Developmental Biology of an Oviparous Shark, *Scyliorhinus canicula*

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Abstract Eggs of the European lesser spotted dogfish, *Scyliorhinus canicula,* were incubated under standard laboratory conditions. The sequence of developmental stages was studied by daily observation of live embryos, light and electron microscopy, biometrical measurements, and chemical analysis (carbon, hydrogen, nitrogen, sulfur, water, urea, Cl^-, Na^+, K^+, Ca^{2+}, osmometry). A new modified version of Balfour's table was proposed, with a chronology of stages at 16°C. About two weeks after the beginning of segmentation, the blastodisc spread out and, besides embryogenesis, the external yolk sac (EYS) formed during the 40 following days. Yolk was first avascular, enclosed by EYS from the anterior side backward, then the vascular area spread from the posterior red streak (suture of EYS) forward, also symmetrically. The resulting increase of blood volume in the embryo allowed further elongation of the external gill filaments 10 days later, which denoted the beginning of the long P stage, during which egg-case apertures were opened by hatching gland enzyme(s). This so-called "pre-hatching", typical of oviparous chondrichthyans, occurred at mid development (about 3 months).

Length growth resumed at that time, following its slowing down during stage P, and was correlated with the onset of yolk transport from the EYS into the gut, which was responsible for the digestion of at least 90 per cent of the yolk. This was confined within the spiral gut by previous closure of the rectum, and digestion lasted until one or two weeks after birth, owing to the storage of yolk by the internal yolk sac (IYS). Reopening of the rectum happened some time before birth in most embryos. The transitory rectal closure, resulting from the adhesivity of opposite epithelial cells, can be considered as an adaptation to yolk digestion, and may also be useful to confine urine and rectal gland fluid in the bladder-like cloaca of larger embryos.

Using freeze-drying for the measurement of water space in various parts of the embryonic system (ES, i.e., embryo + EYS + IYS), and then extracting the osmolytes by 10-fold larger water volumes, the concentrations of ions and urea were shown to be different in each part: almost constant Cl^- and Na^+ in embryos (about 160 mM); a decrease of yolk Cl^- (from 130 to 70 mM), Na^+ (quickly stabilized around 25 mM), and K^+ (65–50 mM); an increase (40–70 mM) and then a slight decrease of K^+ in embryos; 400–450 mM urea in EYS, 430–470 mM in IYS, 320–400 mM in egg cells, 350–390 mM in newborns, and a changing urea concentration in embryos (probably due to changing internal compartmentation). Increase of urea concentration and decrease of yolk ion concentrations during the first part of development may have been compensating mutually. Cl^- and Na^+ ions lost by the yolk during this period may have been partly ejected from the ES by "chloride cells" located on gill primary lamellae from stage P onward, and also by the highly developed rectal gland which could only excrete ions through the cloaca after pre-hatching, as the cloacal slit opened at that time. Osmoregulatory effectors operating at the beginning of development are still unidentified.

The other contents of the egg-case ("jelly") were devoid of urea and closely resembled sea water. It was concluded that the ES was regulating urea and ion concentrations accurately in its various internal compartments. Closure of the esophagus occurred a few hours before the opening

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surface. It is well-known that chondrichthyans reabsorb urea in their kidneys and can—by unknown mechanisms—restrict its diffusion across the gill epithelium, thus being able to maintain their body fluids isosmotic or slightly hyperosmotic to sea water. Despite previous evidence for a high permeability of the egg-case to salts and urea, Smith (1936) proposed that this egg would be of the "cleidoic" type and retains urea at stages where ureogenesis by the embryo could not match osmoregulatory needs. In fact, his theory aimed to explain the evolution towards viviparity which has been achieved in most elasmobranch families. Many textbooks and reviews have adopted his point of view.

However, Read (1968) demonstrated with *Raja binoculata* eggs that urea could be produced very early by the embryo, and it was contained in ES water at concentrations similar to that of the adult's blood. Leakage remained very small. Mellinger and Wrisez (1983) showed that similar urea concentrations exist both in the embryo and the yolk before development of the liver in *S. canicula*. Urea of maternal origin seems to be held back in the yolk by the impermeable egg membranes, and it may simply diffuse into the small embryo. This data led us to examine if the embryo showed morphological adaptations for urea retention. In this respect, the transitory closure of esophagus and rectum will be redescribed, osmolyte concentrations measured, and the development of osmoregulatory effectors in the dogfish embryo will be briefly outlined.

Materials and Methods

Dogfish and eggs were supplied by the Biological Station, Roscoff, located on the French coast of the Channel. They differ biometrically from the Mediterranean population used in our laboratory until 1982 (Mellinger *et al.*, 1984; Muñoz-Chápuli *et al.*, 1984). Shipping by railway to Reims took 24 hours. Our aquarium, built in a dark room in the laboratory's basement, has allowed successful rearing of dogfish since 1968. Mortality for intact, normal eggs was nil.

Eggs were either extracted from dead, freshly caught females in a small fishery in Plougasnou (North of Morlaix), or laid by captive females in the aquarium of the Biological

Table 1. Number of eggs (n) used in each batch (A–F), with age (at 16°C) and total body length (L, in mm) of observed pre-hatching embryos, and newborns.

