

# A Series of Normal Stages for Development of *Scyliorhinus canicula*, the Lesser Spotted Dogfish (Chondrichthyes: Scyliorhinidae)

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**ABSTRACT** By observing numerous living eggs from the lesser spotted dogfish *Scyliorhinus canicula* (L.) caught near Roscoff (France) and reared at recorded temperatures as they developed from first cleavage to hatching, the first reasonably complete developmental table was worked out for this classical material in vertebrate embryology. The successive stages, described and numbered from 1 to 34, correct and replace the incomplete stages A–Q proposed by Balfour (J. Anat. Physiol., 10:555–576, 1876) and other even less complete series later published, and is unique in the inclusion of a timetable at 16°C. The stages can be identified, usually through the cleared eggshell wall, with naked eye or low magnification. This table of normal stages of *Scyliorhinus* can be adapted with slight modification to other chondrichthyan fishes. © 1993 Wiley-Liss, Inc.

Normal-series stages have been published for most of the animal species widely used in experimental embryology, as an aid to others in confirmation of results. An attempt is usually made to mark each stage by the first appearance of some easily recognized structure or event; the usefulness of a series depends on the closeness and obviousness of the steps, and upon a timetable of their appearance under defined conditions such as temperature. For example, landmark series in continued use are those for *Ambystoma* (essentially finished by Harrison in 1927 and widely distributed privately before publication in 1969), for the chick (Hamburger and Hamilton, '51), *Xenopus* (Nieuwkoop and Faber, '56), *Pleurodeles* (Gallien and Durocher, '57), the sturgeon (Dettlaff and Ginsburg, '54), and *Salmo* (Vernier, '69). Others have been published as necessary adjuncts to particular researches on less commonly used species with narrower focus, e.g., Ballard ('86) on *Amia calva*, following earlier studies on several teleost species. Such normal development series for a large number of both invertebrate and vertebrate species has been brought together by Dettlaff and Vassetzky (originally in Russian, later translated to English, '90) but still not including any elasmobranch species.

Earlier such collections usually included wider gaps and lacked timetables, though serving well the consolidation of comparative descriptive embry-

ology. In the first of several volumes edited by Oskar Hertwig and intended to summarize vertebrate embryology, Franz Keibel ('06) re-issued the illustrations of already published normal series of representatives of all classes from cyclostomes to mammals, whose descriptive embryology was being synthesized by the other authors.

At that time the single major study of elasmobranch development was that of Balfour (1876). Despite the high reputation of that author, the defects of his normal series were well recognized. Earliest and latest stages of development were inadequately covered, his Stage E was admittedly abnormal, and he had had to fill gaps in what was otherwise a series for "*Scyllium*" (now *Scyliorhinus*) with specimens of the shark "*Pristiurus*" (now *Galeus melastomus*) and the electric ray *Torpedo*. No time sequence could be offered for any of his haphazard collections.

In spite of this, the Balfour Stages A–Q were regularly and often imprecisely referred to by later investigators of elasmobranch development, whether of sharks or of rays. Proposals for improvement of the Balfour series, with specific and sole reference to the readily available species *Scyliorhinus canicula*, were made by Kastschenko (1888), Wintrebert ('22),

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Kopsch ('50), and Mellinger et al. ('84, '86) but only for the stages which they were studying.

Franz Keibel later edited the series of monographs "Normentafeln der Entwicklungsgeschichte der Wirbeltiere" in which normal series for seven mammals, two birds, one lizard, three urodeles, and four fishes had been published when the effort was discontinued in 1938. Number 12 in the series was the monograph of Scammon ('11) on *Squalus acanthias*, the first and only reasonably complete survey of the development of an elasmobranch since that of Balfour.

Most unfortunately, *Squalus acanthias* is quite useless for experimental embryology, not only because the viviparous female carries just 1–7 embryos through a pregnancy lasting nearly 2 years during migrations of thousands of kilometers in open ocean, but also because it is intolerant of captivity in aquaria, and Scammon could only arrange his randomly collected specimens in the order of their presumed age, with no possibility of guessing how long they might have been developing under controlled conditions.

Both these works of Balfour (1876) and Scammon ('11) are famous landmarks in the history of vertebrate development, but in fact to this date no reasonably complete list of all the easily distinguishable steps in the development of any readily available elasmobranch species has been brought together in one place and one language, including a timetable of their sequence at an optimum constant temperature.

Since *Scyliorhinus canicula* is commonly harvested in large numbers along all the coasts of Europe and is the only elasmobranch species known to us from which any stage of development can be obtained in abundance at any time of year, it seems appropriate to offer a corrected and complete list of stages easily recognized in its development from fertilization to hatching, with a short description of each and a timetable for their sequence at an optimum constant temperature.

Previous reports of development rates in *Scyliorhinus canicula* are limited to a few gastrulation stages described by Kopsch ('50) in "degree-days," and some records of early organogenesis by Vandebroek ('36) which we find wildly different from what we have repeatedly observed in material from Roscoff, where most of his work was done. Our time charts were assembled from continuous observation of living embryos developing in controlled constant temperatures.

We find that many if not all the illustrations of elasmobranch development in the older literature

can be referred precisely to one or another of the closely spaced stages of *Scyliorhinus* herewith, irrespective of the species, shark or ray. Experiments with the appropriate stages of *Scyliorhinus* can thus be more closely compared with earlier work and lead perhaps to resolution of older disputes, for example, about the morphogenetic cell movements and formation and distribution of the germ layers that may have arisen through interpretation of serial sections from less precisely defined embryos of the same or different species.

## MATERIALS AND METHODS

Trawlers and line-fishermen bring to the market large numbers of specimens of *Scyliorhinus canicula* at all the principal harbors of Europe. The species lacks a sharply defined breeding season: at any time of the year many of the females will be found to carry a pair of recently fertilized eggs. Records at Plymouth (Harris, '53) and Roscoff (Wegnez, '76) show monthly variations in catches ranging from 10% of such "pregnant" females in August and September to more than 40% in June and July. Similar observations have been made on *Scyliorhinus* in the Mediterranean (Capapé, '77, '78).

The fish being unloaded by trawlers are dead but the females that are carrying a pair of eggs can be determined by squeezing their flaccid bellies. Not uncommonly a female may be found with an egg already protruding from her cloaca; for the rest it is a quick matter to dissect the pair of them from her oviducts. Well protected within their tough eggshells, they can be handled quite roughly and transported dry for an hour or two without harm, continuing their normal development when restored to cold sea water.

A layer of jelly and the strong elaborate eggshell are secreted over each ovum as it passes through one of the oviducal glands. An egg taken from the anterior end of the oviduct will be found in uncleaved or early cleavage stages, perhaps with the eggshell incomplete at one end and lacking its final, anterior tendrils. An egg in such an unfinished eggshell quickly dies if put into sea water. An egg found in the middle or end of an oviduct will be in quite late cleavage but will not yet have started its morphogenetic cell movements.

*Scyliorhinus* females kept in aquaria may continue to lay pairs of eggs at gradually lengthening intervals over several months (Mellinger, '83). These have been fertilized during their passage through the oviducal glands which harbor long-time potent supply of sperm in their tubules (Metten, '39).

However, aquarium specimens can hardly insure

an abundant or reliable source of embryonic material. Preferably one should meet the boats of the fishermen as they bring their catch to shore, select the "pregnant" females, and take their eggs at once. For instance, one of us, preparing for experiments at Roscoff, harvested more than a thousand of these eggs during January and February of 1983, in series of visits to the workshop of a wholesale fish merchant at Le Diben, Plougasnou, near the Roscoff Biological Station, gathering from 50 to more than 100 eggs per visit. The batches, incubated separately, provided a constant supply of embryos at any desired stage. Eggs that continued to develop normally were on occasion taken from females that had been packed overnight on ice.

Another of us has carried or shipped many such eggs across France to the inland laboratory at Reims, in thermos jugs partly filled with sea water. They were incubated in a closed system of artificial sea water, filtered, and kept at the optimum temperature of 16°C, with negligible loss. Such shipments have also been made to the USA without harm, developing there to successful hatchings several months later.

Sea water from the western English Channel off Roscoff has a salinity of 35‰ (Latrouite and Raguenès, '85), and its temperature near the bottom varies from 10.5 to 12.5°C during the year (Agoumi et al., '83). At Reims, the artificial sea water was adjusted to a density of 1.025–1.030, which corresponds to the natural salinity. The higher temperature we adopted gave entirely normal but faster development, much more convenient for laboratory experiments than the rate set by the running sea water at Roscoff with its winter temperature averaging 10°C.

If an investigator needs to study a particular stage of *Scyliorhinus* development, selected from a batch of eggs for which only the date of removal from the oviducts is known, it should be noted that the timetable given here (see Table 1) may be short by as much as 4 or 5 days from the time of fertilization. However, the stage reached by an individual egg may be determined by making a transparent window in its eggshell (Wintrebert, '20a).

The *Scyliorhinus* yolk mass, an ovoid about 25 mm long, can be made visible within the intact eggshell. The 2 mm embryonic area rests somewhere on its surface, its bright orange color in marked contrast to the light greenish or yellowish yolk, but it can usually be seen through the intact eggshell whose surface is variably pigmented. Moreover, the pigment can be shaved off with a razor (His, 1897; Foulley and Mellinger, '80) leaving the glassy-

transparent inner layers for a closed window through which it can be clearly seen (Fig. 1A). Since the position of the embryonic area is controlled by gravity (Mellinger et al., '86), the search for it can be shortened. When eggs are suspended in a tank by their anterior tendrils, it usually rests at a short distance from the top of the yolk mass, close to either edge of the eggshell. Later on, this optimal position will result in a blastoderm that covers the top of the yolk mass and an embryo that moves freely in the fluid-filled cavity above the yolk. The blastodisc's response to gravity continues until epiboly has started, so that, if the egg is moved, e.g., to the horizontal position, it may drift beyond the window that reveals it. However the window can be enlarged to catch up with it. Culturing the egg by suspension from the tendrils of the square end increases the chance of a successful hatching, which must take place through that end of the eggshell. On the other hand, hanging it by the tendrils of the horned end may make access to the embryo easier for experimental purposes. Wherever the embryo locates itself has no effect on its early development (see Fig. 8).

