Supplementary Text S1

Details of the underwater ultrasound equipment and methods Waterproofed housings, with a maximum working depth of 40 m for ARIETTA Prologue, were co-developed by SSP Ltd. (Matsumae, Japan) and OCA (Fig. 1A). A keypad on the top right side of this housing allowed us to control the power on/off, recording images start/stop, scanning depth and gain while underwater. The probe used in this study was a Convex ARIETTA C251 Transducer in a housing, with 5 – 1 MHz and an ability to reach a scanning depth of 400 mm (Hitachi Ltd., Tokyo, Japan). DICOM file formatted ultrasound images were recorded in the memory installed on the ARIETTA Prologue as a series of successive images (19 fps). We activated the recording function while approaching the shark and turned it off when leaving the shark. The keypad was not handled while the sonogram was taken, as the diver had the probe in one hand and the housing in the other (Fig. 1B). The ultrasound equipment was validated prior to use in whale sharks through the reproductive examination of 14 shark and ray species in the OCA by RM and KM (e.g., Nozu et al. 2018, Murakumo et al. 2020). The ultrasound system was capable of up to 400 mm scanning depth; however, after initial trials we set the depth to 300 mm to reduce artifacts and improve image clarity. The gain was set at the maximum 90 dB (range 10–90 dB). Initially, the probe was placed perpendicular to the surface of the body and moved in a longitudinal fashion between pectoral and pelvic areas on either side of the abdomen, in order to check for artifacts caused by skin, muscle, and liver. We then focused on the area least affected by these factors and scanned transversely from the upper abdomen to the midline of the abdomen (Fig. 1B, C).

References

Murakumo K, Matsumoto R, Tomita T, Matsumoto Y, Ueda K (2020) The power of ultrasound: observation of nearly the entire gestation and embryonic developmental process of captive reef manta rays (*Mobula alfredi*). Fish Bull 118:1–7 https://doi.org/10.7755/FB.118.1.1

Nozu R, Murakumo K, Yano N, Furuyama R, Matsumoto R, Yanagisawa M, Sato K (2018) Changes in sex steroid hormone levels reflect the reproductive status of captive female zebra sharks (*Stegostoma fasciatum*). Gen Comp Endocrinol 265:174–179 https://doi.org/10.1016/j.ygcen.2018.03.006

Supplementary Table

Table S1. Female whale sharks (ID no., sex, total length [TL]) used for ultrasound imagery and blood sampling in 2017 and 2018. a: abdominal area, b: anterior part of pelvic fins, c: post-pelvic distention area.

				Ultrasound				
Individuals	Date	Sex	TL (m)	Start time	Position of scan	Follicle	Scan time (sec)	Blood sampling
Female #1	2017/07/04	F	7	8:09:42	a, b, c		4	
Female #2	2017/07/08	F	12	8:21:01	a, b		9	Yes
				12:05:16	b		20	
Female #3	2017/07/08	F	12	8:24:52	b		14	
				15:54:20	b		4	
Female #4	2017/07/09	F	12	7:56:06	b		20	
				8:02:52	b		12	
				8:04:34	b, c		16	
				8:10:23	a, b		18	
Female #5	2017/07/09	F	12	10:56:56	b		15	
				15:09:20	b		8	
Female #6	2018/09/14	F	12-14	11:29:36	a, b		26	Yes
				13:32:57	a, b		26	
Female #6	2018/09/15	F	12	7:21:47	a, b, c		10	
Female #7	2018/09/15	F	11	7:42:45	a		28	
Female #8	2018/09/15	F	11	10:54:13	b		10	
Female #9	2018/09/16	F	12	7:32:43	b, c		14	
Female #10	2018/09/17	F	11	10:39:11	b		6	Yes
Female #11	2018/09/17	F	11	15:06:13	b		17	Yes
Female #12	2018/09/18	F	11	7:40:34	b, c		30	

Female #13	2018/09/20	F	10-11	7:34:35	b, c		20	Yes
Female #14	2018/09/20	F	12	11:54:09	b	Yes	13	
Female #15	2018/09/24	F	No estimate	7:31:18	a, b		8	
Female #16	2018/09/24	F	11	11:02:59	b		21	
				11:04:22	b	Yes	20	Yes
Female #17	2018/09/24	F	No estimate	11:06:01	a, b		10	
Female #18	2018/09/24	F	No estimate	14:59:33	b		10	
Female #19	2018/09/25	F	No estimate	11:08:52	b		18	
Female #20	2018/09/25	F	No estimate	11:26:34	b		14	
Female #21	2018/09/25	F	12-13	15:14:24	b		15	
				15:25:51	b		6	
Female #22	2018/09/25	F	No estimate	15:28:31	b		8	