Batch	n	Origin	Received	Pre-hatching	Hatching
A	30	laid in Reims, Feb.–June 1983	—	89 days L=39 (n=1)	170–182 days L=102–108 (n=5)
B	50	extracted, near Roscoff, XII.12.1983	XII.16.1983	101–105 days L=39,5–41 (n=6)	194–200 days L=103–109,5 (n=8)
C	50	extracted, near Roscoff, XII.12.1983	I.20.1984	—	—
C'	8	Roscoff, undated	I.20.1984	L=37,5–40	—
D	30	laid in Reims, Dec. '83–April '84	—	85–89 days (n=9)	—
E	61	extracted, near Roscoff, V.10.1984	V.16.1984	—	181–185 days L=101–109 (n=17)
F	28	laid in Roscoff, March 1985	III.19.1985	—	—

Station. Table 1 lists the batches received. There were also two additional batches of eggs laid in Reims. One large batch of eggs, all extracted the same day, is best since the survey of stages is simplified and the synchrony of development can be checked. Mass incubation, i.e., hanging all the eggs together in one tank, was preferred to the traditional method of box incubation, which required frequent water changes and caused snout swelling in the oldest embryos.

Large tanks (1,300 liters each) were built of reinforced polyester. The filtering compartment (300 liters, filled with glass wool) was included at one end, and water was recirculated from its floor into the tank by bubbling. It operated by mechanical sieving and mainly by bacterial degradation of nitrogenous wastes. With eggs, water purity can be perfectly preserved for years, if dust is eliminated with tight covers. Nitrites were always undetectable. The pH, checked with neutral red, was the same as in natural sea water. All walls had smooth, clean surfaces, and neither sand nor stones were deposited on the floor.

Artificial sea water was prepared by dissolving a commercial salt for aquariology ("Wimex", manufactured by H. Wiegandt, Federal Republic of Germany) in tap water. Density (1.025–1.030) and osmolality (1025–1070 mOsm/kg H_2O) were adjusted to those of natural sea water received from Roscoff. Water temperature was regulated at 16°C (± 0.5°C). Populations of protozoa (ciliates) and small invertebrates (copepods, a eunicid worm) were kept to a minimum by almost constant darkness and careful cleaning.

Individual numbering of incubating eggs was accomplished by hanging them at regular intervals under the water surface, on several nylon threads fastened at both ends of each tank. To observe the live embryos easier, eggs were suspended by their knotted tendrils (generally, anterior tendrils) on plastic hooks, which were tied on the threads, so that they could be removed and observed under a dissecting microscope in a Petri dish, or with the naked eye under suitable lighting. The egg-case wall can also be made transparent like glass by carefully peeling patches of the very thin, external pigmented layer with a new razor blade. To avoid punctures, shavings cannot be too large (1.5 mm). Since these "windows" get progressively blurred, parts of the wall surface must be left to prepare new windows. Live embryos were also more closely studied under the microscope when the eggs were opened to fix the ES for histological or cytological treatment, or to perform biometrical and chemical measurements. In this case, the embryo can be immobilized by dissolving minimal quantities of dry tricaine methane sulfonate (MS 222, Sandoz) into the fluid filling the chalazal chamber occupied by the ES in the center of the jelly. The same "anaesthetic" must be used when body length is to be measured.

Morphometric measurements (± 0.5 mm) were performed using dividers. Weights were determined by repeated freeze-drying to a constant weight, using polypropylene tubes as containers (sterile 17–100 mm tubes with caps, n° 2059, Falcon), because their weight proved means. Bleeding was prevented by ligature of the yolk stalk before cutting. Dry weights were determined by repeated freeze-drying to a constant weight, using polypropylene tubes as containers (sterile 17–100 mm tubes with caps, n° 2059, Falcon), because their weight proved to remain constant to the nearest 0.1 mg (at variance with polyethylene containers). Every tube was pre-weighed with its cap, and both were labeled. Freeze-drying was performed by putting several (up to 40) open tubes into the circular chamber of the Lyophilisateur TS1 (Froilabo S.A., France). After one night or more's drying, the chamber was heated to ambient temperature before returning to atmospheric pressure and final quick re-capping of the tubes. To ensure full drying, large yolks, EYSs and internal yolk sacs (IYSs) were ruptured before freezing the tubes in an almost horizontal position. Large embryos and newborns were cut into small pieces after the first freeze-drying session. All dry or fresh materials were preserved in a freezer at -30°C.

Proper separation of egg parts (case, jelly plus fluid, yolk or EYS, embryo, and IYS, if

any) requires practice. The procedure for IYS has already been described (Mellinger *et al.*, 1984). Other parts were put directly into labeled tubes as follows: first cut tendrils off, then cut into the egg-case wall near the anterior end of the egg with fine scissors and follow the edges of the anterior part above the embryo level, cut transversely on one side, take this part of the wall (with part of the jelly) off with forceps (avoid fluid loss), pipet the overflowing fluid into the corresponding tube; insert the caudal extremity of the egg into a pill-container and let stand, which allows microscopical observation if desired; scrape the jelly off the detached part of the wall into its tube, and then also from the open part of the egg; pour the rest of the fluid into the same tube, but be careful to collect the intact yolk in another; finally cut the rest of the egg-case along its edge and collect the last of the jelly. The different pieces of the egg-case were then washed under a water tap with fingers to remove any trace of jelly, wiped, and weighed in a tube. When the embryo could be weighed, the entire ES was first collected in a micrography dish, body length was taken, and separation of the embryo from the EYS was achieved.