Thus exposed, the progress of the embryo toward a desired stage can be checked from day to day by hand lens or low-power microscope. The window will become cloudy over some days but can be made clear again by a slightly deeper shaving, though it must not be perforated. If sea water enters through even the tiniest opening, the young embryo will quickly die.

The prompt death of young stages of *Scyliorhinus* when exposed to sea water is not due to the loss of the osmoregulatory urea content of the egg. The urea may be retained either by the vitelline membrane that lies between the yolk and the surrounding jelly, or by the plasmic membrane of the egg cell, or both. The jelly has an ionic composition identical with sea water and contains no urea (Mellinger et al., '86). The damage is bacterial action that quickly rots the jelly if sea water is allowed to enter through a break in the eggshell.

In any case, it is quite difficult to remove the young embryo from the eggshell because of the brittleness of the vitelline membrane, at least until the embryo has formed a completely vascularized strong yolk sac, shortly before its natural "pre-hatching" time at about 85–115 days (Ouag Te Yio, '31; Mellinger et al., '86; Lechenault et al., '93). After this the embryo and its yolk can be removed without difficulty and cultured in sea water. Hatching occurs at 170–220 days at 16°C.

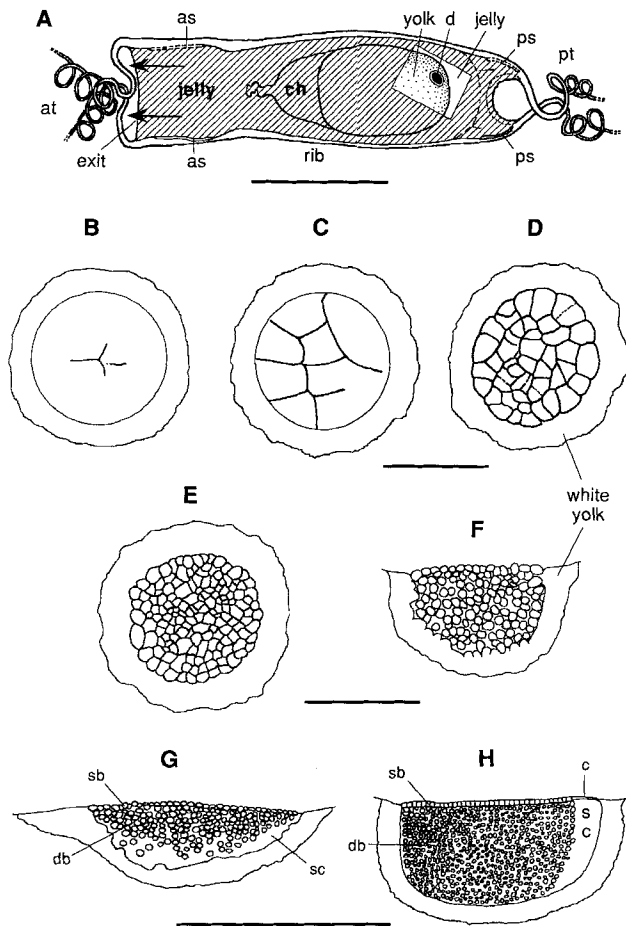


Fig. 1. Egg, eggshell and contents, and earliest cleavages. **A:** *S. canicula* eggshell seen from one side, the yolk mass and germinal disc exposed through a window made by shaving its pigmented layer off. Tendrils at either end, shown only in part, can be stretched up to a meter in length. **B–D:** top views of stage 1 germinal discs resting on their white yolk cups, in earliest cleavages. **E, F:** Stage 2 in top view and cross section, polarity not yet visible. **G, H:** in sagittal sections showing the beginning and the completion of aggregation of the inner blastomeres, with collection of intercellular fluid in a subgerminal cavity and toward the posterior pole under a membranous crescent.

#### Abbreviations

All drawings were made from photographs, with some exceptions where drawing was from live models.

Bar: 1 mm, unless otherwise indicated.

V–VII	placodes or ganglia of cranial nerves V and VII
x	vagus ganglion or placode
4 v.	fourth brain ventricle
A	Anal fin
as	anterior slits of eggshell
at	anterior tendrils of eggshell
bg	buccal groove (the so-called “stomodaeum,” in sagittal sections)
c	posterior crescent
Cl	hyomandibular cleft
C2–C6	other branchial clefts

Embryos to be used for photographs, dissections, or sections were first sketched at recorded magnifications with a drawing tube while alive in situ and then flooded with fixative to harden them before removal from the eggshell. Fixation was then completed, and the embryos again sketched to get a measure of shrinkage. A series was prepared with glutaraldehyde for sectioning, but fixation with Bouin's or  $\text{HgCl}_2$ -acetic solutions was adequate for most purposes.

We have not succeeded in early efforts to culture the embryo aseptically outside the eggshell. Kopsch ('50) reported the survival of a few embryos for 10 days in opened eggs kept in moist chambers. Vandebroek ('36) reported that he cultured embryos for 10 days in opened eggshells resting “dans un baquet couvert, sur un fond d'eau courante” at temperatures up to  $28^\circ\text{C}$ , which we find incredible. Vivien ('54) kept opened eggs in jars, with aureomycin added to the artificial sea water. More recent attempts (Foulley and Mellinger, unpublished), using penicillin, streptomycin, and nystatin, were unsuccessful in preventing the rapid putrefaction of the jelly that still coats the yolk. This technical problem is no doubt soluble.

ch	chalazial chamber (central cavity lying in the jelly-filled eggshell)
clm	cloacal membrane
d	germinative disc
db	deep blastomeres
D1, D2	first and second dorsal fins
egf	external gill filaments
endol	endolymphatic duct
exit	squared end of the eggshell, through which hatching will occur (arrows)
h	hypobranchial muscle sprouts
j	jaws or mandibular arch
kd	kidney duct
lat	lateral line canal or its placode
LC	lower half of the caudal fin
liv	liver
lp	lens placode
md	mandibular head cavity (coelomic)
mff	medial (or sagittal) finfold
olf	olfactory placode or pit
ot	otic placode
ov	optic vesicle
pect	pectoral fin
pro	pronephros
ps	posterior slits of eggshell
pt	posterior tendrils of eggshell
rib	lateral rib, where both halves of eggshell unite
rl	“red line”
sc	segmentation cavity
sb	surface blastomeres
St.	developmental stage

## RESULTS

### *Normal stages*<sup>1</sup>

#### Stages occurring in the oviducts

*Stage 1.* From fertilization to about 100 blastomeres (Fig. 1, B,C,D). The earliest cleavages occur before clearance from the oviducal gland, while the squared end of the eggshell and its tendrils may still be incomplete. More advanced cleavages occur while the eggs are still in the upper parts of the oviducts. In all the stages shown in Figures 1, 2, and 3, the blastodisc is shown surrounded by a ring of white yolk which separates it from the greenish or yellowish general yolk. In sections, the white yolk is seen as a cup.

*Stage 2.* There are more than 100 blastomeres but these are still distinct and individually bulging, as seen with hand lens (12×). There is no subgerminal cavity and the many internal blastomeres are loose and rounded (Fig. 1E,F).

*Stage 3.* Surface blastomeres are now a flattened epithelium and they are difficult to distinguish with a hand lens. Though not visible externally, a subgerminal cavity is beginning to form (Fig. 1G). The eggshell containing the egg now rests in the caudal part of the oviduct.

#### Stages of the laid egg, before onset of epiboly

*Stage 4.* The subgerminal cavity has gathered posteriorly, seen through a thin crescent-like membrane extending along the posterior quarter of the blastodisc margin (Figs. 1H, 2A,B). This stage may sometimes be reached before the egg has been laid.

*Stage 5.* The posterior crescent reaches its maximum development, extending along half the posterior margin of the blastodisc as the inner blastomeres clump more tightly together (Fig. 2C,D).

*Stage 6.* The posterior crescent is reduced by spread of deep migratory blastomeres underneath it. This is the beginning of the morphogenetic cell movements. The external appearance is often similar to that of stage 4 but note the contrast in sections (Figs. 1H, 2E,F, 3).

*Stage 7.* The posterior crescent has vanished but the blastodisc has not yet extended anywhere over the surrounding zone of white yolk. The surface view is similar to that of stage 3, but note the contrast in internal activity (Fig. 1G vs. Fig. 3). This is a stage of short duration.

#### Stages of early epiboly and embryo formation

*Stage 8.* The blastodisc has started to spread caudally, just covering the posterior part of the white yolk ring (Fig. 3).

*Stage 9.* The blastodisc has spread in all directions enough to mask entirely the white yolk ring (Figs. 3, 4). Caudal thickening hardly distinguishable from the rest of the germ ring, which is still usually circular but sometimes oval. Variable temporary folding of excess epithelium begins to appear on expanding blastoderm (Fig. 4).

*Stage 10.* Blastodisc round or more commonly oval, a crescentic embryonic shield forming posteriorly, still without any conspicuous overhang (Figs. 3, 4).

