

A Staging Table for the Embryonic Development of the Brownbanded Bamboo Shark (*Chiloscyllium punctatum*)

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Background: Studying cartilaginous fishes (chondrichthyans) has helped us understand vertebrate evolution and diversity. However, resources such as genome sequences, embryos, and detailed staging tables are limited for species within this clade. To overcome these limitations, we have focused on a species, the brownbanded bamboo shark (*Chiloscyllium punctatum*), which is a relatively common aquarium species that lays eggs continuously throughout the year. In addition, because of its relatively small genome size, this species is promising for molecular studies. **Results:** To enhance biological studies of cartilaginous fishes, we establish a normal staging table for the embryonic development of the brownbanded bamboo shark. Bamboo shark embryos take around 118 days to reach the hatching period at 25°C, which is approximately 1.5 times as fast as the small-spotted catshark (*Scyliorhinus canicula*) takes. Our staging table divides the embryonic period into 38 stages. Furthermore, we found culture conditions that allow early embryos to grow in partially opened egg cases. **Conclusions:** In addition to the embryonic staging table, we show that bamboo shark embryos exhibit relatively fast embryonic growth and are amenable to culture, key characteristics that enhance their experimental utility. Therefore, the present study is a foundation for cartilaginous fish research. *Developmental Dynamics* 247:712–723, 2018. © 2018 Wiley Periodicals, Inc.

Key words: Shark embryonic development; ex ovo culture; fin development; brownbanded bamboo shark; evolutionary developmental biology; staging table; elasmobranchs

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Introduction

Cartilaginous fishes (chondrichthyans), which include elasmobranchs (sharks and rays) and holocephalans (chimaeras), have been key organisms in the study of vertebrate evolution and diversity because of their important phylogenetic position as the sister group of bony fishes (osteichthyans: tetrapods, coelacanth, lungfishes, and ray-finned fishes). Knowledge about cartilaginous fishes is, therefore, necessary to define the characteristics of the gnathostome clade (jawed vertebrates). While cartilaginous fishes have played a crucial role in the context of comparative anatomy and embryology since the 19th century (e.g., Gegenbaur, 1865), there is increasing evidence that cartilaginous fishes can also provide unique insights into molecular studies. One of the strongest advantages is that unlike teleosts, none of the examined species of cartilaginous fishes has been shown to have experienced an additional whole-genome duplication (Jaillon et al., 2004; Kuraku and Meyer, 2009; Venkatesh et al., 2007), facilitating orthologous gene comparisons with tetrapods. Also, the molecular

clock of cartilaginous fish genomes appears to be slower than that of teleosts (Martin, 1999; Martin et al., 1992; Renz et al., 2013). Furthermore, a recent study found that non-coding sequences were more conserved between the genomes of humans and elephant sharks (a holocephalan) than between those of humans and teleosts (Lee et al., 2011; Venkatesh et al., 2006). Indeed, the slow evolution of their genome sequences has increased our understanding of how gene regulatory changes have influenced the morphologies of vertebrates (Onimaru et al., 2015; Sagai et al., 2017; Schneider et al., 2011; Tulenko et al., 2017). Therefore, while teleosts such as zebrafish and medaka are convenient laboratory animals, cartilaginous fishes provide several advantages in comparative analyses of vertebrates.

Despite such unique features of cartilaginous fishes, there has been no promising species that satisfies all of the following conditions critical to molecular developmental biology: the availability of whole-genome sequence information, accessibility of embryos, and a detailed embryonic staging table. Classically, the spiny dogfish (*Squalus acanthias*) was used for shark anatomy and embryology (e.g., Jarvik, 1965), but its ovoviviparous reproduction style, which requires the sacrifice of egg-bearing females, is not convenient to constantly obtain eggs. Recently, the genome

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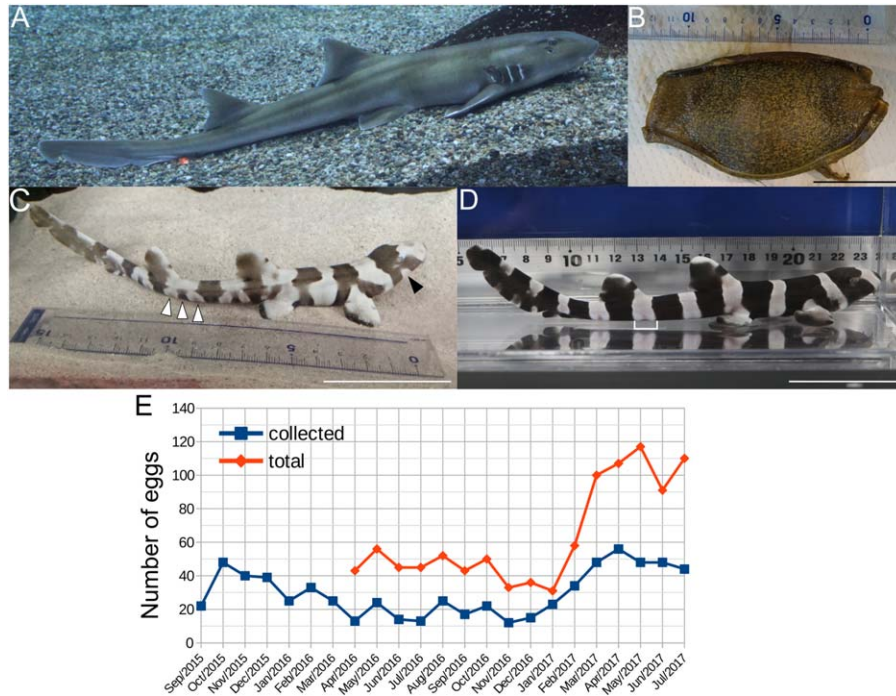


Fig. 1. The brownbanded bamboo shark. **A–C:** An adult (A), an egg (B), and a juvenile (C) from Osaka Aquarium Kaiyukan. **D:** A juvenile with different pigmentation. The photo is provided by Ibaraki Prefectural Oarai Aquarium. **E:** The number of eggs per month over two years; total, the number of eggs including eaten ones; collected, the number of eggs except eaten ones. Scale bars = 5 cm.

of a holocephalan, the elephant shark, has been sequenced (Venkatesh et al., 2014), and there is a description of its embryonic development (Didier et al., 1998). However, elephant shark embryos are rarely available because the habitat range is restricted to southern Australia and New Zealand. On the other hand, an elasmobranch, the small-spotted catshark (*Scyliorhinus canicula*), is a popular choice for studying cartilaginous fish development (Coolen et al., 2008) because its embryonic development has been described in detail (Ballard et al., 1993) and its oviparous (egg-laying) reproduction and captive breeding make eggs accessible. Nonetheless, the large genome size of this species (Stingo et al., 1980) has impeded further molecular studies. In addition, the slow developmental speed of this cold-water dweller (175 days for hatching) (Ballard et al., 1993) is not ideal for practical uses such as drug treatments.

To overcome these limitations, we have chosen the brownbanded bamboo shark (*Chiloscyllium punctatum*) as an alternative species. The brownbanded bamboo shark lays eggs throughout the year and is a relatively common species in aquariums. These factors allow access to embryos without sacrificing wild populations and have led to the use of the bamboo shark in studies of shark development (Atkinson et al., 2016; Dahn et al., 2007; Juarez et al., 2013). It is important to note that its relatively small genome size (Hardie and Hebert, 2003) allows a higher fidelity of various analyses on the genome scale. However, only a partial description of its embryonic development is currently available (Harahush et al., 2007). Therefore, this study defines a formal staging table of bamboo shark embryos with a higher time-resolution. The primary aim of this study is not to describe the details of the embryonic structures, but to define a consensus staging table for any researchers to identify specific time points of developing bamboo shark embryos.