Histology and electron microscopy were performed using conventional methods. The morphological study of embryos (counting of myotomes and visceral pouches or clefts, scale and tooth studies, etc.) was much easier after the use of Bouin-Hollande's fixative because it stains tissues and hard organs.

Carbon, hydrogen, nitrogen and sulfur were microanalyzed in duplicate by two well-established, specialized laboratories. A dry, homogenous powder of newborn body was prepared by us with re-hydrated freeze-dried material, by putting it into a refrigerated mortar and grinding under liquid nitrogen. It was again freeze-dried to a constant weight. Crushing dry EYSs and IYSs with a glass rod worked well to obtain a powder.

Preparation of aqueous extracts for osmolyte (ions, urea) analyses of embyos EYSs, IYSs and jelly (including the chalazal fluid) was done by adding volumes of icy, sterile deionized water (obtained with a Milli-Q apparatus, Millipore Corp.) to the dry material; homogenization (by shaking or with an Ultra Turrax high speed mixer, IKA-Werk, FRG) was followed by centrifugation for 20 minutes at 27,000 g (Sorvall RC–2 refrigerated centrifuge). Water volumes were multiples of the previous water content determined by freeze-drying: 5 × for jelly, 10 × for other materials (20 × for some small embryos). Deproteinization with 10 per cent trichloracetic acid was used only for urea determination, performed on aliquots of the supernatant.

Urea concentration was determined by the diacetyl monoxime colorimetric method using a commercial kit (N° 535, Sigma Chemical Co.) and a single beam spectrophotometer with a circulation cell (Model 42, Beckman). This method was validated by complete digestion of the reacting substance with urease. Osmolality of supernatants was measured with a Fiske "OS" freezing-point depression osmometer using 250 μl samples. Chloride ions were titrated with silver nitrate in an automatic, self-made chloridometer (Laboratoire de Physiologie Comparée des Régulations, CNRS, Strasbourg-Cronenbourg). Sodium, potassium and calcium concentrations were measured with an automatic, clinical flame photometer (FCM 6341, Eppendorf). All results were expressed in grams, milliosmol per kg H_2O, or millimol per liter.

Mean values, standard errors of means (SE) and y-on-X regression lines were computed with a Texas Instruments TI 59 calculator using its statistics module. When kurtosis and/or skewness of univariate distributions departed from Gauss' law, or when variances differed according to the F test, comparisons between them were done by using non-parametric tests (Siegel, 1956).

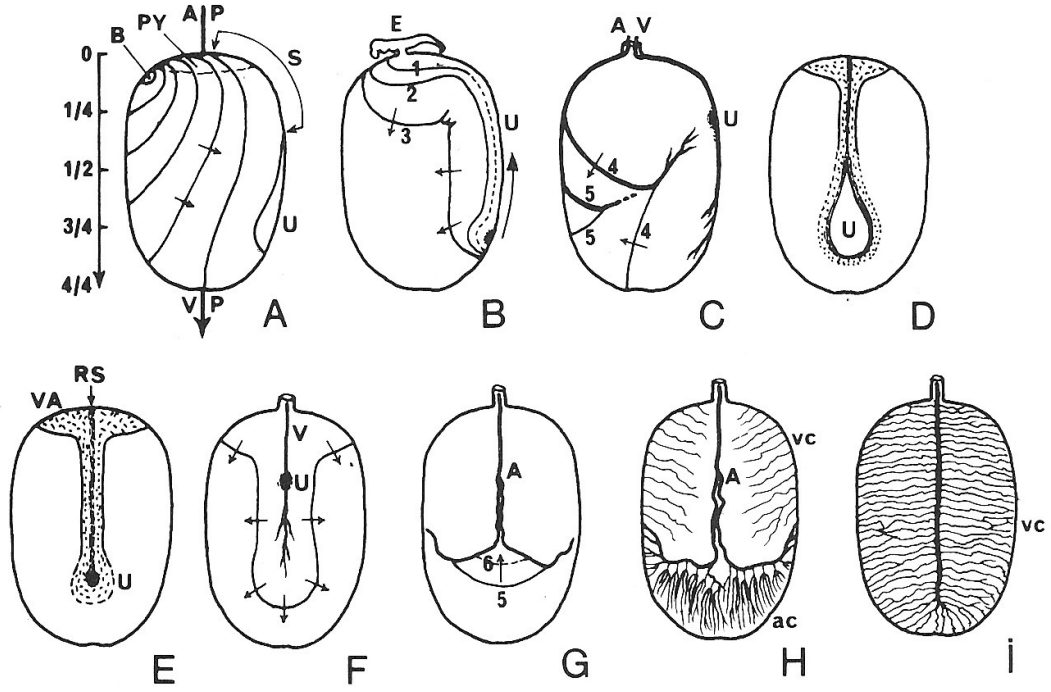

Fig. 1. Polarity and symmetry of dogfish yolk and embryonic system. Development of external yolk sac (A, D, E), subsequent expansion of vascular area (B, C, E–G), and final pattern of vitelline vessels (H, I). Schematic. Orientation: A–C are in profile, D–F and I caudal, G–H cephalic side views of the embryonic system.