*Stage 11.* Definite two-layered epithelial overhang over caudal 45° sector of smoothly rounded margin of the blastodisc (Figs. 3, 4).

*Stage 12.* The embryonic shield includes a V that has its tip directed toward the center of the blastoderm, and a posterior midline "notochordal triangle" (so named by Kopsch, '50) between the bulging arms. This two-layered epithelial triangle comes by extension from the middle of the overhang that first became prominent at stage 11. It forms the roof of a cavity that has a blind end anteriorly and remains wide open caudally. The angle between the right and left arms of the thickened embryonic shield is narrowing to create the embryonic axis. Expansion of the blastoderm smoothes out most of the epithelial folding (Figs. 3, 4).

*Stage 13.* The two arms of the embryonic shield have closed together forming the embryonic axis, which does not yet show any lateral bulges indicating head development. Posteriorly the arms narrow more sharply as they lead to the general germ ring (Fig. 4).

*Stage 14.* The embryonic shield shows a head enlargement, which still lacks a medullary groove (Fig. 4).

*Stage 15.* The embryo shows an open medullary groove and flaring anterior medullary folds (Fig. 4). At the posterior overhang of the shield, its lengthening arms project beyond the otherwise circular border of the blastoderm, where they will form "posterior lobes" (Fig. 4).

*Stage 16.* (Fig. 4). Neural folds begin to close together and the anterior neuropore narrows toward closure. From 9 to 15 pairs of somites are now beginning to be visible in the living embryo. All sectors of the blastoderm are strongly expanding.

#### Metamerization of the pharynx and completion of the somite set

*Stage 17.* The first pair of pharyngeal pouches become detectable by translucence in surface view. At the end of this stage the trunk shows its first motility (Figs. 4, 5).

<sup>1</sup>Abbreviated summary for rapid identification.

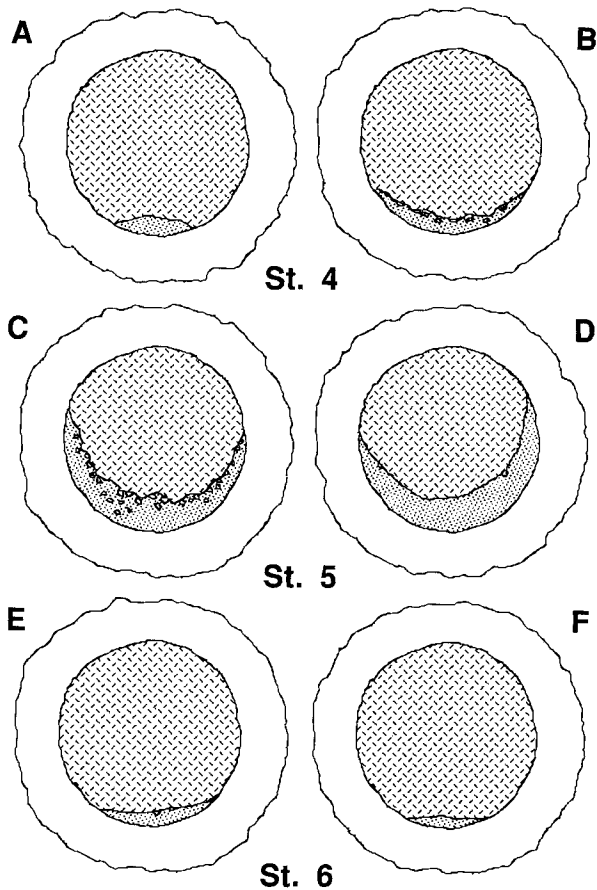


Fig. 2. Later cleavage and onset of epiboly. Top views of blastodiscs, each surrounded by white yolk. **A,B**: the posterior crescent just forming (as in Fig. 1, H). **C,D**: Maximum development of the crescent and start of scattering of deep mesenchyme. **E,F**: Narrowing of the crescent by epibolic spread of the surface epithelium and inner mesenchyme. See Figure 3 also. Much variation in these stages.

**Stage 18.** The first and second pairs of pharyngeal pouches become visible and a shallow buccal groove appears (Fig. 5).

**Stage 19.** The first three pairs of pharyngeal pouches can be seen and the buccal groove deepens (see Figs. 5, 8).

**Stage 20.** The first four pairs of pharyngeal pouches can be seen by translucence but the enlarged pharyngeal cavity still has no opening to the outside (Fig. 5).

**Stage 21.** The second pharyngeal clefts (hyobranchial, C2) and the mouth have opened (Fig. 6).

**Stage 22.** In addition to the second pharyngeal clefts, the first pair (C1, the future spiracles) have opened (see Figs. 6, 8).

**Stage 23.** Pharyngeal clefts C1, C2 and C3 are all open (see Figs. 6, 8).

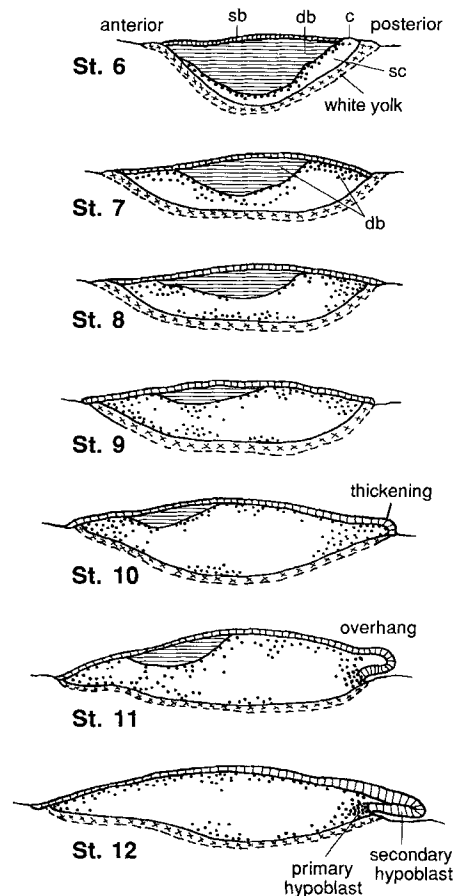


Fig. 3. Early epiboly stages, diagrammed from serial sections to show the surface epithelium (vertical hatching), white yolk cup (cross hatching), consolidated mass of inner blastomeres (horizontal hatching), mesenchyme (dots) spreading through the subgerminal cavity, and appearance of the embryonic shield (to be compared with Fig. 4). All sections are mid-sagittal except the one for stage 12, which is parasagittal.

**Stage 24.** Pharyngeal clefts C1 through C4 are open, and the first gill buds appear in clefts C2 and C3 (Fig. 6). In front view, the mouth opening has become diamond shaped and will remain so until stage 27 (Fig. 7).

**Stage 25.** First opening of pharyngeal clefts C5 (see Figs. 6, 8).

**Stage 26.** The entire set of pharyngeal clefts (C1 through C6) is now open, and gill buds show in all except the C1 spiracular clefts and C6. The final members of the somite series are forming toward the end of the tail. The yolk sac has been enveloped by an almost complete epithelial lining, half of which is underlaid by a capillary network. One can now culture the embryo and its strengthened yolk sac in sea water outside the eggshell.