Results

The Osaka Aquarium Kaiyukan's largest tank, Pacific Ocean, housed 10 male and 11 female adult bamboo sharks as of August 2017 (Fig. 1A). The females constantly lay eggs, but about one-half of the eggs are usually eaten by other fishes. As a result, 10–50 eggs per month are collected throughout the year. Roughly one-third of them do not grow normally, probably due to the failure of fertilization or genetic mutations. We did not find obvious seasonal trends (Fig. 1E). The size of the eggs is around 12 cm x 6 cm (Fig. 1B). Juvenile bamboo sharks have pigmentation bands on their skin (Fig. 1C). We noticed that the pigmentation pattern of juveniles differs between aquariums. For instance, juveniles in the Osaka Aquarium Kaiyukan have thin stripes between the thick pigmentation bands (white arrowheads in Fig. 1C) and non-pigmented regions just beneath the eyes (black arrowheads in Fig. 1C), whereas other juveniles have darker and thicker bands with almost no stripes between them (bracket in Fig. 1D). Therefore, there may be several subgroups within this species. To specify the one that we examined, we sequenced approximately 1-kb-long fragments of the mitochondrial genomes of five individuals. The sequences were identical between them but showed 0.03 nucleotide differences per site from the one deposited in GenBank (JQ082337), suggesting intraspecific variation.

Early Stages

Before stage 9 (ca. 0–4 days post-deposition [dpd]; note that dpd is an estimation and may differ within this species due to genetic variations): The shape of the blastodisc at this stage seems to vary among individuals (Fig. 2A–C, two days before stage 9). Some are circular and have a crescent-like shape (Fig. 2A), whereas some are initially irregular. All of them become a smooth

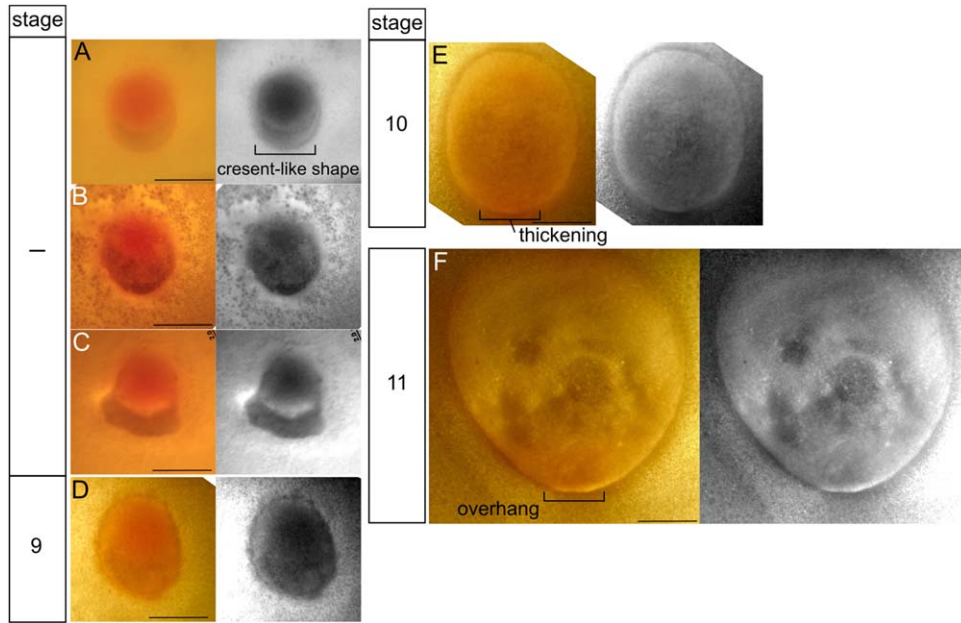


Fig. 2. Live embryos at early stages. **A–C:** The blastodiscs of three individuals two days before stage 9. **D–F:** An embryo from stage 9 to stage 11. Right panels: gray-scale images with high contrast. Note that A–D may not be oriented along with a particular axis of embryos. The brackets indicate the thickened edge (E) and the overhanging edge (F) of the embryonic blastodisc. Scale bars = 1 mm.

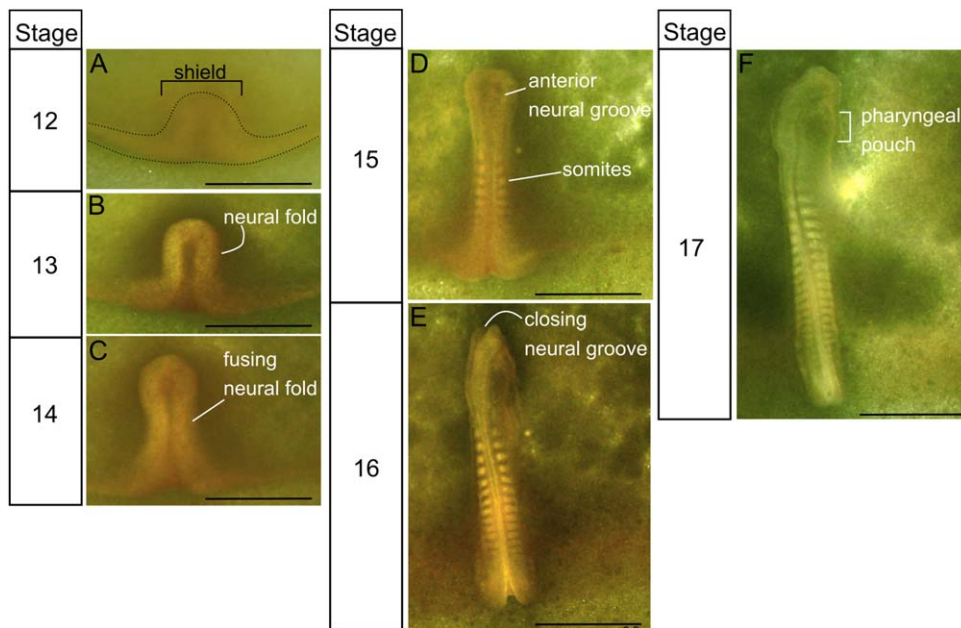


Fig. 3. A live embryo during neural formation. **A–F:** Time-series photos of an embryo at stages 12–17, respectively. Dorsal views; anterior is to the top. Scale bar = 1 mm.

circle or oval by stage 9. This stage takes around 5 days at 25°C after egg deposition.

Stage 9 (ca. 5 dpd): The blastodisc has started to expand (epiboly). The diameter of the blastodisc is about 1.5 mm (Fig. 2D) and progressively expands. It is shaped like a circle, and if the embryo was a crescent-like shape at the previous stage, the shape gradually fades away in this stage.

Stage 10 (ca. 6 dpd): The edge of the blastodisc is partially thickened, forming the embryonic shield (bracket in Fig. 2E).

Stage 11 (ca. 7 dpd): The embryonic shield shows further thickening (bracket in Fig. 2F) because the edge of the embryonic shield overhangs toward the outside of the disc as seen in catshark embryos at stage 11.

Neural Fold Closure

Stage 12 (ca. 8 dpd): The thickened edge forms a U-shaped or V-shaped neural fold (the embryonic shield) (Fig. 3A).