A) Polarity of egg cell marked by the presence of a whitish polar yolk (PY) cap around the animal pole (AP), most conspicuous in green-colored egg cells. The blastodisc (B) is thus rejected in the upper corner and generally behind one edge of egg-case. Curves are the successive steps of extraembryonic blastoderm spreading (arrows). Its growth toward the vegetal pole (VP) can be scaled as indicated on the left (see Fig. 2 for the resulting graph). U, open umbilicus. S, suture, growing from the AP on midline.

B) Yolk sac completely closed, with a red spot on the closed umbilicus (U), which rises during the following days (arrow). Subsequent expansion of vascular area (arrows; three steps: 1, 2, 3). Embryo (E) now in polar position (note its orientation: head turned leftward).

C) Further steps of vascular area expansion (4, 5), with arterial lining (A; note orientation of arterioles toward umbilicus).

D) Caudal view of the open umbilicus (U), with the yolk sac suture above, and vascular area (islets shown as dots), lined in part by branches of presumptive vitelline artery (solid, symmetric lines).

E) Umbilicus just closed (U), vascular area (VA) still unexpanded. Proeminent "red streak" (RS) marking the suture of yolk sac: this pigment (hemoglobin?) already existed along blastoderm edge during its spreading over the yolk.

F) Same structures during step 3 of vascular area expansion (arrows). Risen umbilicus (U) crossed by vitelline vein (V).

G) Steps 5 and 6 terminating vascular area expansion. A, vitelline artery, bifurcated.

H) Full development of vitelline artery (A). Distal bundles of arterial capillaries (ac) are emitted by both arterial branches, while blood is drained by venous capillaries (vc) to the opposite side.

I) Opposite side (caudal). Vitelline vein and the dense network of venous capillaries (vc).

Results

Polarity and symmetry of yolk and embryonic system

There are two kinds of eggs, which differ by pigmentation of the yolk: green or yellow. Yolk polarity was more easily observed in the former. When eggs were suspended, with the longitudinal axis of the egg-case vertical, re-equilibration of the yolk along its density gradient

after several hours occurred, presumably by rotation. A distinct, whitish and veined, polar yolk marked the place of the animal pole. The blastodisc slowly followed the movement, but it was arrested by the edge of the polar yolk. The resulting relationship determined the symmetry: the embryo always appeared on the blastodisc margin which contacted this yolk, and its tail was turned toward the animal pole. During a two-week period, as embryogenesis proceeded, the embryo moved backwards, finally to sit over the animal pole. Fig. 1 shows this orientation, and the accurate symmetrical development of the avascular EYS, followed by that of the vascular area, can be observed very easily. The opposite closing of the EYS and the vascular area is an amazing feature of this egg.

One remark should be added. The shape of every yolk drawn in Fig. 1 corresponds to a side view, where the yolk is more compressed crosswise owing to the flat shape of egg-case. In sketches D–I, the plane of symmetry of the ES is also crosswise, although it is shown in profile in sketches A–C. In fact, the blastodisc tended to be located behind one ridge of the egg-case (not shown, but see A). Later on, the ES turned more or less around the vitelline gradient, probably under the effects of EYS development, while the embryo was still minute. Thus, the umbilicus could often be seen from the side (D). But its real position varied from one egg to another. After the completion of the extraembryonic vascular system, the increasing stiffness of the EYS and the strength of the embryo's swimming movements enabled active rotation.

As Hochstetter (1905) noticed in *S. stellaris,* a red line runs parallel to the edge of the blastoderm and gives a prominent red color to the suture. This red streak was replaced by the vitelline vein, but angiogenesis has not been studied in detail. There was a curious migration of the red umbilical knot toward the animal pole (Fig. 1B, E, F), with a simultaneous shortening of the streak, before the vein appeared.

Development table and chronology at 16°C

The A–H stages defined by Balfour (1878) and partly revised or subdivided I– Q stages are listed in Table 2. Daily observation of live eggs were done with batch C in a preliminary way, and then more thoroughly with batch F (Table 1). The latter allowed us to track the chronology of development until pre-hatching (Fig. 2). Batches B and C were used to estimate the delay between egg extraction and Balfour's A stage (19 days at 16°C), in order to supply Fig. 2 with a likely origin of times. This adjustment may have introduced a small error. Results obtained with batch C were mainly biometrical (Fig. 3; see also the following sections). Despite the constant and uniform environment provided during egg incubation, differences in the development length were still observed between the batches (Table 1). However, with regard to the long duration involved, there was a remarkable synchrony in pre-hatching and hatching. Twin eggs were even pre-hatching on the same day. Thus, the claim for large individual variation in the developmental speed, made by earliest dogfish embryologists, must be qualified.