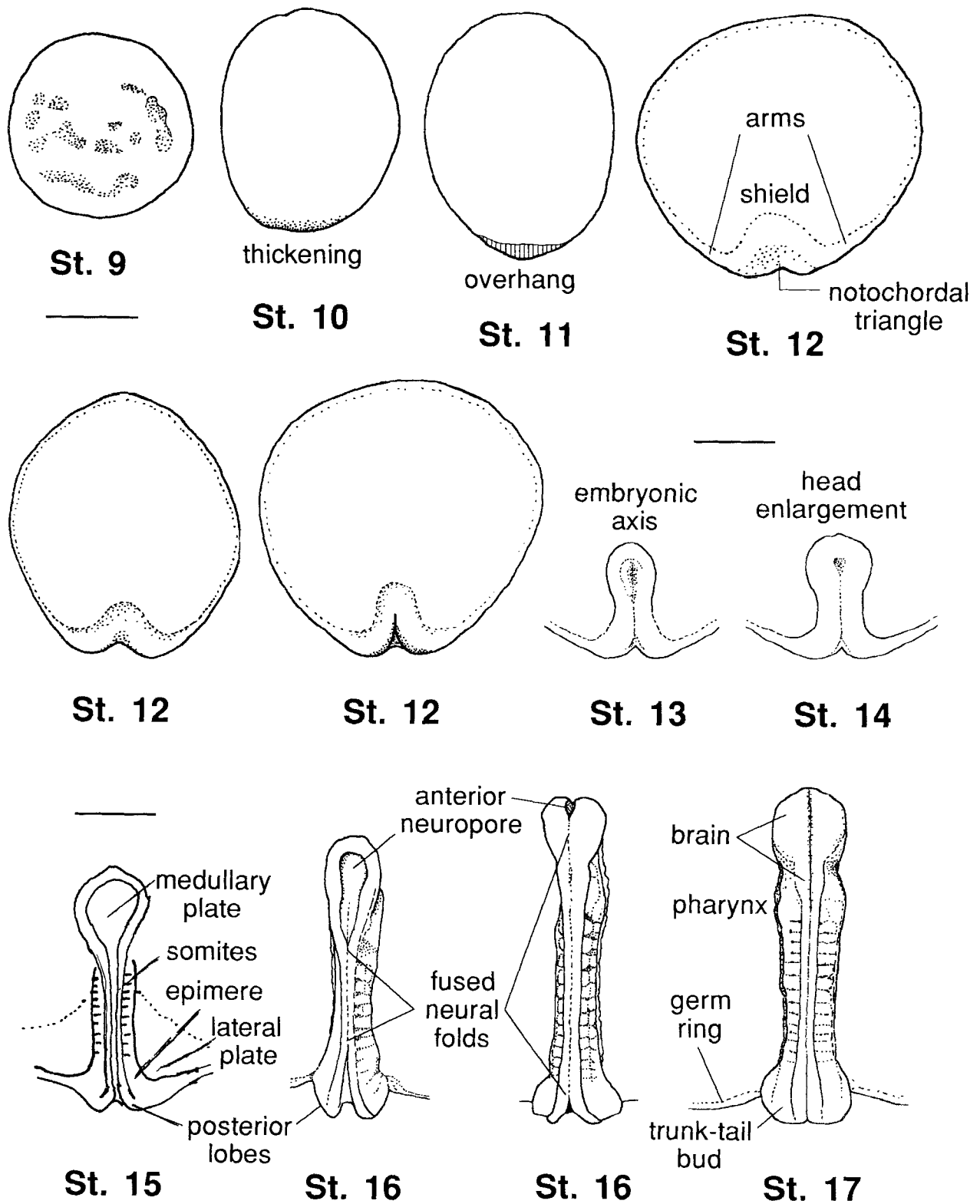


Fig. 4. Principal morphogenetic cell movements and earliest organogenesis shown in top views. Shading in the Stage 9 figure represents depressions in the wrinkled surface epithelium (variably seen in this and several succeeding stages). Compare stages 9–12 with sectional diagrams of Figure 3. Stage 12 is seen in rapidly succeeding early, middle, and late forms. Early and late stage 16 drawings show rapid closure of neural folds.

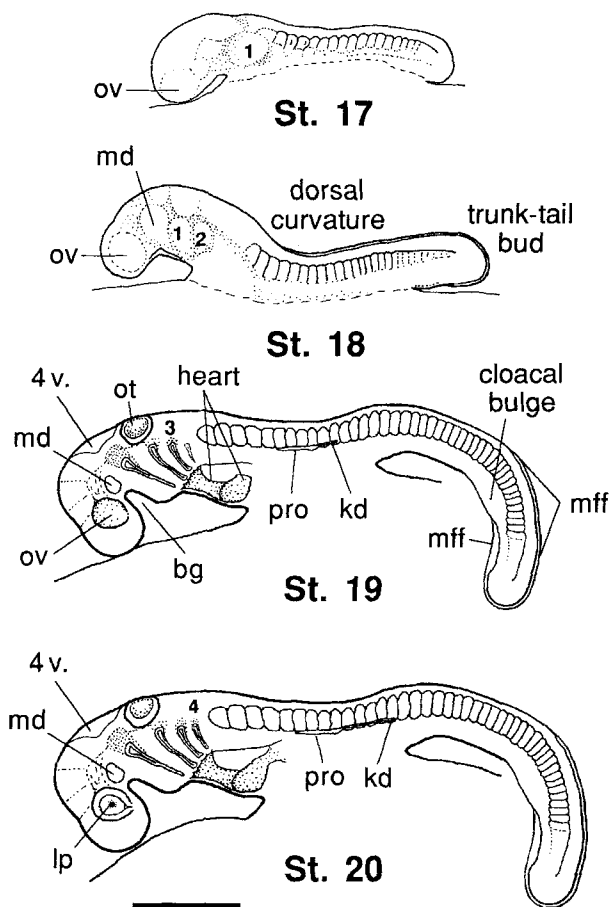


Fig. 5. Side views of lengthening embryos at same scale during the appearance of successive pharyngeal pouches before any clefts break through.

#### Development of external gill filaments. Stages leading to pre-hatching

*Stage 27.* Buds of gill filaments finally appear in the spiracular clefts (Fig. 6). The mouth opening is still diamond shaped (Fig. 7).

*Stage 28.* The mandibular arches reshape themselves, converting the mouth opening from diamond-shaped to a traverse oval (Fig. 7).

*Stage 29.* The lengthened and joined mandibular arches crowd into the mouth opening, which thereby converts the oval space to an arched line.

*Stage 30.* Buds of the caudal embryonic scales begin to show, bulging in dorsal and ventral rows on either side of the tip of the tail. Eyeballs circled with black pigment. Shallow cuts in the low sagittal finfold mark the posterior limits of the emerging two dorsal fins and the anal fin. There is still no sign of a rostral protrusion.

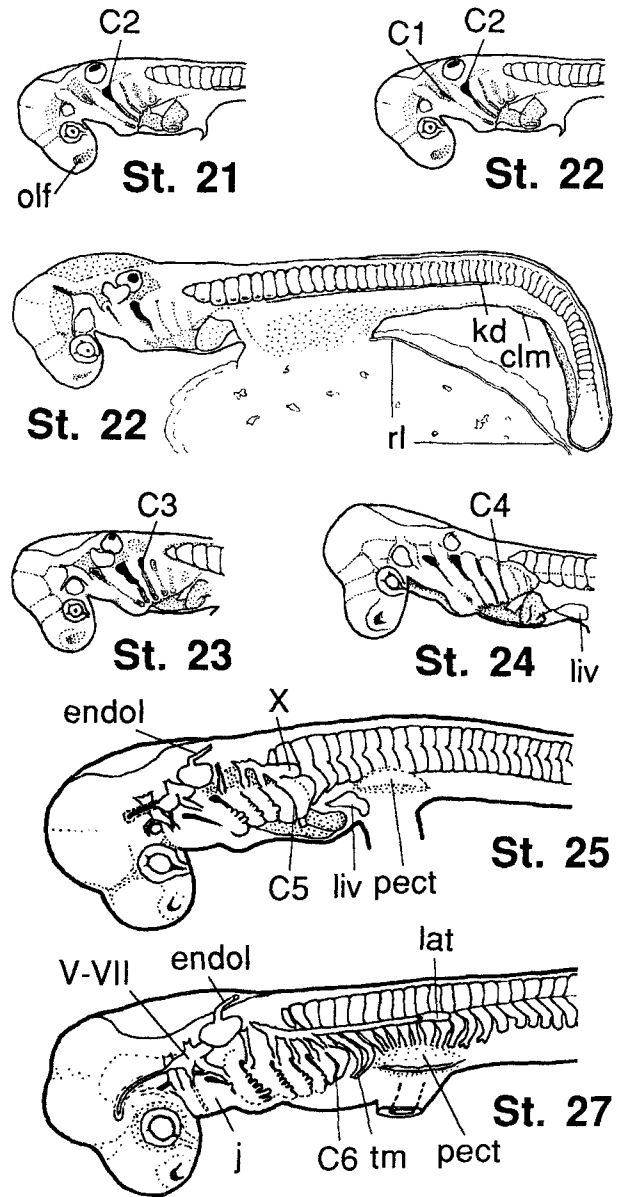


Fig. 6. Formation of the pharyngeal clefts, the stages all shown at the same magnification.

*Stage 31.* The rostrum is a detectable protrusion medial and anterior to the mouth and at right angles to the body axis (Fig. 7). Yolk begins to be transferred from the external yolk sac to the internal yolk sac and the spiral intestine at the time of pre-hatching, which marks the middle of this stage. During the pre-hatching period both dorsal fins and the anal fin grow out so that their posterior edges form acute angles with the fully grown general finfold.



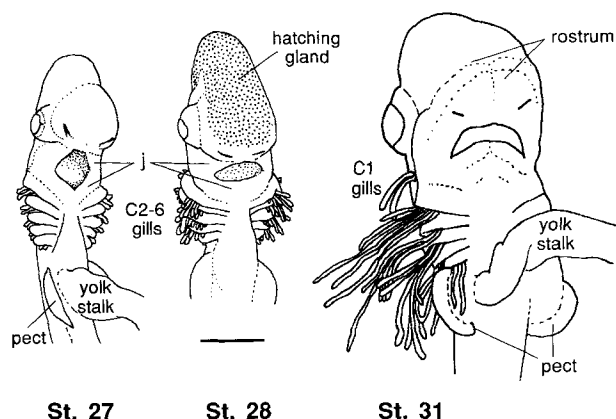


Fig. 7. Ventral views to show changes in the mouth, gills, and pharyngeal region, etc.

### Stages from pre-hatching to hatching

**Stage 32.** Though yolk has started to move inward through the vitelline duct the external yolk sac diameter has not yet decreased. The rostrum becomes prominent, decreasing its angle with the body axis to less than  $90^\circ$ .

**Stage 33.** With continued movement of yolk into the embryo, the external yolk sac is obviously shrinking in size.

**Stage 34.** The external yolk sac is practically empty, button-like, or hanging on its shrunken vitelline duct. The fetus, much enlarged, fills and is immobilized within the eggshell.

### Timetable

The normal delay for *Scyliorhinus canicula* between ovulation and egg laying in the open ocean is not known, but evidence suggests a minimum of 5 days. Captive females held at  $16^\circ\text{C}$  may continue to lay pairs of eggs, but the interval between two layings has never been less than 5 days, and progressively increases for each female (Mellinger, '83). Of the hundreds of females we have examined, only two individuals have ever been found carrying two pairs of eggs at once (by J.M. in April at Banyuls-ser-Mer). It must be normal that a first pair of eggs is laid before the next two are ovulated.