Supplementary Figures

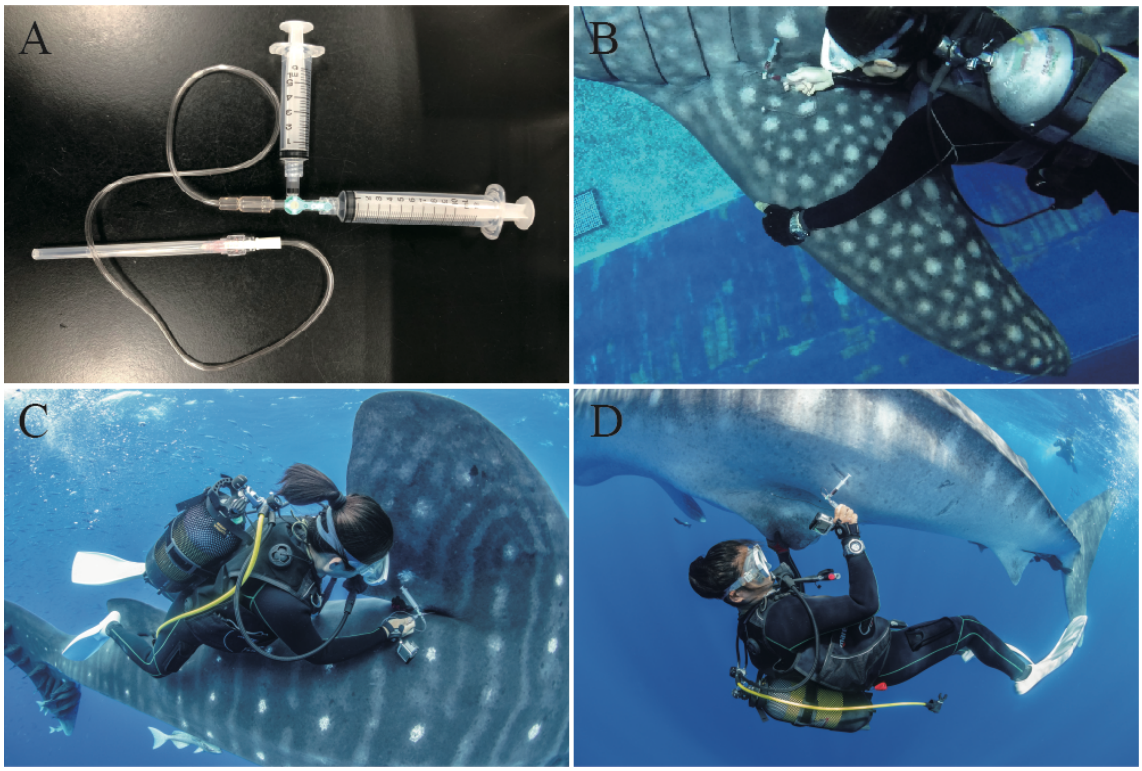

Figure S1. Blood sampling tools consisting of (A) two syringes, connected by a 3-way stopcock, an extension tube, and 18 gauge and 70 mm long needles, were used for collecting blood. Blood extracted from (B) the dorsal surface of the pectoral fin, (C) the posterior base of the first dorsal fin and (D) the dorsal surface of the pelvic fin.