Development of the unpaired fins was linked to the presence of a sagittal fold of the skin which already existed at stage H. It remained very low until stage N, when these fins began to arise, and the height shown on Balfour's sketch referring to stage O was not attained until stage P. Thus, Balfour's sketches of stages N–P cannot be trusted. As this fold grew, it became interrupted by three cuts, one behind each dorsal fin and one behind the anal fin. The angles of both dorsal cuts became progressively more acute, providing a measure for re-defining stage P. This stage was also characterized by longer gill filaments (4–5 mm, compared with 1 mm at the beginning of stage O), and also by the completion of mouth folds, nasal valve and sensory canals on the snout.

It has been shown once again that the growth in length of this embryo has a diphasic nature (Figs. 2, 3): it slowed down before pre-hatching and hatching (see Alluchon-Gérard and

Table 2. Developmental table of the dogfish (modified from Balfour, 1878).

Stage	Features
A:	early gastrulation (bump on blastodisc posterior margin)
B:	early neurulation (notch on blastodisc posterior margin)
C:	neural folds parallel (no brain enlargement)
D:	medullary groove with brain enlargement and caudal lobes
(E:	no normal stage)
F:	neural tube closed in trunk region
G:	first pair of visceral pouches (P1); neural tube completed
H:	P1-2
I1:	P1-3
I2:	P1-4, neither visceral clefts nor mouth opening
K1a:	second pair of visceral clefts (C2) and mouth
K1b:	C1 (future spiracles) and C2
K2:	C1-3 (mouth losange-shaped until stage M)
K3:	C1-4
L:	C1-5
M:	C1-6
	(M1: no branchial buds on mandibular arch)
	(M2: one or two branchial buds on mandibular arch)
N:	mouth triangular (mandible edge transverse)
O:	mouth curved (mandible protruding); unpaired fins-forming fold with obtuse cuts
	(O1: buds of embryonic caudal denticles absent on tip of tail)
	(O2: buds present on tail tip)
P:	snout distinct but not protruding ahead; acute cuts in the fully grown, unpaired fins-forming fold
	(P1: before pre-hatching)
	(P2: after pre-hatching)
Q:	snout protruding more and more ahead
	(Q1: external yolk sac still well developed)
	(Q2: external yold sac more or less reduced)
	(Q3: external yolk sac empty; embryo filling its egg-case).

Mellinger, 1971; Foulley and Mellinger, 1980a). The resumption of growth at about pre-hatching time (Fig. 2) started very soon after the onset of yolk entry into the spiral gut, which was observed in the same embryo.

Egg and newborn components

The eggs from Roscoff varied in shape, pigmentation, weight, length, and width, like those from Mediterranean, but they were twice as heavy. Length, measured along the mid-line of the egg-case, varied from 50 to 65 mm. Width stayed within narrower limits (19–24 mm), which is typical of nidamental gland function (Foulley and Mellinger, 1980a).

Twenty-three eggs (mainly from batches C and F) were weighed in order to determine the relative amounts of yolk, jelly, and egg-case materials at the beginning of development, i.e., after the stabilization of total egg weight (Foulley and Mellinger, 1980a), and before yolk consumption by the embryo began. Yolk weight showed only a weak correlation with egg weight (Fig. 4).

Egg-cases from wild females contained 53–56 per cent water, and jellies 94–95 per cent. Water contents of embryos and yolk plus membranes (EYS, IYS), varied during development, and this is depicted in Fig. 5. During stage O1, when the fresh weight was 32–41 mg, the embryo contained 89.6–93.3 per cent water; from stage Q3 to several days after birth, 74–76