From one large number of females trawled on a single day, 13 eggs were found just freeing themselves from the oviducal glands at stage 1. Almost all the eggs resting at the cloacal end were at stage 3 with only several starting stage 4. These fish are trawled at depths of 70 to 80 m where the temperature varies from  $10.5$  to  $12.5^\circ\text{C}$  according to the season (Agoumi et al., '83). Embryos collected at stage 1 and incubated at somewhat higher temperatures,

took 3 days to reach stage 3, and several more to reach stage 4. The great majority of stage 3s that had been found at the cloacal ends of their oviducts must have been retained there for several days, perhaps longer in the colder waters.

The ages of the hundreds of embryos used for Table 1 are given in days from "Day 1" when they were removed from the uterus or laid naturally. Any given batch of eggs sharing a common day 1 would include specimens varying by 5 or so days in actual age from fertilization. However, cultured at Roscoff in running sea water at somewhat variable temperature, they could be roughly sorted into groups of the same actual ages by segregating each day the group that had reached the most advanced stage. This was necessary for the continuing supply of embryos arriving together at a particular stage for study of a particular aspect of the morphogenetic cell movements, and it worked well. Such segrega-

TABLE 1. Timetable of *Scyliorhinus canicula* development at  $16^\circ\text{C}$

Proposed stages	Former stages	Gross duration (in days, d)	Range (age in d)	Numbers of eggs observed
3	—	1	1–2	12
4	—	1	1–3	12
5	—	1	2–4	46
6	—	2	3–7	41
7	—	0.5	4–8	15
8	—	1	5–9	33
9	—	1	6–10	41
10	A	1	7–10	81 + 21
11	—	1	7–10.5	70
12	B	2	8–13	25 + 21
13	C	1	10–14	10 + 21
14	D	1.5	11–15	7 + 21
15	F	1.5	12–16	8 + 21
16	—	2	14–18	2 + 21
17	G	2	16–20	21
18	H	3	18–22	19
19	I-1	1.5	22–24	16
20	I-2	2	23–26	15
21	K-1a	1	26–27	14
22	K-1b	1	27–28	13
23	K-2	2	28–30	12
24	K-3	1.5	30–31.5	12
25	L	7	31–38	10
26	M-1	4–5	37–42	9
27	M-2	4	42–46	9
28	N	3–5	46–51	9
29	O-1	4	49–53	7 + 5
30	O-2	7–8	52–60	7
31	P	15	60–80	6 + 4
32	Q-1	40–50	75–125	4 + 13
33	Q-2	30	115–155	7
34	Q-3	8–18	145–175	19

tion of like-developed embryos daily for a week after Day 1 yielded, for instance, an assured supply for 5 or 6 days of the stage 10 specimens needed each day for a particular experiment. This also was managed at Reims with the added benefit of 16°C constant temperature. For instance, a group of 21 embryos was carried through to hatching, all having reached stage 8 together. In these series of records, there was less variation in the times embryos spent within particular stages than in the spread of stages achieved after a common "Day 1."

A timetable of this sort was published earlier for the stages from 10 on (Mellinger et al., '86, Fig. 2, p. 318) but it starts with an error in the presumed age of Balfour's Stage A. This has been corrected in the Table 1 herewith. Accumulation of sufficient records as to the duration of stages 32–34 at 16°C is proceeding slowly and will probably lengthen these periods; there may be seasonal variations in them also.

#### *Further details on the stages*

*Stage 1.* Segmentation is slow, asynchronous, and often irregular (Fig. 1B,C,D). Cleavages lag behind mitoses, so that for some time numerous nuclei can be found in a temporarily syncytial blastodisc. During the winter if the youngest eggs are suspended in running sea water at temperatures as low as 6–8°C they may achieve no more than 16 blastomeres in three days, and no more than 100 in a week.

The germinative disc, about 1.8 mm diameter, has a slightly scalloped margin and is bright orange in color. It rests in a shallow cup of whitish yolk that shows in surface view as a white ring around it. In eggs from different females, the rest of the yolk mass varies from light greenish to yellowish in color. For locating the germinative disc in the unopened egg, see Materials and Methods and Figure 1A.

*Stage 2.* The blastodisc of 100 or more mounded external cells lies in a depression inside the white yolk cup and both of these have somewhat more regular margins. Numerous deep blastomeres are forming from tangential cleavages, or some of them may be pinching off from the white yolk. These internal cells are spherical and the spaces between them are bathed with liquid which has not yet been collected in a single subgerminal cavity (Fig. 1E, F).

*Stage 3.* Eggs extracted from the caudal ends of the oviducts have usually reached this stage. They remain thus at least a week when water temperatures are close to 10°C. The superficial cells have become flattened and epithelial. The large numbers of deep cells mound this sheet up slightly above

the surrounding white yolk, and they begin to adhere to each other so that the intercellular fluid is accumulating in a subgerminal cavity of variable extent and irregular outline, visible only in sections (Fig. 1G).

*Stage 4.* Most eggs at this stage have already been laid spontaneously. Eggs taken from the oviducts reach stage 4 within 4 days at 18°C, but not for 8–15 days at 10°C. In colder water they may remain at this stage for more than a month.

The newly formed "caudal crescent" (Figs. 1H, 2A, B) consists of a translucent ooplasmic layer containing a few yolk particles and yolk nuclei, thinned out perhaps by pressure of the accumulating subgerminal fluid. It bridges the gap between the posterior quadrant of the blastodisc and the white yolk ring. In external view this first shows the bilateral symmetry of the embryo, whose body axis will have its posterior end in the direction of the center of the crescent.

*Stage 5.* The blastodisc may now have become oval, averaging  $1.6 \times 1.2$  mm wide after fixation. The membranous roof of the subgerminal cavity (Fig. 2C,D) is a much broader crescent and is easily broken during fixation for histologic processing. Such an aperture was wrongly described as a blastopore by Hoffmann (1896) and His (1897).

*Stage 6.* The external aspect is generally the same as at stage 4 but the caudal crescent is now narrow due to the migration of cells along its lower surface from the densely wadded population of deep blastomeres (Figs. 2E,F, 3).

*Stage 7.* This is a very brief stage with an aspect quite similar to that of stage 3, the crescent having narrowed and disappeared. For the moment, the blastodisc is again circular. In sections one observes many more deep blastomeres migrating toward the posterior edge of the blastodisc, though the central clump of such cells remains dense (Fig. 3).

*Stage 8.* Epiboly now first starts. On the living egg it can be seen that the blastodisc has extended across the caudal sector (but this only) of the white ring (Fig. 3). Sections or dissections show that the mass of deep blastomeres has been diminished by the scattering of individual cells or randomly arranged cluster of them under the surface epithelium. As a consequence the subgerminal cavity has become wider and more shallow. Where the former transparent crescent was seen, the underlying sheet of blastomeres is thin and may be sufficiently translucent so that in the living embryo the subgerminal cavity shows faintly through a gray area.

**Stage 9.** The blastodisc has increased in diameter to about 2 mm, and it is more pale in color, fairly flat, and circular or perhaps slightly elliptical. A posterior sector of it is detectably thickened. Most of the non-epithelial deep blastomeres have scattered from the central mass, individually or in random clusters (Fig. 3).

The epithelial surface of the blastoderm begins to show randomly oriented folds that become more prominent in stages 11 and 12, possibly serving as a reserve of epithelium for the quickening of epiboly. Such folds commonly disappear during fixation.

**Stage 10.** The thickening posterior area of the blastodisc quickly spreads on both sides forming a crescentic embryonic shield (Fig. 4). Balfour (1876) admittedly exaggerated this area in his "Stage A" drawing. Kastschenko (1888) named his similar stage V the *rüsselförmige Keimscheibe*, i.e., the "blastoderm with a snout-like appearance."

The blastodisc (now properly called a blastoderm) is now underlaid by a shrinking remnant of the consolidated mass of inner blastomeres, from which more and more cells are dispersing as mesenchyme. The thickened posterior area, including its lateral bulges, contains many more of these motile deep cells than elsewhere.

**Stage 11.** The thickened posterior rim of the blastoderm now has obviously begun to extend caudad, as a two-layered epithelium, overhanging the yolk mass (Figs. 3, 4). The amount of overhang is difficult to see except in perpendicular sections or dissection.

This stage marked the beginning of "gastrulation" for previous authors, but no consensus has been reached as to how the morphogenetic cell movements are taking place in this or any other elasmobranch fish. Both surfaces of the overhang are epithelial, the lower surface often called the "secondary hypoblast" to distinguish it from the non-epithelial deep cells above the subgerminal cavity, called the "primitive hypoblast" (Fig. 3, stage 12). Swaen (1887) assumed that the lower epithelium came partly from the upper layer (called epiblast) by a dorsal-lip type of turning-under, augmented anteriorly by "primitive hypoblast" cells that pushed outward and became epithelial. Rückert (1887) however interpreted similar serial sections to mean that the "secondary hypoblast" arose exclusively by the emigration and transformation of "primitive hypoblast" cells. Desai ('32) proposed that the line where the overhang starts remains there as a stationary growth center, spinning off hypoblast in two directions, anteriorly into what will become the head and posteriorly toward the future trunk. Other investigators

have varied and expanded such theories, trying to relate this uniquely elasmobranch behavior to the gastrulation of other vertebrates. Only two of them have tried to watch clusters of suitably labeled living cells as they took part in these morphogenetic movements: Vandebroek ('36) and Kopsch ('50 and earlier). Where their results can be compared they are contradictory. There is no record yet, of any experimental confirmation of these or other explanations as to how the diagnostic events of stages 11 through 16 are brought about in elasmobranchs.

**Stage 12.** The cavity under the double-layered overhang (Fig. 3) remains wide open caudally and ends blindly inside. It was called the alimentary canal by Balfour (1878). Other authors have called it the gastrocoele, the archenteron, the gastric cavity, or even the digestive intestine. Whatever name is to be preferred, the question remains open as to what morphogenetic cell movements are going on in its roof. Its floor is merely a thin layer of cytoplasm that retains the yolk.