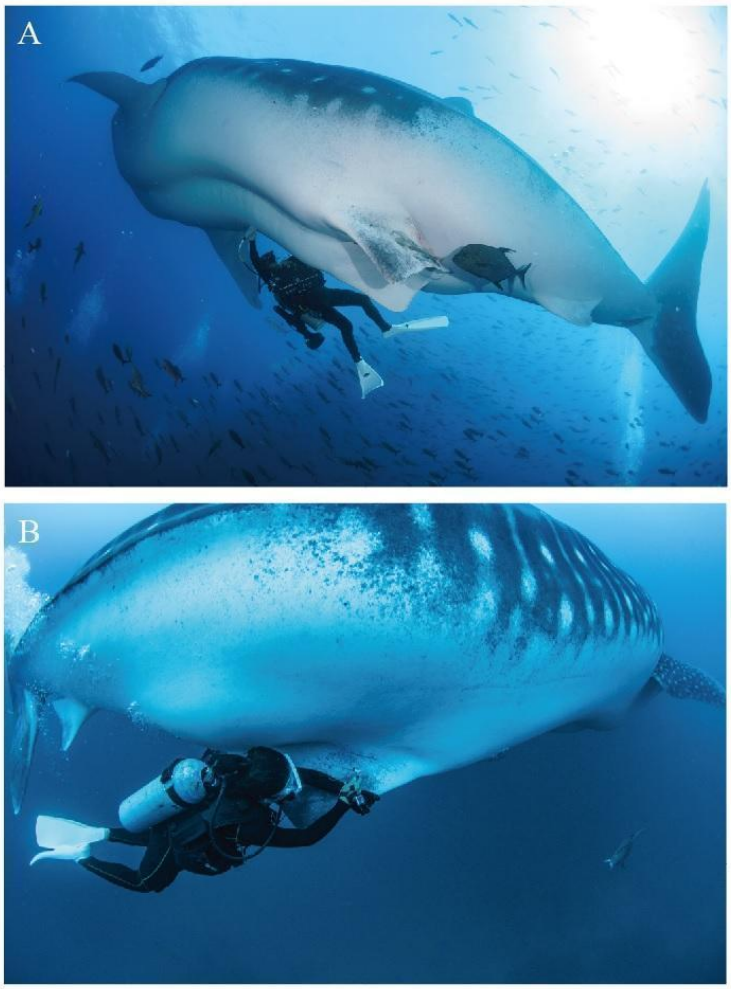

Figure S2. Photos of (A) underwater ultrasonography and (B) blood sampling from the female whale sharks in the Galapagos.

