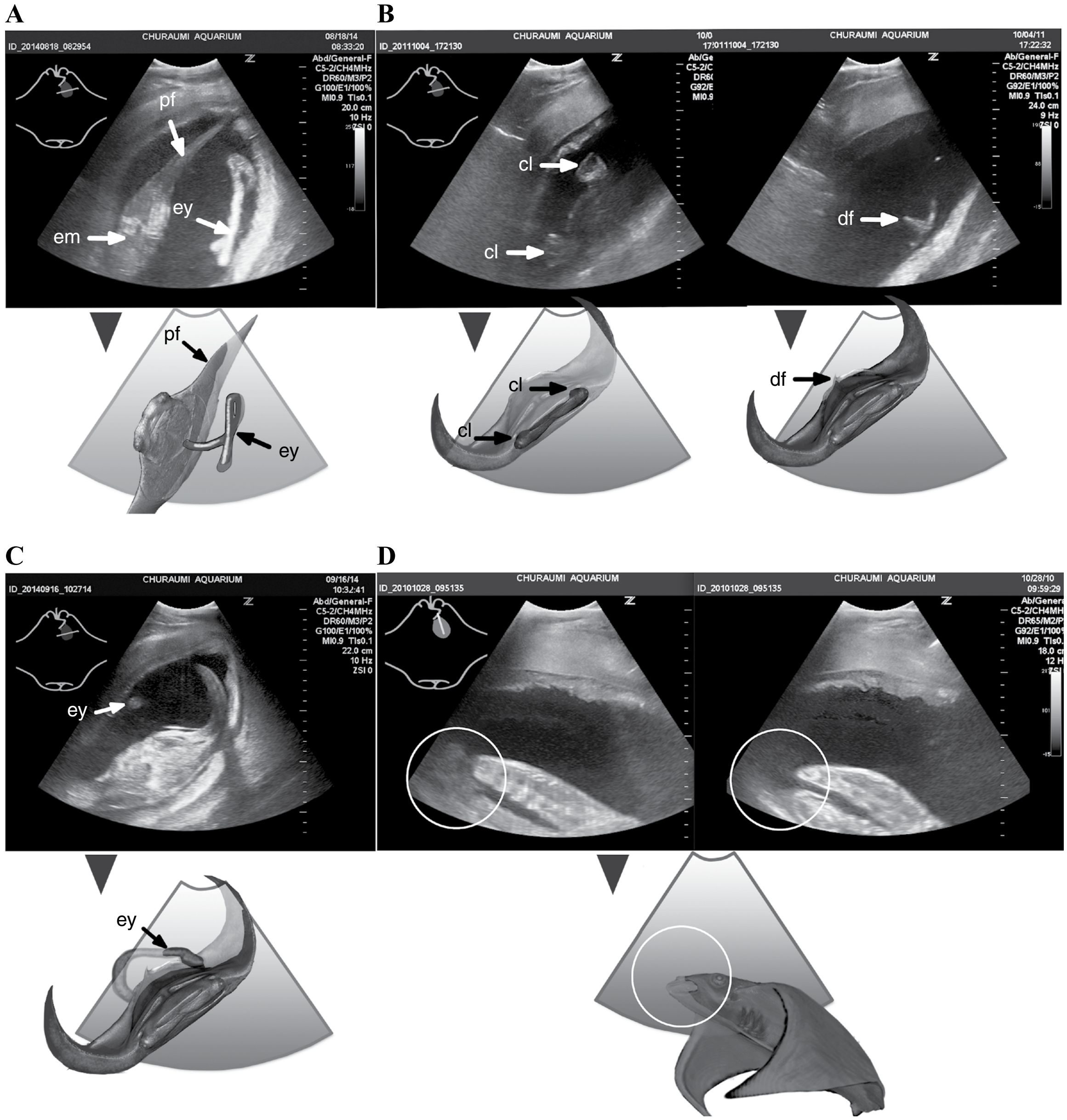

Figure 4

Ultrasound flat sections (top images) and 3-dimensional images (bottom images) of embryos in the middle developmental stage (80–150 d after copulation) in 2 captive reef manta rays (*Mobula alfredi*) at the Okinawa Churaumi Aquarium in Japan. (**A**) Both images of an embryo (em) at 81 d after copulation (in specimen 2 on 18 August 2014) show the large pectoral fin (pf) and embryonic yolk (ey). (**B**) In images of an embryo at 103 d after copulation (in specimen 1 on 4 October 2011), the right and left cephalic lobes (cl, left images) and dorsal fin (df, right images) are visible. (**C**) The images of an embryo at 110 d after copulation (in specimen 2 on 16 September 2014) show well-developed and non-overlapping pectoral fins. (**D**) In the images of an embryo at 125 d after copulation (in specimen 1 on 28 October 2010), the cyclic opening (left) and closing (right) of the mouth can be seen.