**Stage 13.** The two thickened arms of the embryonic shield have closed together to form the embryonic axis. Each arm is seen to continue laterally into the germ ring of its own side by a progressively sharper turn (Fig. 4).

Contrary to the statements of both Balfour ('87) and Vandebroek ('36), the anterior end of this embryonic axis is not now being pushed toward the center of the blastoderm. Instead the lengthening of the axis is being accomplished by the posterior growth and joining together of the right and left arms of the embryonic shield. Extension at the anterior end of the axis does not occur until later when organogenesis is advancing. During the present stage the shield varies in length from 1.1 to 1.5 mm, averaging 1.3 mm.

More important events at this stage occur internally, invisible in the living embryo. Two or three pairs of somites take shape, and the notochordal strand segregates from the somewhat thinned epithelial roof ("secondary hypoblast") of the overhung cavity.

**Stage 14.** At their anterior ends, the arms of the embryonic shield form slight lateral bulges, suggesting the start of the head, but there is no trace of a medullary plate between them. The blastoderm spreads to a diameter of 4 mm and its embryonic axis may vary from 1.2 to 1.8 mm long (average 1.4 mm). Sections show that the notochord is sharply defined in the lower layer of the axial overhang.

**Stage 15.** The embryo, now 2 to 3.5 mm long, has a broad medullary plate ringed around by a rounded medullary fold (Fig. 4). Farther back the folds are

drawing together or fusing except at their posterior ends where they diverge into ridges that may overshoot the posterior curve of the blastoderm. Since these ridges will produce trunk structures as well as the tailbud, Balfour's name for them, "caudal lobes," must be rejected. We call them "posterior lobes."

The later fusion of these lobes and their medullary folds will not only enclose the neural tube but at the same time also close the external opening of the "archenteric" cavity under them. As a consequence the future alimentary canal and the neural tube will remain in communication through a neurenteric canal that persists until stage 23. For the present this communication is a groove open to the outside. Internally, the overhung cavity is lengthening and widening, and the mesodermal strips lateral to the notochord have formed 5–7 pairs of somites though these have to be seen in sections or dissections. Thin strips of lateral plate mesoderm lie more laterally narrowing to the germ ring on each side (Fig. 4).

*Stage 16.* The length of the embryo may be from 2 to 4 mm and there are from 9 to 15 pairs of somites (average 11). As Wintrebert ('20b, '22) noted, the number of somites varies too much to determine this or later stages, though during stages 16 to 18 it passes fairly regularly from about 10 to 30 pairs at 16°C at an average rate of 6 pairs each 24 hours. Fusion of the medullary folds completes itself except for the anterior neuropore and the space between the posterior lobes (Fig. 4).

*Stage 17.* The body of the embryo has become quite transparent so that the 17 to 25 pairs of somites are obvious in external view. The first contractions begin in the most anterior somites (Wintrebert, '20b; Harris and Whiting, '54; Harris, '55). The embryo is straight as seen from above though the anterior half of its head is bending ventrad (Figs. 4, 5). The optic vesicles begin to bulge and the otic placodes are barely visible.

The lateral walls of the pharynx have begun to inflate, and from either side one can detect a single pharyngeal pouch, by translucence. Anterior to it a mandibular head cavity is also dimly visible. The neural tube's posterior neuropore and the open end of the "archenteron" have closed, completing the neurenteric canal. The broad trunk-tail bud, which has now formed through the fusion of the posterior lobes, still only slightly overhangs the yolk sac.

This stage was described in considerable detail and figured by Wintrebert ('20b), who considered it equivalent to Balfour's Stage G.

*Stage 18.* The embryo has a length of 4 to 5 mm and variously shows 21 to 30 pairs of somites. It can bend itself to one side or even whip to both sides rhythmically. Its back is now usually concave in side view, the head and the trunk-tail bud somewhat elevated (Fig. 5). Posteriorly its blunt end prominently overhangs the yolk but there is not yet any narrowing to form a definitive yolk stalk or vitelline duct. The forebrain makes a 90° "cephalic angle" with the hindbrain but the buccal groove remains shallow. The fourth ventricle of the hindbrain begins to inflate and the otic placodes have become conspicuous. The second pharyngeal pouches appear by translucence in the lateral walls of the pharynx, and a pair of mandibular head cavities also show just posterior to the optic vesicles and about their same size, through translucent windows.

Toward the end of the stage, the anlage of the cloaca appears even if there is still no cloacal membrane. The heart can be seen as a straight tube, still not beating. Blood islands are maturing in the lateral plate mesoderm. This was Balfour's Stage H, which Wintrebert ('20b) has figured in some detail.

*Stage 19.* The 5 to 6 mm long embryo is beginning to separate itself from the yolk mass by a still wide yolk stalk (Fig. 5). The tail has lengthened and is usually bent down; it is fully as long as the head, and bears traces of dorsal and ventral finfolds. The blastoderm has spread out so as to encompass about half of the yolk mass and blood islands are conspicuous along its rim (see Fig. 8). The forebrain has extended outward and downward, deepening the buccal cavity behind it, limited posteriorly by the lengthening mandibular arches. Now there are three pharyngeal pouches visible by translucence on each side of the pharynx (Desaive, '32). There are about 40 pairs of somites.

Pronephric tubules are swelling alongside somites 6–9, and their longitudinal kidney ducts extend as far back as the 14th somites. The heart tube has begun to bend, and its pulsation has started. Erythrocytes are leaving the blood islands and have reached the first pair of aortic arches.

Distinguishing stages 20–26 by looking at the live embryos through the unopened eggshell windows as described in "Materials and Methods" is seldom successful since you need to count the numbers of visible pharyngeal pouches and clefts, and the actively thrashing embryos will give you only fleeting glimpses of the sides of their heads. However you can see evidence through the same windows or the translucent eggshell that such stages are being passed through. Look for the "red line" (see Figs.

6, 8, stage 22). Blood flow rapidly increases during this period and the right and left halves of the blastoderm are closing together under the trunk and tail. Each half of the blastoderm is bordered by blood-forming tissue, the edge of which becomes an efferent vitelline vein. As these veins are brought together and fill with blood, they fuse together forming a conspicuous red line that first lies under the trunk and tail but quickly extends toward the posterior yolk pole (see Fig. 8) following the shrinking yolk plug.

*Stage 20.* The 6.5 to 7.5 mm long embryo now has about 50 pairs of somites. Four pairs of unopened pharyngeal pouches show themselves by translucence (Fig. 5). The lens placodes are pressing into the optic vesicles which have become creased by their choroid fissures. The olfactory placodes become visible.

Serial sections show that the thyroid gland and Rathke's pouch are forming. The esophageal lumen has been obliterated, and it will remain so until stages 33 or 34 (Mellinger et al., '87).

*Stage 21.* At approximately 7.5–8 mm body length the second pharyngeal pouches (the hyobranchials) open through their clefts (C2). The first (C1, hyomandibular or spiracular) clefts are still unopened (Fig. 6). At the same time the buccal membrane begins to perforate and thereafter vanishes more or less rapidly. The lens rudiments are open to the outside, the olfactory placodes are depressed, and the otic vesicles are still widely open. The cloacal membrane has formed, and the longitudinal kidney ducts have nearly achieved their contacts with the cloacal cavity. The heart has become fully S-shaped and drives a strong circulation, via the first aortic arches and the dorsal aorta, out toward the tail. Blood from the caudal vein goes to a subintestinal vein anterior to the cloaca, and so at early stages to the posterior cardinal veins. As first noted by Balfour (1876) the selachian yolk sac (in contrast to that of teleosts) is supplied through a vitelline artery given off by the dorsal aorta at pronephros level and drained by veins formed at the closing right and left borders of the blastoderm, gradually uniting in a single midline vitelline vein, the "red line" (see Figs. 6, 8) (Mellinger et al., '86), now visible beyond the tail. The longitudinal kidney duct projects from the pronephros almost to the cloaca.

*Stage 22.* During this swift stage no other conspicuous biometrical or anatomical advances occur except the delayed opening of the C1 or spiracular pharyngeal clefts. Wintrebert ('22) included and diagrammed embryos in his K-1 stage that included

specimens that we distinguish in our stages 21 and 22 (see Figs. 6, 8).

The mouth also begins to open at this stage but large pieces of the buccal membrane persist. The "red line" (vitelline vein) projects beyond the tail. The longitudinal kidney duct projects from the pronephros almost to the cloaca.

*Stage 23.* The fifth and even the sixth pair of pharyngeal pouches, the last of the series that become visible by translucence, now appear (Fig. 6), along with the breaking through of the third pair of pharyngeal clefts (C3). The mouth is fully open. Ganglia of the Vth and VIIth cranial nerves become visible. The ear placodes have sunken inward forming shallow cups with narrowing edges dorsally. A second pair of aortic arches begins to carry blood and the dorsal aorta has extended itself as far back as the 50th somites. The longitudinal kidney ducts have lengthened almost or quite to the cloaca, and mesonephric tubules are taking shape along them. Except for the 1st pair of nephrostomes, the future funnels of the müllerian ducts, the pronephroi have regressed.

The embryo's connection with the yolk sac has narrowed to a wide yolk stalk whose opening spans the levels of somites 7 to 11. The blastoderm has extended over most of the blastoderm and the "red line" fusion of the vitelline veins has variably extended beyond the tip of the tail. The still uncovered yolk plug (see Fig. 8) is slowly diminishing.