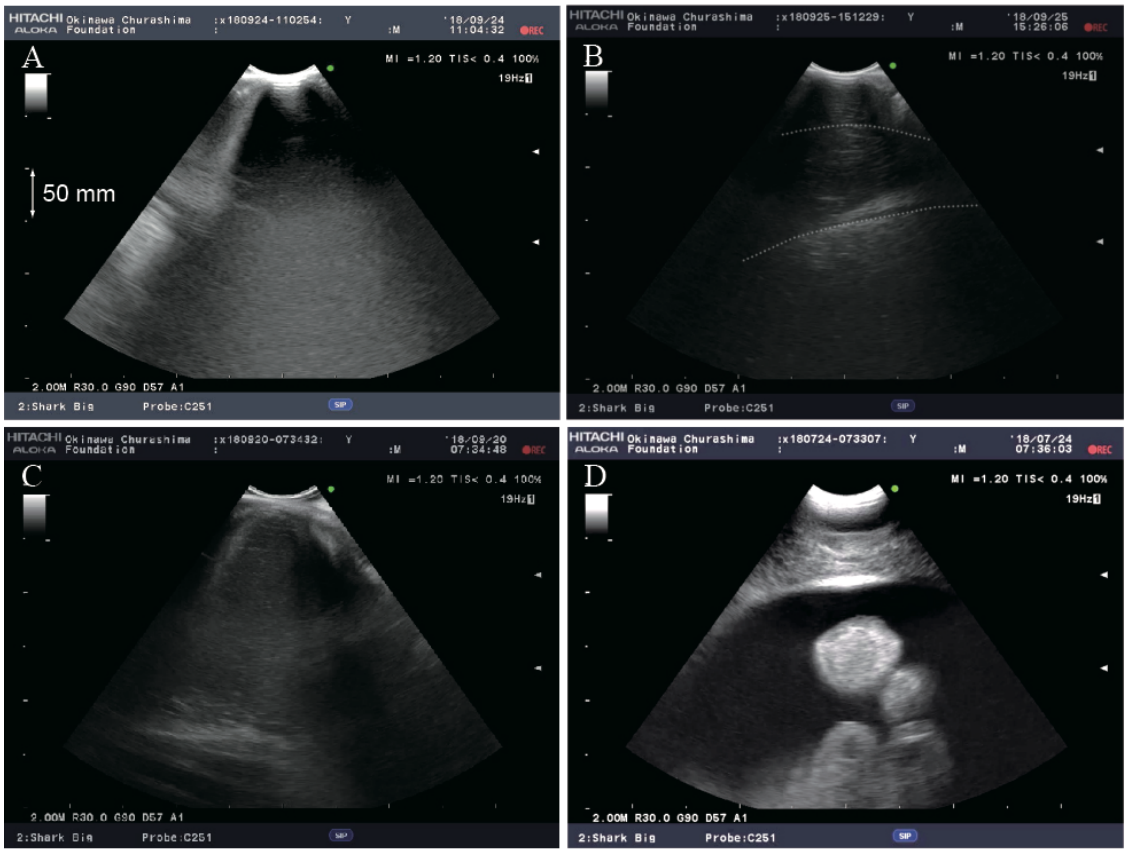

Figure S3. Same ultrasound images as Fig. 2A-D with boundaries removed. (A): Ovarian follicles and (B): Skin, muscle and liver in front of the pelvic fins on the transverse plane, and (C): Skin and muscle in the area of the post-pelvic distention on the transverse plane in the mature female whale sharks in the Galapagos, and (D): Skin, muscle, intestine, rectal gland, unexpanded right and left uteri without ovarian organs in front of the pelvic fins on the transverse plane in an immature female in the OCA.

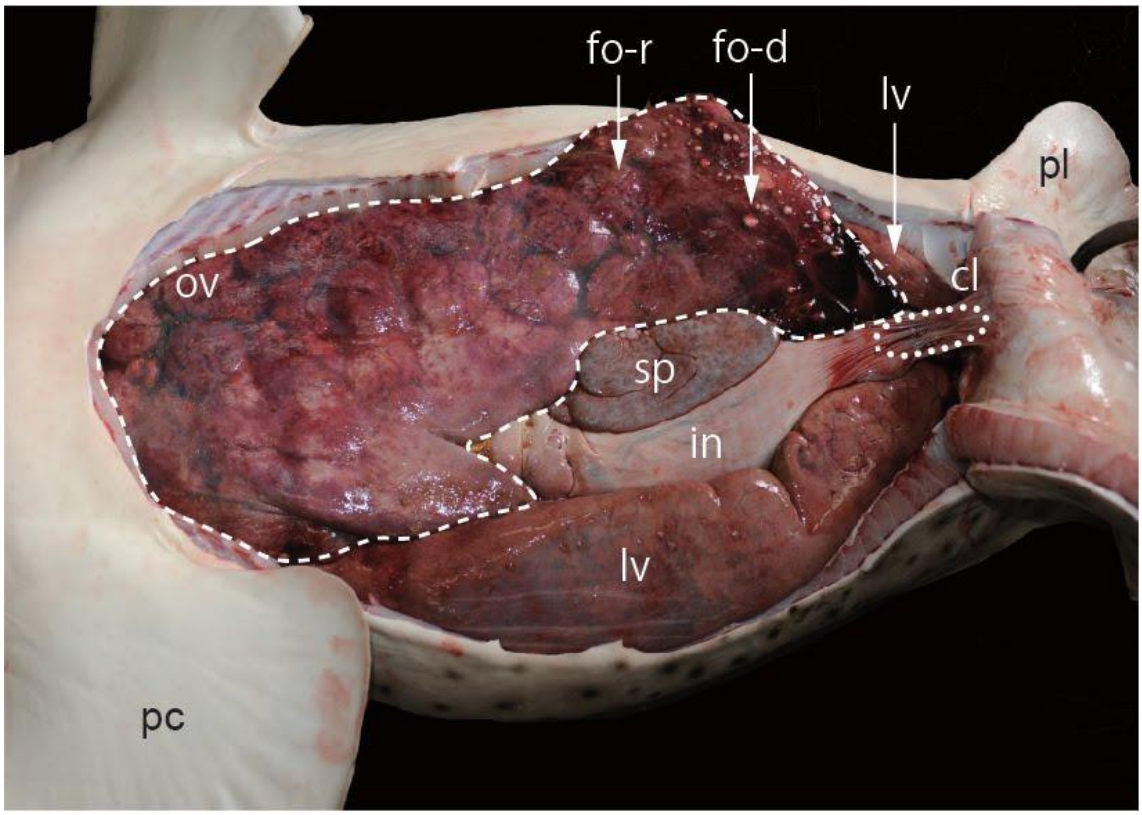

Figure S4. Anatomical photo of a female zebra shark (223 cm TL) during reproductive season. Left ovary (ov) enclosed dashed line which ranges between pectoral (pc) and pelvic (pl) fins, is filled with a lot of ripe follicles (fo-r) and developing small follicles (fo-d) overlapping other organs including liver (lv), intestine (in) and spleen (sp). Dotted circle indicates the original position of cloaca (cl) before peeling skin and muscle layers caudally.