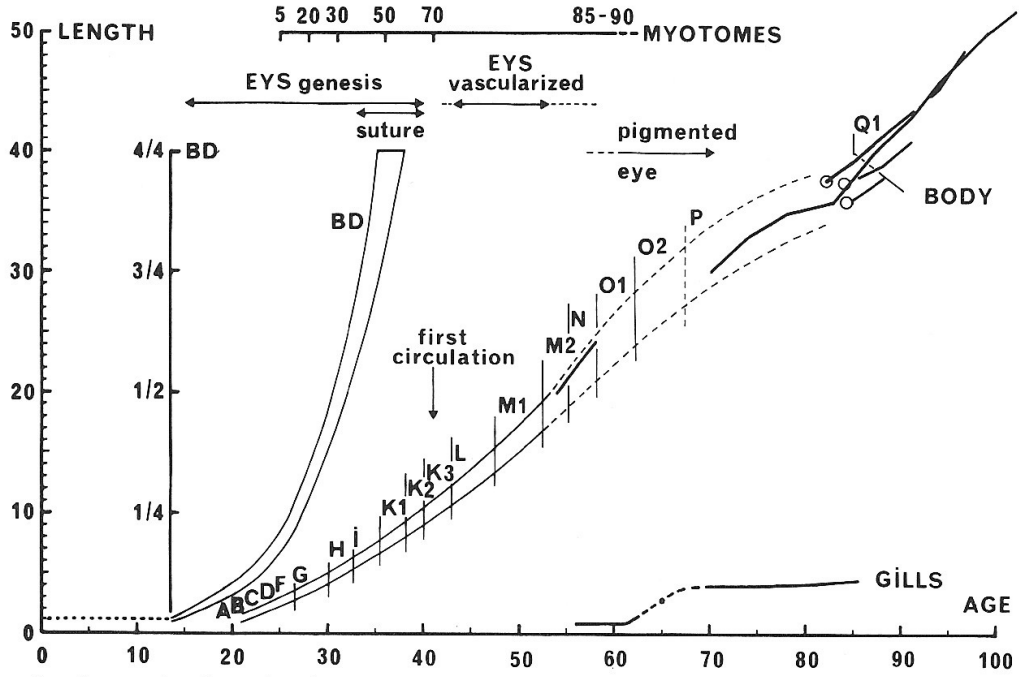

Fig. 2. Synopsis of egg development during the first half of incubation at 16°C (daily survey, 21 eggs of batch F). Age (abscissae, in days since egg extraction) was deduced from the previous survey of synchronous batch C. Length (ordinates, in mm) was measured on live embryos, mostly through the transparent egg-case. Thirteen embryos were also measured more accurately before fixation. No individual measurements are shown, but only the range of all data (thin, solid lines).

Approximate limits of development stages A-Q1 (see Table 2) are indicated along the relationship. After stage M1, only a rough estimate of body length could be made (dashed lines). However, two embryos were kept outside their egg-cases and measured after immobilization by MS 222 (thick lines; the smallest died on day 62). Three additional embryos were measured from pre-hatching on (open circles). Transition from short to long gill filaments is shown on the small graph (bottom).

The separate scale labeled BD refers to the spreading of blastoderm anterior margin (graph BD; only by range). It has been defined in Fig. 1A. Timing of other developmental events and myotome production are indicated on top.

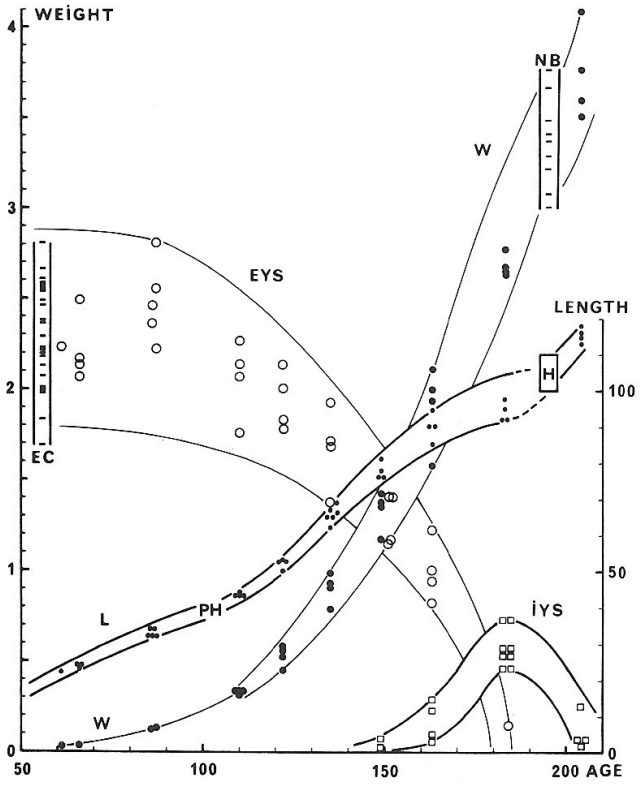

Fig. 3. Biometrics of embryo (L, W), external (EYS) and internal yolk sac (IYS) during the synchronous development of batch C (see Table 1). Age, expressed in days since egg extraction, deduced from batch B growth curve (not shown). Length (L) in mm, fresh weights (W, EYS, IYS) in grams. Weights of embryos (W) do not include IYS weights (same for Fig. 5). Weight ranges for egg cell (EC) and newborn (NB), from various batches are shown (dashes, between vertical lines), and also the range of body lengths at hatching (H, rectangle). In this batch, neither hatching nor pre-hatching (PH) have been watched. Distributions delineated by eye.

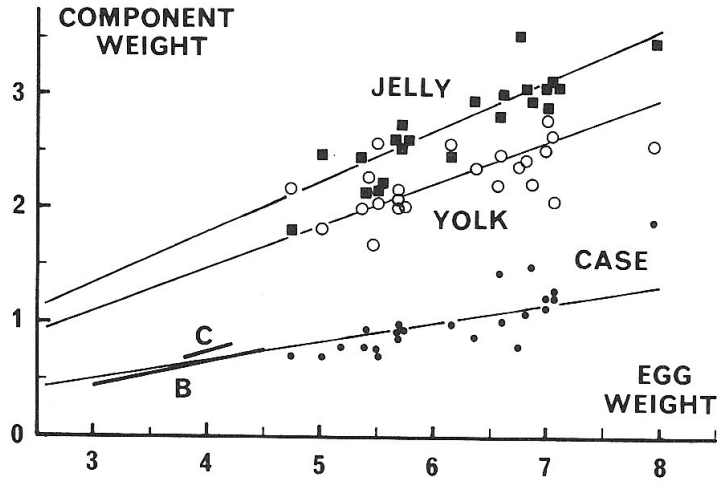

Fig. 4. Weights of the three component parts of the dogfish egg (egg-case, dots; yolk, open circles; jelly, solid squares) vs. total egg weight (in grams). Jelly weight was calculated by substracting yolk plus egg-case from total weight. Tendrils were cut off. Thick lines B and C correspond to egg-cases laid by two females of Mediterranean origin (Mellinger, 1983): they do not differ. Thin lines were drawn from the origin of scale through the mean point of each set of bivariate data. Although linear regressions were found significant by t-test, these proportional relationships fit the best (except to yolk data). Calculated means were: 6.20 g for egg, 1.03 g for egg-case, 2.31 g for yolk, 2.72 g for jelly. Correlation coefficients (r) were: 0.77 for egg-case, 0.69 for yolk, 0.88 for jelly.

per cent. In the yolk until stage O1, there was about 50 per cent water, and this decreased to about 38 per cent from stage Q3 onward.