time when the pregnancy becomes obvious from external observations and because the occurrence of this overlap is relatively easy to detect by ultrasound, the feature is useful as an indicator of the late stage of pregnancy. Similar fin overlap has also been reported to occur in dead embryos of other *Mobula* species, such as the giant manta (K. Ueda and Y. Matsumoto, personal observ.) and the smoothtail mobula (*Mobula thurstoni*) (Notarbartolo-di-Sciara, 1988), indicating that the same stage criteria could be used for other *Mobula* species. Even though the developmental staging of *Mobula* species was not established in a previous study, the middle and late stages

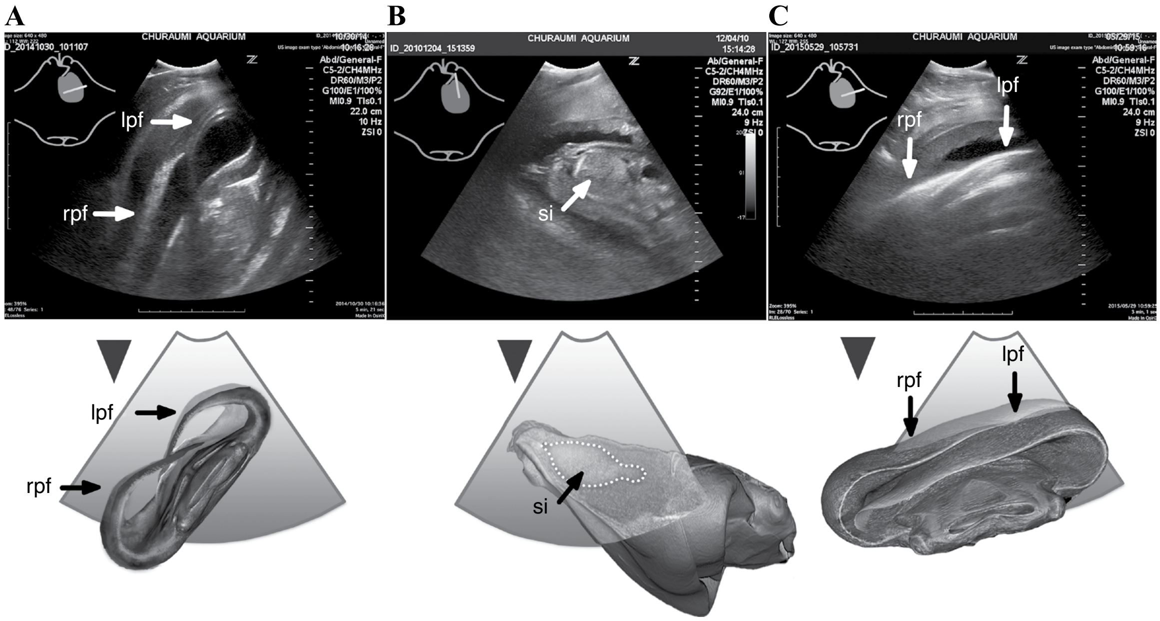

Figure 5

Ultrasound flat sections (top images) and 3-dimensional images (bottom images) of embryos in the late developmental stage (150–360 d after copulation) in 2 captive reef manta rays (*Mobula alfredi*) at the Okinawa Churaumi Aquarium in Japan. (**A**) In both images of an embryo at 154 d after copulation (in specimen 2 on 30 October 2014), the overlapping right pectoral fin (rpf) and left pectoral fin (lpf) are visible. (**B**) The images of an embryo at 162 d after copulation (in specimen 1 on 4 December 2010) provide a vertical cross section of the spiral intestine (si). (**C**) The images of an embryo at 365 d after copulation (in specimen 2 on 29 May 2015) show the rpf and lpf overlapping.

recognized in this study may correspond to stages 32 and 33 of the oviparous winter skate (*Leucoraja ocellata*), during which the anterior tips of the pectoral fins reach the anterior-most point of the head (Maxwell et al., 2008). The main difference between stage 32 and 33 is the presence of external gill filaments at stage 32; however, this feature was not examined in this study.

Our methods, which do not require constraining animal movements, may be useful for studying wild individuals. In previous studies, ultrasound experiments for wild elasmobranchs were conducted by placing specimens on or near the research vessels (e.g., Sulikowski et al., 2016). The constraints used to place specimens on or near a vessel are potentially stressful for the specimens and are almost impossible to use in studying large elasmobranchs, such as manta rays.

Despite its advantages, the use of underwater ultrasound had some limitations in our study. The main limitation was the low resolution of the ultrasound images. During early gestation, the small embryo and its movements are difficult to detect. Previous studies have shown that sex hormones can be used for detecting pregnancy in viviparous elasmobranchs. For example, concentration of plasma progesterone is generally elevated during the early stages of pregnancy and declines in the later stages (Tsang and Callard, 1987; Fasano et al., 1992; Manire et al., 1995; Snelson et al., 1997; Tricas et al., 2000). Therefore, the combined use of ultrasound and other methods (e.g., blood analysis) likely represents the best strategy for more precisely studying the reproductive cycle and embryonic development of manta rays.

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