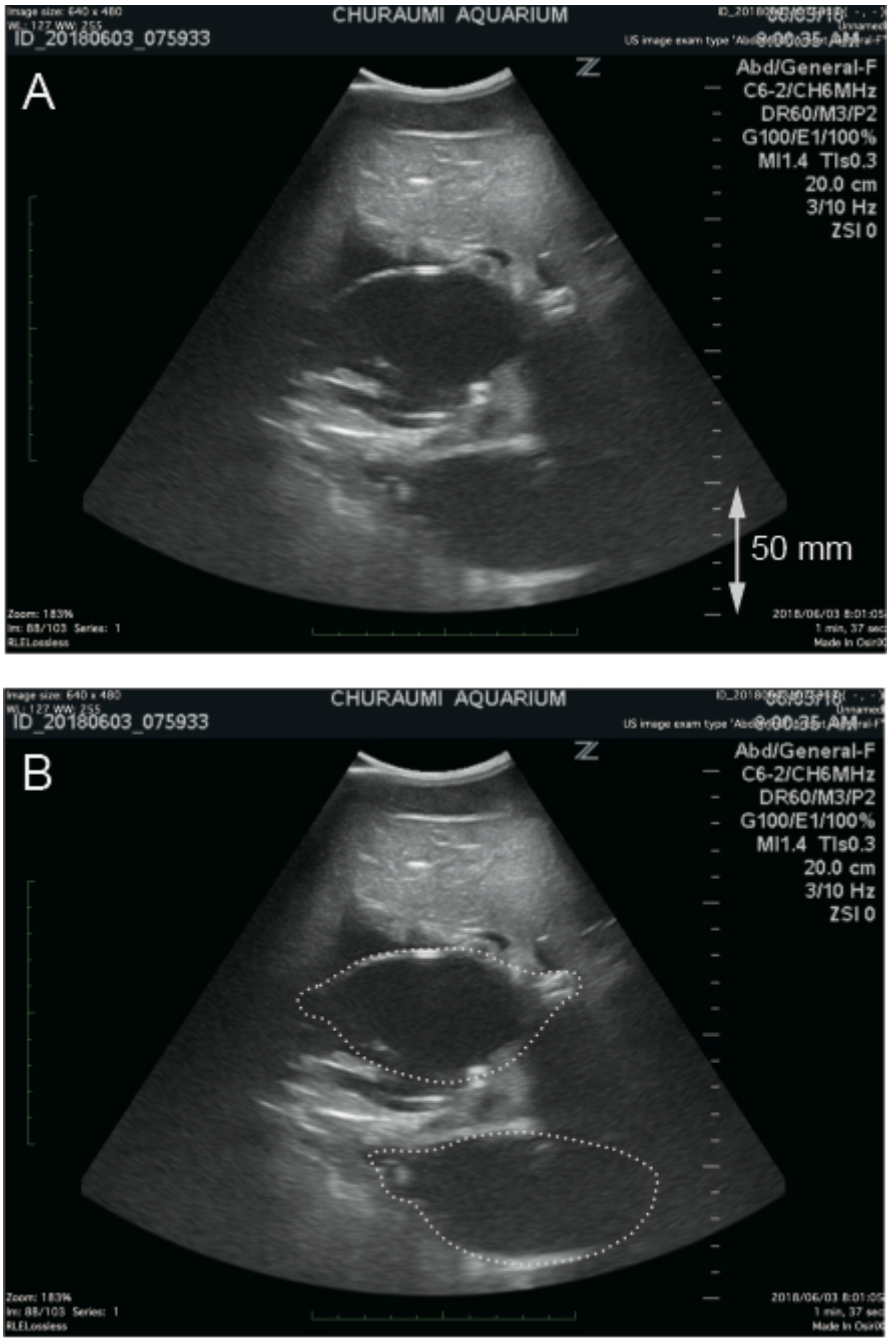

Figure S5. Cross sectional ultrasound images of egg cases within the uterus in a zebra shark. (A): Original image and (B): Two egg cases enclosed dotted lines.