*Stage 24.* The first four pairs of pharyngeal clefts (C 1–4) are now open to the outside (Fig. 6). The large mouth opening becomes diamond shaped and will remain so until the end of stage 27. The stalk of the yolk sac continues to narrow. By now the elongated and straightened trunk and tail posterior to this stalk have become longer than the part of the body anterior to it. The number of somite pairs averages about 70 but varies from 64 to 78.

The first few buds of gill filaments appear upon the anterior borders of pharyngeal clefts C2 and C3, and then C4. The olfactory placodes have sunken in as pits. The lenses become free of the surface epithelium and press into the eyeballs. The otic vesicles have begun to enlarge but their openings are constricting where the endolymphatic ducts are beginning to lengthen.

Though the mesodermal head cavities are reducing, the pair bordering the mandibular arches, smaller than the eyes now, can be seen by translucence, supporting the ophthalmic nerves sprouting forward past the trigeminal ganglia. The post-otic ganglia of nerves IX and X have become conspicuous.

The pectoral fin rudiments appear merely as ec-

todermal crests (Vasse, '71), later to be filled out with somatic mesodermal sprouts. Up to 30 pairs of mesonephric tubules are lined up transverse to the mesonephric ducts. Downgrowths of the somites are now obscuring the mesonephric ducts from view. The liver begins to bulge slightly posterior to the heart.

*Stage 25.* Pharyngeal clefts 1–5 are all open (Fig. 6). There are usually about 80 pairs of somites, and they are becoming V shaped. The pectoral fin borders have extended as ridges wide enough to obscure the lower parts of the nearest somites. Just behind the pharynx, several of the most anterior somites begin to send processes ventrad where they will later differentiate as the hypobranchial sets of muscles. The upper parts of the mandibular arches become larger.

Lateral line nerve sprouts are visible, extending from their pre- and post-otic ganglia, the posterior ones reaching as far back as somites 7–9. Pelvic fin rudiments can be detected near the cloaca. Buds of gill filaments have appeared on pharyngeal arches 2–5.

The yolk plug finally closes completely over the yolk, entirely enclosing it in a cellular yolk sac which is still half vascularized. Only the upper half of the yolk sac is covered by a bed of vitelline capillaries (see Fig. 8).

*Stage 26.* All six pairs of pharyngeal clefts are now open. Some specimens have already completed the full adult set of 85–90 pairs of somites at this stage, while others do not finish this process until stage 27.

The rounded dorsal borders of the pectoral fins reach above the ventral borders of somites 6–14, all of which are sending myotomic downgrowths into them, as well as somites 15–18. Sprouts forming the posterior lateral lines have reached to somite 5 or even 10 on each side. The mesodermal head cavities can no longer be distinguished externally in surface view, though they are still fully developed internally.

*Stage 27.* (distinguished as M2 by Mellinger et al., '84a, '86). This stage completes the vascularization of the yolk sac. Throughout its length the conjoint vitelline vein drains the capillary network of both sides of the sac. The capillaries themselves are fed by a wide-branching single vitelline artery that has branched from the dorsal aorta at pronephros level.

One or two buds of gill filaments show on the first (mandibular) and finally on the 6th pharyngeal arches. On the intervening arches, gills may have blood flowing through them. The hatching gland

begins to show over the protruding surface of the head. The olfactory placodes have sunken in but are still wide open ventrally. The upper part of the mandibular arch thickens and presses against the eye. The whole pharyngeal complex pushes caudad so that the first several pairs of somites are now found dorsal to it. Several of them are sending sprouts down behind the pharynx, turning forward to form hypobranchial muscles.

Serial sections show rapid development of the spiral intestine, the formation of parachordal cartilages at the base of the hindbrain, and appearance of gonad ridges.

*Stage 28.* The reshaping of the ventral ends of the mandibular arches converts the mouth opening from diamond-shaped to oval (Fig. 7). Each nasal organ, while sinking in, narrows its opening and throws its nasal epithelium into folds. The supra-orbital lateral lines have extended dorsal to the eyeballs and then turned downward, but have not yet met the infraorbital lines. The paired pelvic fins have rounded out and lengthened their margins laterally. By slight projections the sagittal finfold begins to show which parts of it will form the anal fin and the two dorsal fins.

All six pairs of aortic arches are carrying blood, and the branchial ones are supplying capillary loops to the elongated gill rudiments. The hatching gland now slightly roughens the skin over the forebrain and midbrain.

*Stage 29.* The dorsal parts of the right and left mandibular arches have met in the midline, while the extension of their ventral parts toward each other crowds them into the mouth opening. This gives the mouth its curved final shape. There is an incomplete circle of pigment over the eyeballs, but no pigment anywhere else on the body. Downgrowths of the trunk myotomes begin to invade the belly walls.

*Stage 30.* Each eyeball now bears a complete circle of black pigment. External gill filaments, engorged with blood, are growing rapidly; some are already 4–5 mm long. The belly wall has been 3/4 invaded by the downgrowths of the myotomes. The two dorsal fins and the anal fin have grown out posteriorly to form obtuse angles with the persistent parts of the sagittal finfold.

A set of precocious embryonic placoid scales, characteristic at least of Scyliorhinid embryos, may now be seen in two rows on each side of the tail, by use of the dissecting microscope. Germs of about 9–13 of the scales appear in each dorsal row, and 5–10 in each ventral row. These will erupt during the pre-hatching period, considerably earlier than the placoid scales in the rest of the skin.

*Stage 31.* Its beginning may be set at first detectable appearance of the rostrum as a slight bulging ridge anterior to the nasal organs, making an angle of  $90^\circ$  to the body axis (Fig. 7). Stage 31 cannot be conveniently subdivided because the numerous changes that occur are gradual and on-going.

At a time when the embryo is about 40 mm long, the four slits at the corners of its eggshell (Fig. 1A) are rather suddenly opened up by the activity of the hatching gland, whose secretions digest away the jelly content of the eggshell, including the special cement that has sealed the slits tight until now. Once these are open, sea water circulates through them, owing to the movements of the embryo. This crucial change generally occurs in the middle of this stage. It can be anticipated by observing the moving border of the undigested jelly in the squared-off end of the eggshell (photographed by Foulley and Mellinger, '80). And its completion is easy to check by gently pressing the eggshell between one's fingers.

Meanwhile, the sagittal finfold ceases its growth and at the same time the anal fin and the two dorsal fins extend their posterior borders so as to change from obtuse to acute the angles they make with its remnant (see Fig. 9). The myotomes of the trunk finish their spread toward the midline of the belly. The external gill filaments reach their maximum development. The main posterior lateral line nerves reach their final terminus at the bases of the special scales of the ventral rows along the tail, which are in process of erupting. Germs of another set of special embryonic scales become detectable in two dorsolateral rows along the trunk (see Fig. 9). Ordinary placoid scales of the general skin are not visible until much later (for a description of scale development, see Mellinger and Wise, '92). The male specimens become externally recognizable by the gradual appearance of their pelvic claspers or pterygopodia.

#### **Notes on the pre-hatching period**

In other fish and in amphibia, when the hatching gland starts to secrete, it digests a hole in the protective envelope of the egg or at least loosens it, allowing the developing individual to escape almost at once. Not so, in the oviparous sharks, rays, and holocephals. These animals remain in their very large, very sturdy eggshells, big enough to hold them and their large yolk sacs, for an extraordinarily long time before hatching, making enormous growth.

The tightly closed eggshells of *Scyliorhinus* are suspended in a current where they will not be

smothered in silt, and they tangle with seaweeds. Yolk and embryos are kept safe inside, free from physical damage and microbial infections. Nevertheless the fetus would soon run into respiratory difficulties, held within such thick closed walls, even after developing a circulatory system and then augmenting it with a richly vascularized yolk sac (Fig. 8) and greatly elaborated gill surfaces (Fig. 9). Provision must be made also for its escape before its food supply is quite used up.

These problems are all solved at the right time by the onset of secretion by the hatching gland. First, this digests the jelly content of the eggshell giving the embryo much more room to grow into. Then it digests the cement that up to then has sealed the slits in the corners of the eggshell so as to allow the thrashing fetus to pump a continuing supply of sea water through them for breathing. Finally, it loosens the attachment of the two surfaces of its squared-off end, preparing the escape hatch through which the young fish can push its way out to freedom.

By this time the hatching gland's work is completed and it entirely disappears long before the time of hatching. The *Scyliorhinus* fetus still has the second half of its development to accomplish. During the next 3 months before it escapes, it will continue to benefit from the protection of the unlocked but fairly closed tough eggshell. Even crabs and starfish cannot get at it.