Fig. 5 also shows the chronology of dry weight changes in the embryonic system. Whereas newborns were always heavier than egg cells on a fresh weight basis (Fig. 3), here we observe that their mean dry weight was less than the mean of egg cell dry weights. The balance of water and dry matter during dogfish development is presented in Table 3A.

Plotting the sum of EYS and IYS against time in Fig. 5 allows demonstration of the rapid final course of yolk consumption, which ends about ten days after birth. Although vitelline circulation was well-developed since day 55, yolk consumption remained practically nil until day 100 (about the time of pre-hatching). This is not surprising, since the dry weight of the embryo (about 35 mg) was negligible compared to yolk amounts. Intestinal digestion thus disposes of at least 90 per cent of the yolk supply.

Table 3B shows the results of egg cell and newborn microanalysis. Means of duplicate or triplicate determinations were computed for each analyzed item. No differences between both laboratories appeared, but sulfur microanalysis and IYS analyses have not been repeated. Since the statistical distributions of data were homogenous, means have been computed for each sample. Precision varied according to each sample and element, but was satisfactory. Percentages differed significantly except for carbon in IYS vs. EC. Even small discrepancies between IYS and EC with regard to hydrogen and nitrogen seemed to be significant. Carbon and hydrogen losses were clearly larger than nitrogen loss. Thus, nitrogen appears to be conserved to a certain extent. No distinction can be made between organic sulfur and sulfate ions.

Osmolyte concentrations

The osmolality of aqueous extracts was low and constant for each dilution adopted: about 220 mOsm.kg^{-1} H_2O for 5-fold diluted jelly extracts, 100–110 mOsm in the 10–fold diluted water space of body, EYS and IYS, and 40–50 mOsm for 20–fold diluted water of the

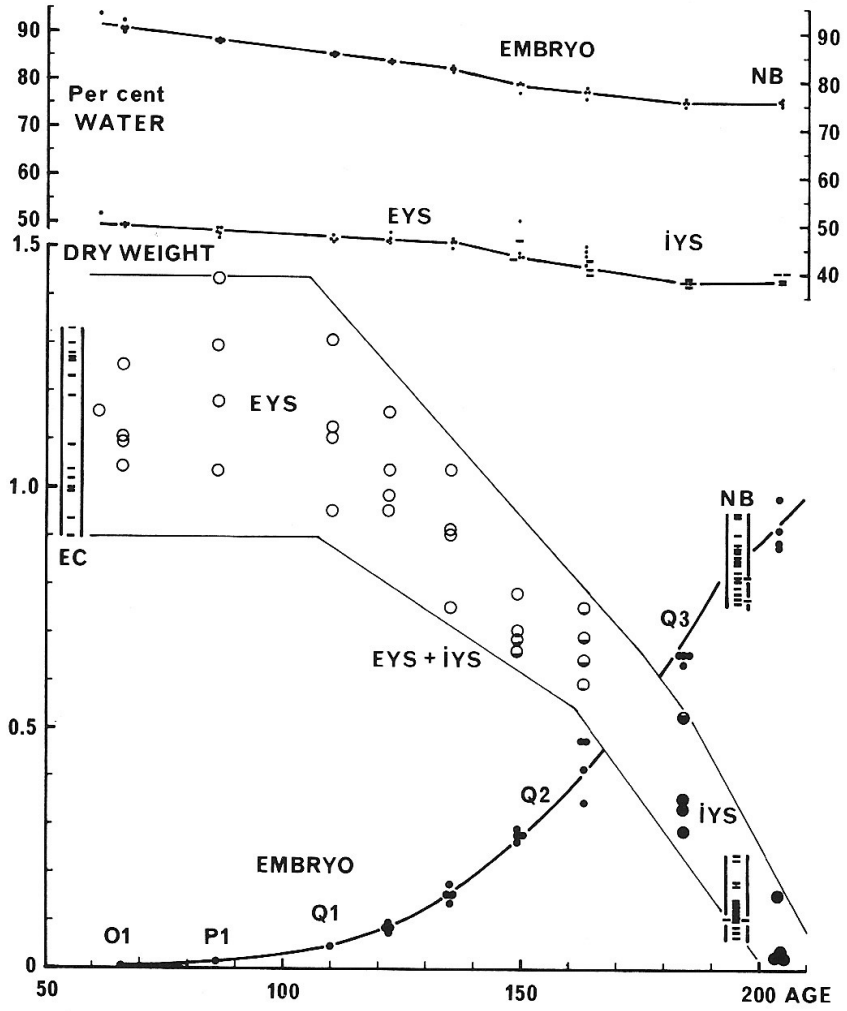

Fig. 5. Dry weights (in grams, lower graph) and water contents (in percent of fresh weight, top) during batch C synchronous development. Age, expressed in days since egg extraction, adjusted to batch B growth curve like in Fig. 3 (same abbreviations; ranges of EC and NB dry weights are also shown for comparison, plus the range of IYS data for the NB; but, in Fig. 5, EC do not include data from batch C). Developmental stages are indicated. Lines and curves fitted by eye.