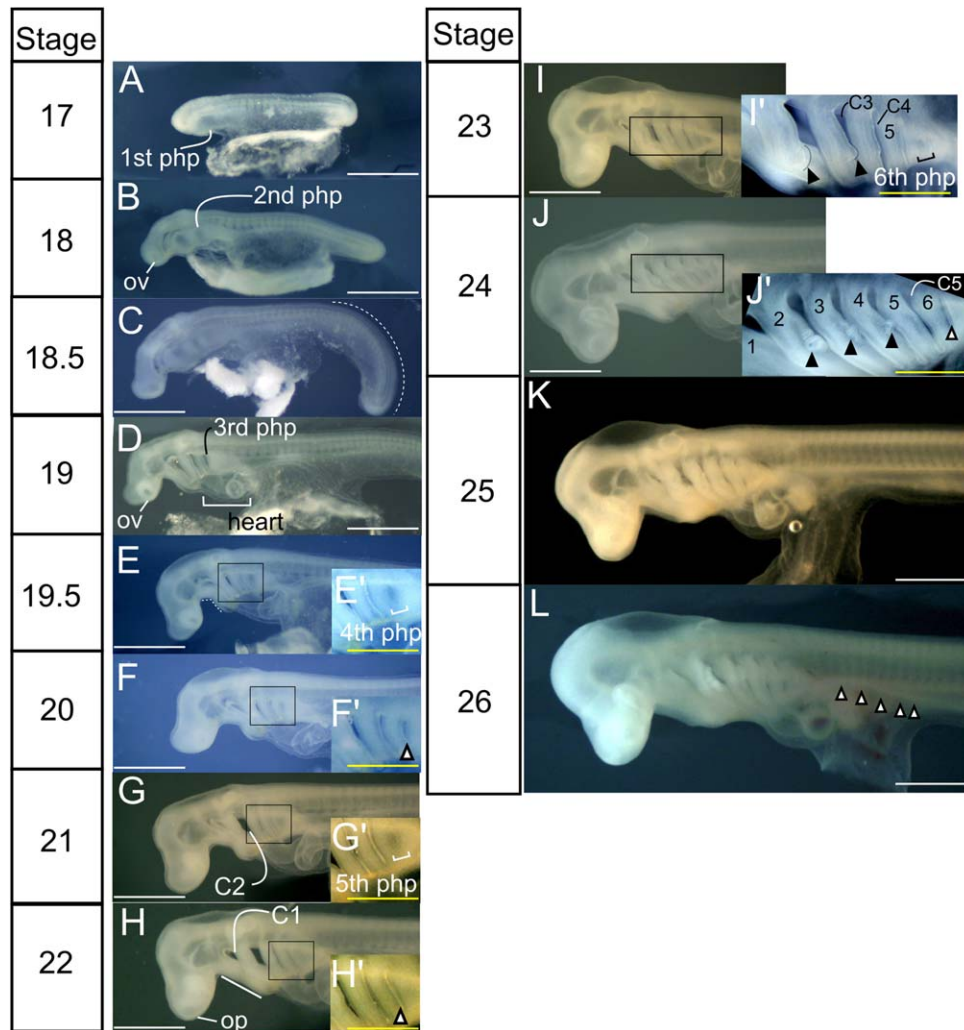


Fig. 4. Fixed embryos during pharyngeal formation. **A–L:** The lateral views of fixed embryos at stages 17–26, respectively. **E'–J':** Magnified views of boxes in E–J. The white arrowheads in F', H', and J' indicate newly forming pharyngeal pouches. The dashed line in C indicates a bending tail. The solid lines in E and H indicate bending and straightening mandibular arches, respectively. The black arrowheads in I' and J' indicate gill buds. The white arrowheads in L indicate pectoral fin buds. C1–5, pharyngeal cleft; op, olfactory placode; ov, optic vesicle; php, pharyngeal pouches. White scale bars = 1 mm. Yellow scale bars = 500 μ m.

Stage 13 (ca. 8.5 dpd): The embryonic axis becomes apparent as the neural fold extends toward the center of the blastodisc (Fig. 3B).

Stage 14 (ca. 9 dpd): The neural fold starts to fuse at the middle of the anterior-posterior axis, resulting in a keyhole-like shape (Fig. 3C).

Stage 15 (ca. 10 dpd): A neural groove remains at the anterior region of the embryo (Fig. 3D). The neural fold progressively fuses at the posterior end until stage 17. Due to the opacity of the embryo, it is very hard to see the internal structure; however, 3–9 somites are visible.

Stage 16 (ca. 11 dpd): 10–15 somites. The anterior neural groove is gradually closing to form the brain (Fig. 3E).

Stage 17 (ca. 12 dpd): 16–19 somites. The anterior neural groove is completely closed, and the neural fold at the posterior end is also closed by the end of this stage (Fig. 3F). The first pharyngeal pouches are visible in fixed embryos (Fig. 4A). The embryo starts moving by the end of this stage.

Pharyngeal Formation

From stage 18 onward, live imaging is not feasible because the embryos are transparent and move. Therefore, fixed embryos are shown in Figures 4–8. For these same reasons, counting somites in live embryos is extremely difficult. Thus, from this stage, the range of somite numbers indicates observations from fixed embryos, but not the range of a stage. Embryos with in-between ranges of somite number may be included in the closest stage or be designated by adding “early” or “late” if required.

Stage 18 (ca. 14 dpd): 20–26 somites. The second pharyngeal pouches are visible as translucence (2nd php in Fig. 4B). The brain primordium is divided into the fore-, mid-, and hindbrain compartments. The optic vesicles have begun to form (ov in Fig. 4B). The straight heart tube becomes visible by the end of this stage.

Stage 18.5 (ca. 15 dpd): 27–36 somites. The tail of the embryo starts curving toward the ventral side (Fig. 4C).

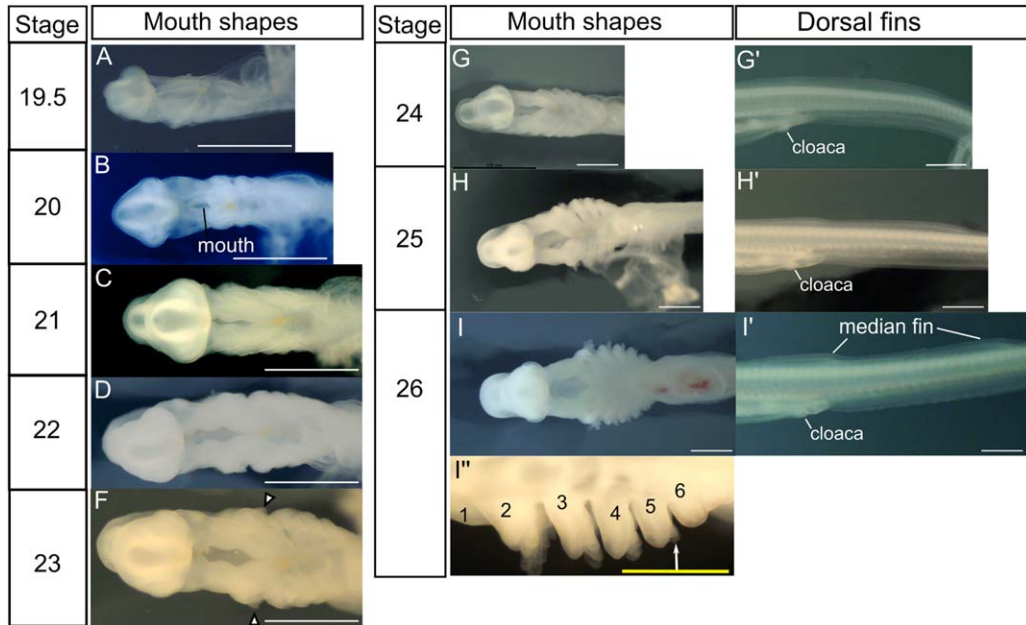


Fig. 5. The shape of mouths and dorsal fins during pharyngeal formation. **A–I:** Ventral views of head regions at stages 19.5–26, respectively. **G'–I':** Lateral views of a posterior part of embryonic trunks. **I'':** A dorsal view of the left pharyngeal arches of the embryo in I. The arrowheads in F indicate budding external gills. The arrow in I'' indicates a gill bud in the fifth pharyngeal arch. White scale bars = 1 mm. Yellow scale bars = 500 μm .