It is not only the invention of this long pre-hatching period that is impressive here. There are other examples in the animal kingdom where a variety of structures and functions of both parents and the offspring act in concord both in time and space to ensure the survival of the species, but this one, unique to chondrichthyans, is unique. Their very large and very fragile eggs could not survive in the absence of any one of three perhaps separately evolved elements in their proper sequence: 1) the copulatory apparatus and behavior of the male parent; 2) the multiple and precisely timed operations of the female's oviducal glands, successive parts of which serve to store a perennial supply of sperm for the internal fertilization, then to surround the zygote with soft loose jelly, and finally to secrete the elaborate eggshell around these, provided with its anchoring tendrils and cemented apertures; and also, much later 3) the re-adaptation of the eggshell to the changing needs of the fetus, by action of its own hatching gland, which has lost any function it may once have had in the hatching process itself. The assembling and perfection of all these components in such a novel way reminds us how



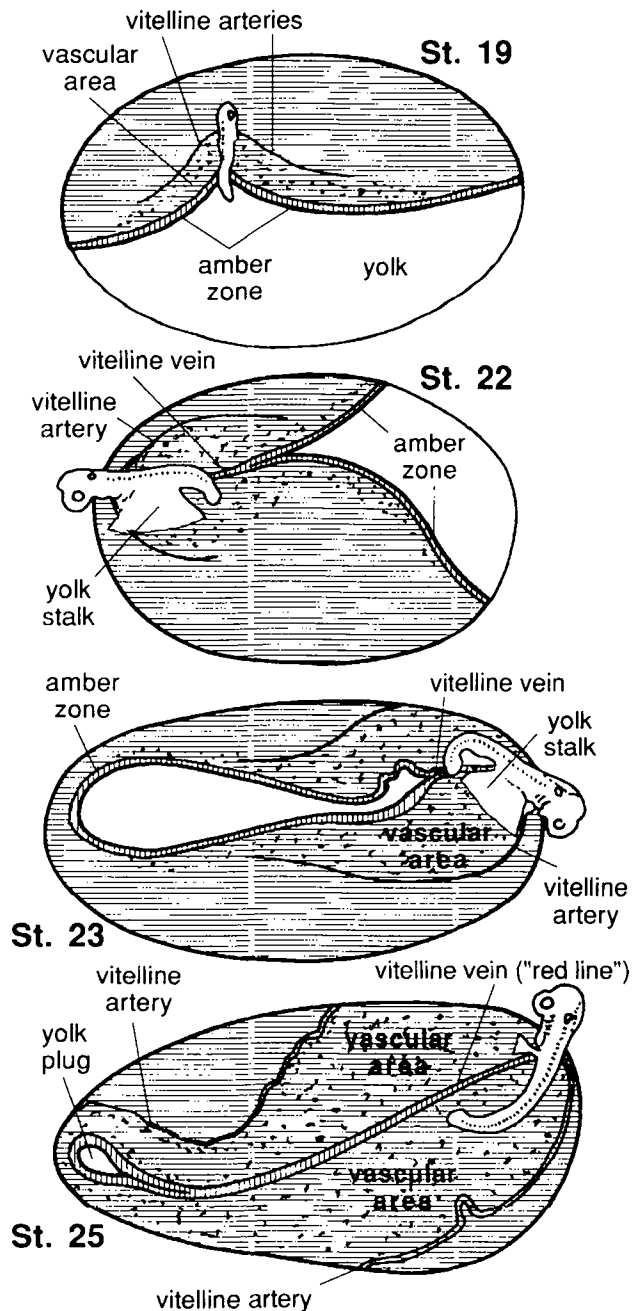


Fig. 8. Formation of the external yolk sac by spreading of the blastoderm (horizontal hatching) while the embryo is increasing in average length from 6 to 12.5 mm. Mottled zones show where blood islands and capillaries are developing. They spread from earlier amber zones (vertical hatching) in which the vitelline veins form and join. The still uncovered yolk mass is left white. Each of these embryos was removed from its egg-shell whose squared-off end was at your right. They show how gravity affects the embryo's location on the yolk sac. The stage 19 specimen was cultured in a horizontal position, the others vertically, the Stage 22 specimen with the horned end up, and those of Stages 23 and 25 with the squared-off end up, more convenient for hatching.

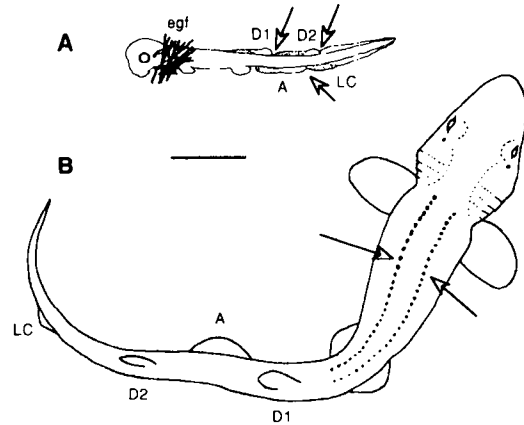


Fig. 9. Early prehatching fetus and hatchling at same magnification as an indication of growth attained during the prehatching period of about 3 months. External gill filaments regress entirely. Stippled areas of the sagittal finfold are thin membrane (later lost). The plain parts are the thicker rudiments of the two dorsal fins, anal fin, and upper and lower parts of the caudal fin. The newly hatched fish has two rows of special placoid denticles along its back (arrows). These and the four rows of special caudal denticles (not shown) all appear before hatching.

much we still have to learn about the process of evolution.

*Stage 32.* Again, a long stage during which numerous changes takes place gradually at variable rates. However it can be conveniently subdivided by reference to the angle that the rostrum makes with the body axis, as this organ narrows it down from 90° until it has extended itself straight forward above and beyond the mouth. The yolk sac has not yet appreciably diminished in volume, though its dry weight begins to shrink (Lechenault et al., '93).

An internal yolk sac is developing into the body cavity by extension from the upper lining of the vitelline duct. For some time it remains empty; any yolk flowing in through the duct at first flows directly to the spiral intestine, which is beginning to swell and equip itself for digestion. There is no evidence that the rich capillary bed on the surface of the external yolk sac is there for picking up nourishment from the contained yolk: in fact its endoderm lining remains very flat. Its main function is respiratory.

Pharyngeal respiration starts at about body length of 65 mm, along with regression of the external gill filaments. Pigment covers the eyeballs and begins to appear in the skin generally. All parts of the sagittal finfold regress completely except for the anal fin, the two dorsal fins, and the dorsal and ventral parts of the emerging caudal fin.



*Stage 33.* The sign of this stage is that the external yolk sac is obviously shrinking in size as yolk actively flows into the fetus. Much of this is temporarily stored but not digested in the internal yolk sac, which becomes so engorged that it expands caudally along the right side of the coelom all the way to the cloaca. Perhaps half of the yolk had already been digested before shrinkage of the external yolk sac became obvious. Finally it decreases to a small "button" or dangles empty on a shriveled vitelline duct. For further details of this transformation see Mellinger et al. ('86), Mellinger and Wisez ('89), and Lechenault et al. ('93).

*Stage 34.* After the emptying of the external yolk sac and the shriveling of its duct (otherwise it might be torn away during the difficult hatching process), there may be a waiting period of 8 to 18 days before the fetus can escape. Its belly is inflated so that it can scarcely move inside the shell. If taken from the eggshell during this delay, it squirms clumsily and swims reluctantly. The yolk stored in its internal yolk sac is used up within 10 days after hatching, whereupon if it does not succeed in catching food it quickly starves to death. The ordinary placoid scales only erupt just before hatching, roughening the skin at that time.

## DISCUSSION AND CONCLUSIONS

Table 1 shows that our *Scyliorhinus* stages are nothing like a series of photographs. Instead they point to successive periods of development, during which numerous events happen at their own and perhaps variable rates. How much variation there may be between different individuals of this species has not been measured, let alone the variabilities among elasmobranchs in general. The stages of organogenesis in *Scyliorhinus* (19 to 31) were first worked out by Mellinger et al. ('84) for the Mediterranean race, and these were augmented and confirmed with insignificant differences from collections at Roscoff, together with other details including dissections and serial sections. How well can these stages correspond to developments in other sharks and rays, as recorded in the literature?

Scammon's ('11) monograph on *Squalus acanthias*, hitherto unrivaled in its completeness, records in handsome drawings, wax reconstructions, and serial sections the anatomy of dozens of randomly collected but closely spaced specimens, lacking chiefly sufficient material before somite formation and specimens beyond our stage 31. There is remarkably close correspondence with our *Scyliorhinus* stages. His specimen 2 corresponds to our stage

10, his specimen 3 is very like our stage 11, and his 4 to 6 fit with our stages 13 and 14. Further close matches are

<i>Squalus</i>	<i>Scyliorhinus</i>
7	14
8-9	15
10-14	16
15	17
16-18	18
19	19
—	20-21
20-22	22
23	23
24-25	25? 25?
26	25
27-30	28
31-32	31

Even the details from his serial sections follow ours with insignificant exceptions, e.g., his specimens 16-18 show the pronephros and our 18 does not. The rich information in the *Squalus* monograph applies to the development of *Scyliorhinus* quite well except for the missing stages, cleavage, the onset of morphogenetic movements, and the unavoidable absence of a timetable.

Few other comparisons can be made from the scattered literature on other kinds of elasmobranchs. Cleavage and gastrulation stages of the electric ray *Torpedo marmorata* as illustrated by the Zieglers (1892), His (1894), Rückert (1889), and Emmert (1900) are easy to match with our *Scyliorhinus* figures. Mellinger et al. ('87) found that *Torpedo* specimens fit our stages 19-25 as well.

Of course, during its development any elasmobranch species may take on structural features peculiar to its own class, order, family, or genus that are too specific to be useful in a generalized system of developmental stages. For instance the appearance of precocious sets of scales mentioned at our stages 29 and 31, as found in Scyliorhinids, may not help in the search for equivalent stages in other sharks, even *Squalus*. Neither would the unique late-forming oddities of hammerhead sharks or the special batoid characteristics of flattened body, grossly expanded pectoral fins, and electric organs that develop so late in *Torpedo*. However these are localized growth modifications seen much later, of organs, tissues, and regions which can be identified at much earlier stages according to a schedule characteristic of elasmobranchs in general. Even the gross differences of the oviparous *Scyliorhinus* at hatching, compared with the viviparous *Squalus* at birth, do not invalidate that they both pass through stages like those of our stages 28-34.

We propose therefore that the *Scyliorhinus* stages described herewith are probably applicable, with quite minor changes, to the development of any elasmobranch species.

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