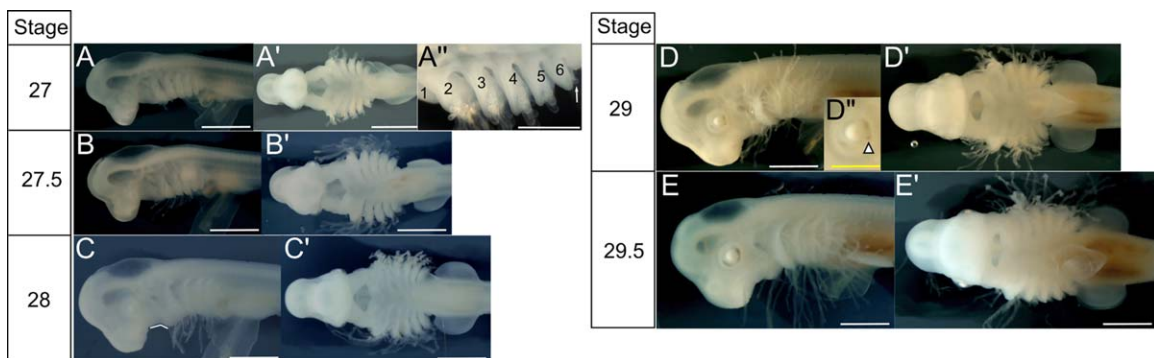


Fig. 6. Fixed embryos during early post-pharyngeal formation. **A–E, A'–E':** Lateral views and dorsal views of bamboo shark embryos at stages 26–29.5, respectively. **A'':** A dorsal view of the left pharyngeal arches of the embryo in A. The arrow indicates gill buds in the sixth pharyngeal arch. **D'':** A magnified view of the left eye of the embryo in D. The arrowhead in D'' indicates eye pigmentation. White scale bars = 1 mm. Yellow scale bars = 500 μm .

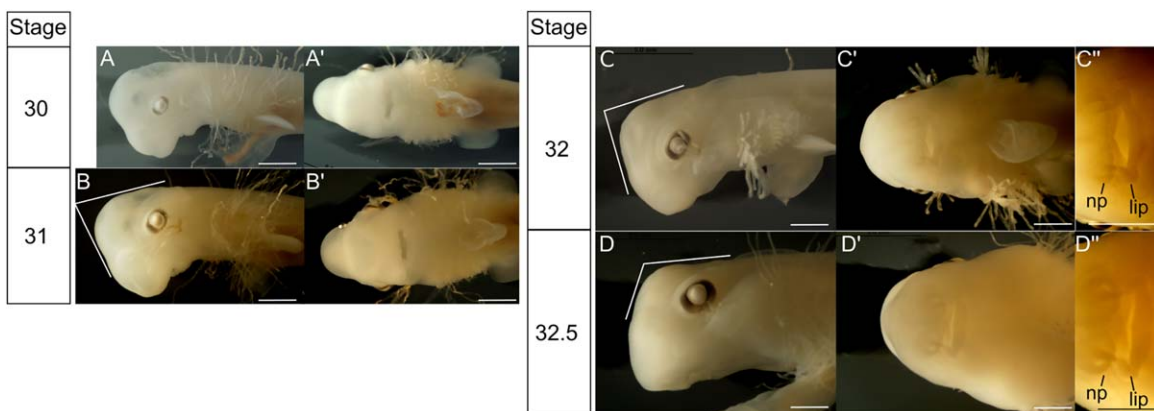


Fig. 7. Fixed embryos during late post-pharyngeal formation. **A–D, A'–D':** Lateral views and dorsal views of bamboo shark embryos at stages 30–32.5, respectively. The white lines in B, C, and D indicate the head angles. np, nasal process. Scale bars = 1 mm.

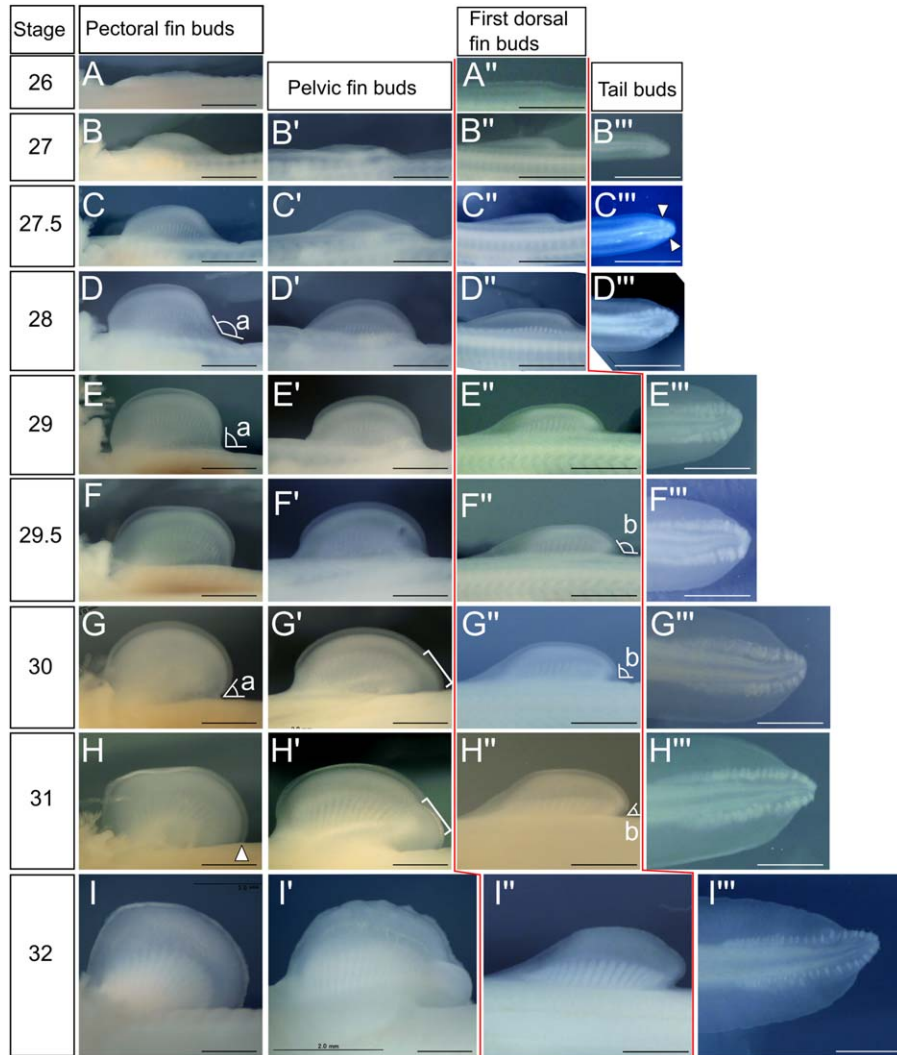


Fig. 8. The fins of fixed embryos during post-pharyngeal formation. **A–I, B'–I', A''–I'', B'''–I'''**: The pectoral fins, the pelvic fins, the dorsal fins, and the tails of bamboo shark embryos at stages 26–32, respectively. The “a” in D, E, and G denote the angle of the posterior edge of the pectoral fin bud toward the body axis. The arrowhead in H indicates the posterior edge of the pectoral fin. The brackets in G' and H' indicate a growing clasper. The “b” in F', G', and H'' denote the angle of the posterior edge of the first dorsal fin bud toward the body axis. The arrowhead in C''' indicates the primordia of denticles. Scale bars = 1 mm.

Stage 19 (ca. 16 dpd): 40–43 somites. The three pairs of pharyngeal pouches show slit-like shapes, although they are not open yet (3rd php in Fig. 4D). The heart tube starts looping. The optic vesicles become apparent (ov in Fig. 4D).

Stage 19.5 (ca. 17 dpd): 48–50 somites. The fourth pharyngeal pouches are visible as translucence (4th php in Fig. 4E'). In lateral view, the first pharyngeal arch bends, forming an obtuse angle (dashed line in Fig. 4E). The mouth is not yet open (Fig. 5A).

Stage 20 (ca. 18 dpd): 54–57 somites. The fourth pharyngeal pouches appear as slit-like shapes (arrowhead in Fig. 4F'). The mouth begins to open (Fig. 5B).

Stage 21 (ca. 19 dpd): 60–64 somites. The fifth pharyngeal pouches are visible as translucence (5th php in Fig. 4G'). The second pharyngeal pouches have opened widely with bending of the second pharyngeal arch (C2 in Fig. 4G), though the timing of opening may vary among individuals. The mouth has elongated and shows a slit-like shape (Fig. 5C).

Stage 22 (ca. 21 dpd): 66–72 somites. The fifth pharyngeal pouches appear as slit-like shapes (arrowhead in Fig. 4H'). The first pharyngeal pouches are opened (C1 in Fig. 4H). The timing of the third pharyngeal pouch opening seems to vary among individuals. From the lateral view, the shape of the first pharyngeal arch is becoming straight again (white line in Fig. 4H). The olfactory placodes become slightly visible (op in Fig. 4H). The curve of the tail is the greatest in this stage, and the tail gradually becomes straight again by stage 26.

Stage 23 (ca. 22 dpd): 77–79 somites. The sixth pharyngeal pouches are visible as translucence (6th php in Fig. 4I'). The third and fourth pharyngeal pouches have opened (C3 and C4 in Fig. 4I'). Small external gill buds appear on the second and third pharyngeal arches (black arrowheads in Fig. 4I').

Stage 24 (ca. 24 dpd): The fifth pharyngeal pouches appear as slit-like shapes (white arrowhead in Fig. 4J'). Small external gill buds appear on the fourth pharyngeal arches (black arrowheads in Fig. 4J'). From this stage, due to the opacity of embryos, the

somite number cannot be counted precisely from the external appearance.

Stage 25 (ca. 26 dpd): The mouth is changing its shape to pentagonal (Fig. 5H). Whereas in catshark embryos at stage 25, pectoral fin buds are visible as a ridge (Ballard et al., 1993), there is no sign of them at this stage in the bamboo shark.

Stage 26 (ca. 28 dpd): The first and second dorsal fin buds appear (arrowheads in Fig. 5I'). The pectoral fin buds become visible. The tail has become straight again. The sixth gill slits are open by stage 27. External gill buds appear on the fifth pharyngeal arches (arrow in Fig. 5I'').

Post-pharyngeal Formation

Stage 27 (ca. 30 dpd): At this stage, the first and the sixth pharyngeal arches start budding their external gill filaments (arrow in Fig. 6A''), showing the sixth gill buds). The pectoral fin buds appear as round projections, and the pelvic fin buds start to grow (Fig. 8B,B').

Stage 27.5 (ca. 32dpd): The optic vesicles become circular. The primordia of dermal denticles start forming in the tip of the tail bud (Fig. 8C''').

Stage 28 (ca. 34 dpd): Convergence of the left and right maxillary processes mark this stage, resulting in the transition of the mouth shape from pentagonal to rectangular (Fig. 6C'). In the lateral view, the mandibular arch is bent again (white line in Fig. 6C). At this stage, the eyes do not have pigmentation.

Stage 29 (ca. 38 dpd): Pigment first appears in the eyes (arrowhead in Fig. 6D''). The mouth shape becomes oval (Fig. 6D'). Muscle migration can be seen as stripes in the first dorsal fin (Fig. 8E'').

Stage 29.5 (ca. 40 dpd): The eye pigment becomes thicker (Fig. 6E). Although the pelvic fin buds emerged after the pectoral fin buds, now both sets of buds are of similar size (Fig. 8F,F').

Stage 30 (ca. 42 dpd): From the dorsal view, the posterior edge of the pectoral fin buds shows an acute angle to the body wall (a in Fig. 8G), and that of the first dorsal fin shows an orthogonal angle (b in Fig. 8G''). In male embryos, the primordia of the claspers start to bud from the posterior part of the pelvic fin buds (bracket in Fig. 8G',H'). Some of the primordial dermal denticles on the tail tip become sharper.

Stage 31 (ca. 46 dpd): The eyes are now completely surrounded by black pigment (Fig. 7B). The proximal-posterior ends of the pectoral fin buds have moved to the ventral side of the body and are obscured by the trunk when viewed dorsally (arrowhead in Fig. 8H). The posterior edge of the first dorsal fin now forms an acute angle with the body (b in Fig. 8H'').

Prehatching Period

Stage 32 (ca. 50 dpd): The nasal process becomes prominent (np in Fig. 7C'',D''). A lip-like structure also surrounds the mouth (lip in Fig. 7C'',D''). The dorsal angle of the head (solid line in Fig. 7C) is approximately orthogonal. In live embryos, the edge of median fin folds often becomes white and starts to reduce from this stage (Fig. 9A'), but sometimes from stage 31. The yolk sac becomes white as well. However, such white color of the fin edges is not recognizable in fixed embryos.

Stage 32.5: The angle formed by the anterior and dorsal surfaces of the head has changed from a right angle to an obtuse angle (compare solid lines in Fig. 7C,D).

Stage 33 (ca. 58 dpd): The second dorsal fin shows pigmentation (arrowhead in Fig. 9B').

Stage 34 (ca. 66 dpd): The reduction of the median fin folds has completed. The stripe pigmentation, which is characteristic of young bamboo sharks, starts from the posterior part of the tail (arrowheads in Fig. 9C).

Stage 35 (ca. 73 dpd): The external gill filaments have apparently reduced but still remain in the posterior pharyngeal slits (bracket in Fig. 9D'). The main eight pigmentation bands (arrowheads in Fig. 9D) on the skin also appear.

Stage 36 (ca. 80 dpd): The external gill filaments have now completely disappeared (Fig. 9E). Pigmentation starts gradually on the posterior part of the pectoral fins.

Stage 37 (ca. 90 dpd): Small spot pigment appears in the anterior part of the pectoral fins (arrowheads in Fig. 9F,F').

Stage 37.5 (ca. 104 dpd): The yolk is dramatically shrinking, and the trunk of the embryo becomes wider, likely due to of the absorption of the yolk nutrients (Fig. 9G). The color of the stripes on the skin becomes darker.

Stage 38 (ca. 111 dpd): The yolk is almost completely absorbed but there is still a remnant of the yolk sac (arrowhead in Fig. 9H').

Stage 39 (ca. 118 dpd): The remnant of the yolk sac has been internalized (arrowhead in Fig. 9I').

Measurements of the Developmental Time Scale

To measure the time scale of each stage, eggs were incubated in artificial seawater at 25°C. We first tried to culture embryos without their egg cases in artificial seawater. We succeeded in reliable cultures of embryos only from stage 31 without the egg case due to the fragility of yolks in early-stage embryos. In stages 26–27, the egg case can be partially removed. The surrounding gel of the yolk seems to be required until stages 29–30 to protect the yolk from shearing by the bottom of the culturing dishes. Until stage 25, even a partial removal of egg cases is lethal for bamboo shark embryos if the eggs are surrounded by seawater. The reason seems to be more than just the fragility of the yolk, because any small hole causes the embryos to die. Therefore, to visualize young embryos, only the outer layers of egg cases were removed as much as possible. From stage 18, to stop embryos from moving, eggs were cooled on ice for 10–20 min before taking pictures. In addition, we also measured the developmental speed of embryos from stage 19.5 to stage 26 by removing a part of egg cases in a culture medium that we developed (see the next section for details). We measured at least two samples per stage. The results of the measurements are indicated in Table 1. Because we removed egg cases from stage-31 embryos to visualize them, we could not determine the exact hatching timing. Instead, we considered the complete absorption of their yolk sacs or the first defecation as hatching. The estimated duration until the hatching period is around 118 days, which is approximately 1.5–1.8 times as fast as that of the small-spotted catshark, indicating an experimental advantage for working with bamboo shark embryos.

Culturing Early Embryos of the Bamboo Shark

Culturing embryos without their egg cases is convenient for visualizing them as well as performing experiments such as cell-lineage tracings and drug treatments. However, as mentioned above, bamboo shark embryos are vulnerable to seawater until

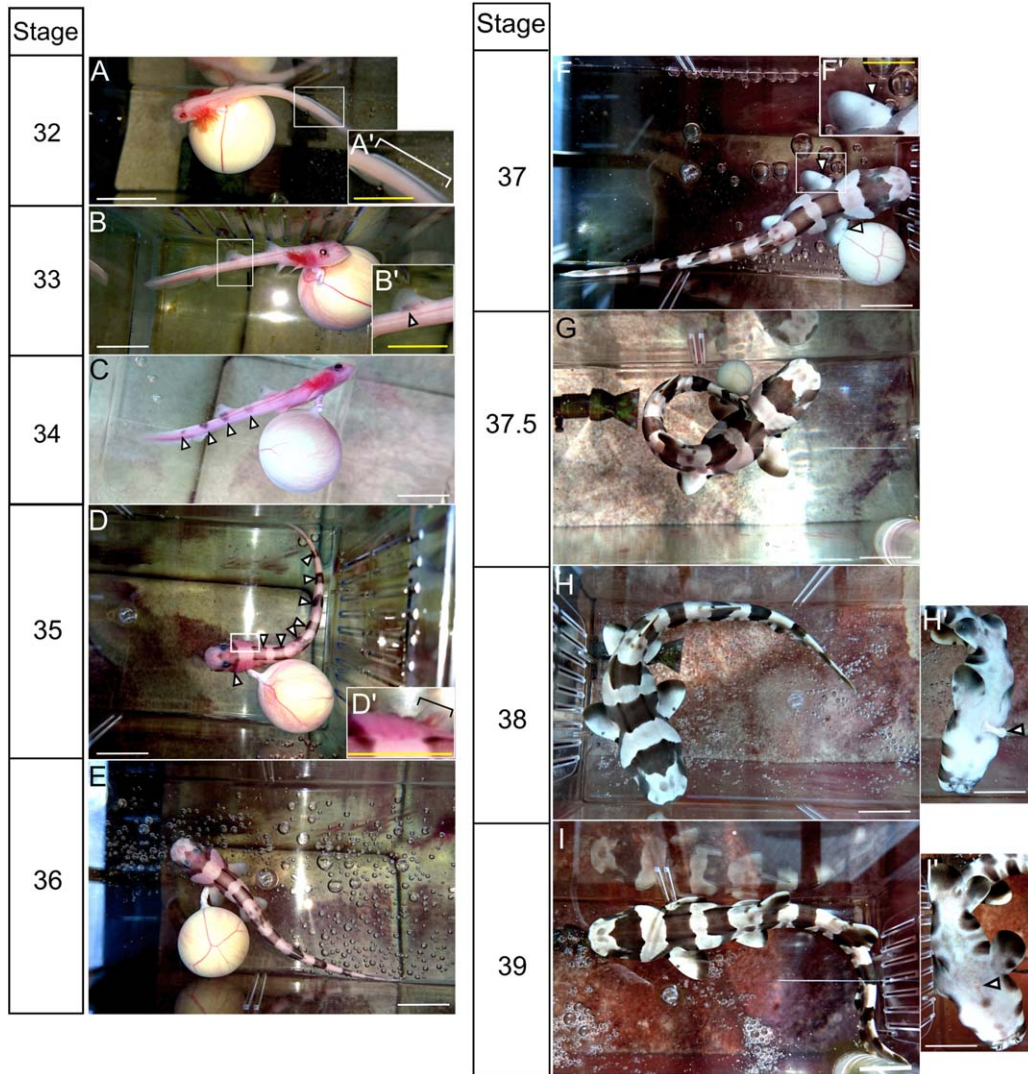


Fig. 9. A live embryo during prehatching period. **A–I:** Bamboo shark embryos at stages 32–39, respectively. **A', B', D':** Magnified views of regions indicated by boxes in A, B, and D, respectively. The bracket in A' indicates a white fin edge. The arrowheads in B', C, D, and F indicate skin pigmentation. The bracket in D' indicates remaining gill filaments. The arrowheads in H' and I' indicate reducing yolk sacs. White scale bars = 2 cm. Yellow scale bars = 1 cm.

stage 25. To address this problem, we tested several conditions. As the main cause of the lethality in catshark embryos was suggested to be bacterial infections (Ballard et al., 1993), we first added four antibiotics—gentamicin, amphotericin B, penicillin, and streptomycin (Poyer and Hartmann, 1992)—into filtered artificial seawater at the density of 1026 kg/m^3 (nearly equal to that of the aquarium water), which we refer to as “antibio seawater.” We placed partially opened eggs in small containers filled with the antibio seawater (Fig. 10) and found that early embryos ranging from stages 9 to 19 grew for at least seven days in this condition ($n = 4/6$), and one of them kept growing more than 14 days from stage 19. Because commercial seawater often does not have a detailed table of components, we also developed a more defined solution. To this end, we simplified a solution previously used to culture elasmobranch cells in vitro (Poyer and Hartmann, 1992) by reducing nutrients and lowering the concentration of urea to adjust the osmolality to approximately 1 Osm/L , which we named the “new shark solution” (NSS). In addition, we tried a simpler

solution, the NSS2, which contains only NaCl, a buffering agent, and the antibiotics. The NSS and NSS2 also allowed early embryos to grow more than seven days ($n = 4/5$ and $n = 3/5$, respectively), and two embryos cultured in NSS beginning at stages 15 and 18, respectively, developed normally at least until stage 26. Because one-third of eggs were usually mutants, as we noted above, we could not distinguish whether the abnormal development was caused by the culturing conditions or intrinsic factors. Together, the antibio seawater and the NSSes provide opportunities to study the early development of cartilaginous fish embryos.

Discussion

We have defined a staging table for bamboo shark embryos. This description is the first detailed and formal staging table of the embryonic development of the bamboo shark. It takes around 118 days for embryos to reach the hatching period when they are

TABLE 1. Summary of the Staging Table of the Brownbanded Bamboo Shark embryos^a

Stage	Duration (days)	Observed deviation (days)		Estimated days post deposition	Catshark stage
		minus	plus		
Before stage 9	5	0	0		4–8
9	1	0	0	5	9
10	1	0	0	6	10
11	1	0.5	1	7	11
12	0.5	0	0.5	8	12
13	0.5	0	0	8.5	13
14	1	0.5	1	9	14
15	1	0.5	0	10	15
16	1	0.5	0	11	16
17	2	0	1	12	17
18	1	0	0	14	18
18.5	1	0	0	15	
19	1	0	0	16	19
19.5	1	0	0	17	
20	1	0	1	18	20–22
21	2	0	1	19	21–22
22	1	0	1	21	22
23	2	0	1	22	23–24
24	2	0	1	24	24–25
25	2	0	0	26	24–26
26	2	0	0	28	24–28
27	2	0	0	30	25–30
27.5	2	0	2	32	
28	4	0	2	34	28–30
29	2	0	0	38	29–31
29.5	2	0	2	40	
30	4	2	0	42	30–31
31	4	0	3	46	30–31
32	8	2	2	50	32
33	8	1	0	58	32
34	7	0	1	66	32
35	7	1	0	73	32
36	10	0	6	80	32
37	14	0	0	90	33
37.5	7	0	3	104	
38	7	0	1	111	33
39	N/A			118	34

^aThe duration indicates days to reach the next stage. The observed deviations indicate maximum deviations from the duration of each stage. The estimated days post-deposition is calculated by the cumulative sum of the median durations. The rightmost column indicates small-spotted catshark stages that share similar morphological characters with bamboo shark stages.

cultured at a stable 25°C in artificial seawater (Table 1), which is approximately 1.5 times as fast as catshark embryos (see Fig. 11 for a comparison). Our staging table is largely based on the catshark stages for cross-species consistency in numbering stages. Particularly, stages 9 to 21 of bamboo shark embryos share similar morphological characteristics with the corresponding stages of catshark embryos. Stages 22 to 31 of bamboo shark embryos are also similar to those of catsharks, but they show some

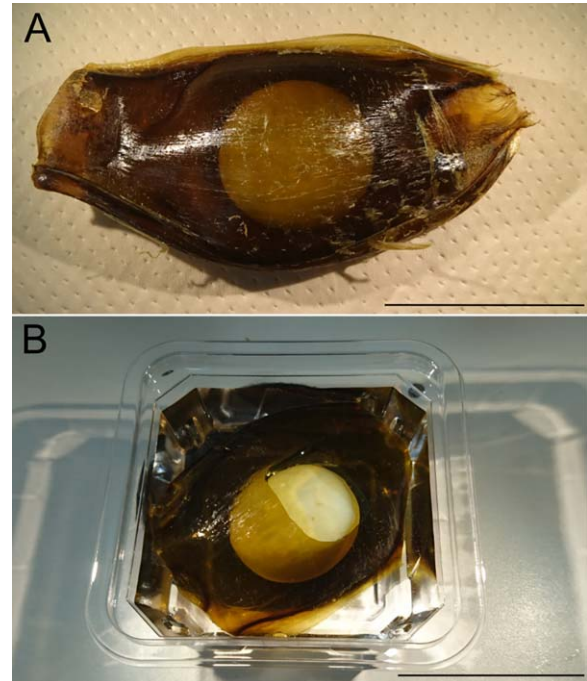


Fig. 10. Culturing embryos. **A:** A bamboo shark egg after the outer layers were removed (compare with Fig. 1B). **B:** An example of embryo cultures.

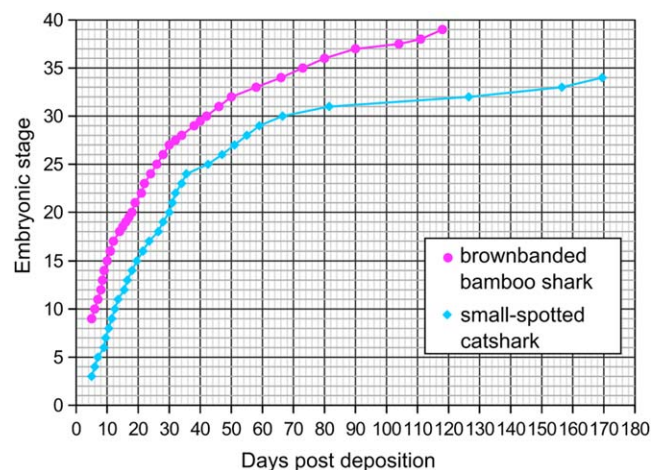


Fig. 11. A plot of the embryonic stage progressions of the bamboo shark and the catshark. The horizontal axis, days post deposition; vertical axis, embryonic stages. The stages between the bamboo shark and the catshark are not directly comparable to each other, yet the plot visualizes the different progressions of stages between species. Note that bamboo shark embryos reach the final hatching stage 50 days faster than catshark embryos.

differences in the timing of organogenesis. In addition, our staging table includes mid-stages (e.g., stage 27.5), when the landmarks used to define a stage are subtle. After stage 31, we have described more details than the catshark staging table, which raises the highest stage number to 39. Regarding embryo earlier than stage 9, we may not be able to obtain blastomere (early cell cleavages) stages because they may grow in maternal oviducts. We obtained several bamboo shark embryos that may correspond

to catshark stages 4 to 8. However, unlike catshark embryos, the shape of the blastodisc at these stages shows substantial variation. Therefore, we did not separate it into several stages. Overall, our staging table consists of 38 separate stages, including mid-stages and “before stage 9.”

For a similar reason to the Hamburger and Hamilton stages of chick embryos (Hamburger and Hamilton, 1951), external morphological characters of embryos serve as landmarks for our staging table. Landmark-based staging tables are more robust than time-based ones because the developing speed of embryos depends on the culturing temperature and individuals. Indeed, in the previous study (Harahush et al., 2007), there is an apparent mismatch between dpd and the degree of embryonic growth (the embryo at 55 dpd in Fig. 4 is younger than the one at 42 dpd in Fig. 5A in this report). This mismatch is probably caused by the temperature variation of their tank. Thus, our morphological landmark-based staging table would be more useful for specifying a precise developmental time point.

In this study, we found appropriate culture conditions for early embryos of the bamboo shark with a partial opening of their egg cases. The difficulty of ex ovo cultures with young embryos is a known issue in catsharks (Ballard et al., 1993; Yonei-Tamura et al., 2008). The possible causes may depend on species and include the fragility of yolks, infection, osmotic and/or component differences between seawater and the liquid in egg cases, or combinations of these factors. As antibiotics improved the viability of embryos, the difference of the components between seawater and the liquid in egg cases may not be a major problem in the bamboo shark. Although we were able to culture early embryos of the bamboo shark, whether the same conditions can be applied to other species such as catsharks remains to be confirmed. Together, our findings potentially extend the capacity for experiments with bamboo shark embryos to include cell-lineage tracing analyses, drug treatments, electroporation, and so on.

We obtained bamboo shark embryos from only one aquarium. Therefore, a potential concern is that bamboo shark embryos from different areas or aquariums may show some heterochrony of developmental events due to natural variation within this species. Because the external embryonic characters even between the small-spotted catshark and the bamboo shark are similar from stage 9 to stage 31, natural variation within a species may not occur in these stages. However, there seems a variation in the skin pigmentation pattern of juveniles. Interestingly, analysis of mitochondrial genes also suggests that the brownbanded bamboo shark consists of subspecies (Naylor et al., 2012). Although it is uncertain if skin color variation is caused by environmental or genetic differences, the sequence of pigmentation onset in the prehatching period may differ within the species.

The rightmost column in Table 1 shows a rough comparison between the staging tables of catshark and bamboo shark embryos. Although the cross-species comparison of developmental stages is quite difficult and sometimes uninformative, we can recognize some conserved time-sequence of developmental events. In particular, stages 9 to 20 may be comparable between them, in which embryos form the main axis, the somites, and the anterior portion of the pharyngeal arches. On the other hand, before stage 9 and after stage 20 we observed several interspecific and intraspecific variations of developmental events. An obvious example is the timing of median fin growth. Whereas in the catshark, median fins start growing after pectoral and pelvic fin buds appear (Ballard et al., 1993), in the bamboo shark they

appear before pelvic fin buds. Before stage 9, bamboo shark embryos show substantial variation even among individuals.

Since the late 19th century, embryonic development has been described for several cartilaginous fishes, including sharks (*Chiloscyllium punctatum*, *Chlamydoselachus anguineus*, *Heterodontus japonicus*, *Heterodontus portusjacksoni*, *Odontaspis taurus*, *Squalus acanthias*, and *Scyliorhinus canicula*) (Balfour, 1876; Ballard et al., 1993; Gilmore et al., 1983; Gudger, 1940; Harahush et al., 2007; Rodda, 2000; Scammon, 1911; Smith, 1942), batoids (*Leucoraja ocellata*, *Raja brachyura*, *Raja eglanteria*, *Rhynchobatus djiddensis*, *Rhinobatus halavi*, and *Torpedo*) (Balfour, 1876; Clark, 1927; Luer et al., 2007; Maxwell et al., 2008; Melouk, 1949) and chimaeras (*Callorhynchus milii* and *Hydrolagus colliei*) (Dean, 1906; Didier et al., 1998) (see Didier et al., 1998 for a comparison of those staging tables). The descriptions are often sparse, probably owing to the opacity of egg cases and the poor accessibility of eggs in many species. A few studies, mostly on oviparous (egg-laying) species, almost cover the complete series of embryonic development (Ballard et al., 1993; Dean, 1906; Luer et al., 2007; Smith, 1942). In particular, Ballard et al. (1993) is an exceptional study because they described a complete series of catshark embryos developing under a fixed temperature (16°C) with a time scale. Also, Luer et al. (2007) not only described embryonic development of the clearnose skate (*Raja eglanteria*), but also attempted artificial insemination. Although our staging table does not contain blastomere stages, we believe that it covers a wide range and a fine time-resolution of bamboo shark development that will be useful for researchers studying the developmental biology of this species. We also note that focusing on a few species would lead to biased conclusions in any research because the above well-described species may not represent the common characteristics of cartilaginous fishes (e.g., only 7 of 56 families in sharks are oviparous) (Conrath and Musick, 2012). In addition, cartilaginous fishes are important not only for evolutionary and developmental biology, but also for various fields, such as marine ecology, physiology, biological resources, and aquarium exhibitions. For these fields, it would be informative to know the hatching time, incubation temperature, and culture conditions without egg cases, as well as protocols for artificial insemination. Together, we hope that our work will be a valuable addition to knowledge about cartilaginous fishes.

Perspective

Even though jawed vertebrates consist of only two major groups, cartilaginous fishes and bony vertebrates, there have not been enough studies focusing on the former. One of the advantages of the bamboo shark is that its genome assembly, which will be released by our laboratory, has better continuity and coverage than that of other elasmobranchs, such as the whale shark and the little skate (Read et al., 2017; Wyffels et al., 2014; S. Kuraku, unpubl. data, 2017). We hope that our present study also will support diverse research with this species. One of the limitations of this species is that although experimental perturbations of shark embryos by drug treatments have been demonstrated (Cooper et al., 2017; Onimaru et al., 2016; Onimaru et al., 2015), there is still no way to directly modify their genomes. Methodological development for genetic modification that can be applied to this species will be one of the demanding challenges in the future.

Experimental Procedure

Animals

The eggs of the brownbanded bamboo shark, *Chiloscyllium punctatum*, were obtained from Osaka Aquarium Kaiyukan; the tank that kept adult bamboo sharks was maintained at 23°C. After transferring bamboo shark eggs to our laboratory tanks, they were cultured at 25°C in artificial seawater. The artificial seawater was prepared by dissolving commercial sea salt (Marine Art Hi, Tomita Pharmaceutical Co., Ltd.) in pure water at the density of 1026–1028 kg/m³. Because the intact egg cases were completely opaque, the surface layers of the cases were removed with a knife and forceps to view the embryos. For storage, embryos were fixed with 4% paraformaldehyde/phosphate buffered saline (PBS) at 4°C overnight, dehydrated with PBS/methanol, and stored in 100% methanol at -20°C. The photos of fixed embryos were taken in PBS after rehydration. Animal experiments were conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee (IACUC), RIKEN Kobe Branch.

Shark Solution Preparations and Culturing Early Embryos

The antibiotic seawater for one liter was prepared by filtering artificial seawater at the density of 1026 kg/m³ with a 0.2- μ m mesh (Acrodisc® Syringe Filters with Supor® Membrane, Pall); 50 mg gentamicin (Sigma-Aldrich), 50 mg amphotericin b (Sigma-Aldrich), and 10 ml 100 x penicillin-streptomycin (Thermo Fisher Scientific K.K.), were added to the seawater. The NSS for one liter was prepared by dissolving the following reagents in ultrapure water: NaCl, 18.8 g; NaHCO₃, 3.7 g; KCl, 0.4 g; glycine, 0.76 g; glycerol, 1.8 ml; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Nacalai Tesque), 6 g; urea, 5 g; trimethylamine *N*-oxide (TMAO)·2H₂O (Sigma-Aldrich), 6 g; gentamicin, 50 mg; amphotericin b, 50 mg; 100 x penicillin-streptomycin, 10 ml. The NSS2 for one liter was prepared with 28.6 g NaCl, 6 g HEPES, and the same concentrations of the antibiotics. After adding the antibiotics, the solutions were stored at 4°C.

To culture the bamboo shark embryos, the outer layers of the egg cases were removed (Fig. 10A) and wiped with 70% ethanol. The egg cases were cut with sterile scissors to fit in a sterile plastic container (approx. 300 ml). A small window (approximately 2 cm x 2 cm) was opened on each egg case with a sterile knife. The container was filled with above media and closed with the cover (Fig. 10B). The embryos were cultured in an incubator at 25°C.

Mitochondrial Genome Analysis

DNAs were extracted from individual embryos with DNeasy Blood & Tissue Kit (Qiagen). Partial sequences of the mitochondrial genome were amplified with polymerase chain reaction (PCR) using the following primers: 5'-ATCTGAGGTGGATTCTCAGTAGAC-3' and 5'-TATTCTCGAGCAGTAACCAGATGC-3'. The sequence information was deposited in GenBank under the accession number MF801629.

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