Yolk consumption was undetectable before day 100, as shown by drawing of EC + EYS total range. Only EYS water contents were declining slowly, until day 135. Subjective drawing of limits (thin lines) for the whole distribution of EYS data (open circles), EYS plus IYS (partly filled circles), and IYS (solid circles) suggested that yolk consumption started about day 100, which agrees with the first entry of yolk into the gut occurring a few days before pre-hatching. Nearly half the yolk was already digested when the IYS began to load up (on day 150).

Note the very parallel decrease of water contents in both embryo and yolk plus its membranes (top graph; EYS data shown as dots, IYS data by dashes).

smallest embryos. Because of the lack of proportionality of osmolality to osmolyte concentrations in complex mixtures, and the lack of sensibility of osmometry at low concentrations, this constitutes only a gross indication of the internal medium being isosmotic to sea water.

Ions and urea were determined directly with 10–40 μl aliquots of the extracts, except Na^+ of yolk and embryo which required freeze-drying of 1 ml aliquots in order to reconstitute the original undiluted medium (yolk water space), or a 2–fold diluted medium (embryo), to get a sufficient concentration for reliable Na^+ measurement. The samples were re-analyzed for simultaneous Na^+ and K^+ determination with an Instrumentation Laboratory (model 343)

Table 3. Composition of "egg cell" (EC) and newborn (NB): the chemical balance of development. Mean weights ± SE, in grams. IYS: internal yolk sac of newborn.

A. Results of dry weight determinations.

Sample	n	Fresh weight	Dry weight	Water	% water
(a) EC	22*	2.302 ± 0.058	1.167 ± 0.032	1.148 ± 0.033	49.8
(b) NB minus IYS	14	3.318 ± 0.060	0.8439 ± 0.0154	2.475 ± 0.045	74.6
(b) − (a)		+1.016 (+44.1%)	−0.3231 (−27.7%)	+1.327 (+215%)	
(c) NB with IYS	14	3.535 ± 0.057	0.9705 ± 0.0260	2.564 ± 0.049	72.5
(c) − (a)		+1.233 (+53.5%)	−0.1965 (−16.8%)	+1.416 (+223%)	
(d) IYS alone	14	0.2172 ± 0.0231	0.1338 ± 0.0141	0.0834 ± 0.0090	38.4

* n = 23 for fresh weight.

B. Results of carbon, hydrogen, nitrogen and sulfur microanalysis on dry samples.

Sample	n	C	%C	H	%H	N	%N	S	%S
(a) EC	6	0.594 ± 0.030	52.8	0.091 ± 0.004	8.0	0.196 ± 0.007	13.0	0.0106 ± 0.0005	0.94
(b) NB minus IYS	8	0.388 ± 0.009	45.2	0.061 ± 0.001	7.1	0.118 ± 0.003	13.8	0.0098 ± 0.0002	1.14
(b) − (a)		−0.206 (−34.6%)		−0.030 (−32.9%)		−0.028 (−19.1%)		0.0008 (−0.8%)	
(c) NB with IYS	8	0.464 ± 0.015	—	0.072 ± 0.002	—	0.136 ± 0.004	—	—	—
(c) − (a)		−0.130 (−21.8%)		−0.019 (−20.8%)		−0.010 (−6.8%)		—	
(d) IYS alone	9	0.081 ± 0.010	53.5	0.012 ± 0.001	8.2	0.019 ± 0.002	12.5	—	—

Table 4. Osmolyte concentrations ($mmol.l^{-1}$) in egg jelly. Figures are mean ± SE (range). Urea concentration (n = 24) was always 0.

Batch	n	Cl^-	Na^+	Cl^-/Na^+	K^+	Ca^{2+}
C	12	581 ± 1.4 (571–587)	486 ± 1.0 (482–492)	1.195*	10.6 ± 0.17 (10–12)	13.9 ± 0.32 (12.7–16.7)
F	6	620 ± 6.6 (595–643)	520 ± 1.9 (511–526)	1.192*	11.0 ± 0.05 (10.8–11.2)	14.4 ± 0.09 (14–15.6)

* In natural sea water, the Cl^-/Na^+ ratio is 1.166.

flame photometer; the 10–fold higher K^+ values obtained worked as an internal standard. The series has yet to be completed with small embryos and IYS. Calcium was only measurable in sea water, jelly, large embryos and newborns. A few sea water samples were collected, but they did not give as reliable results as the egg extracts. Repeated extraction of centrifugation pellets showed the recovery being over 95 per cent, so results were not corrected.

Table 4 gives the ionic composition of jelly, which closely resembles sea water. Urea was always undetectable. Differences between batches C and F with regard to Cl^- and Na^+ probably resulted from the use of improperly diluted sea water for the incubation of C eggs: one undiluted sea water sample was found afterward to be as low as 980 $mOsm.kg^{-1}$

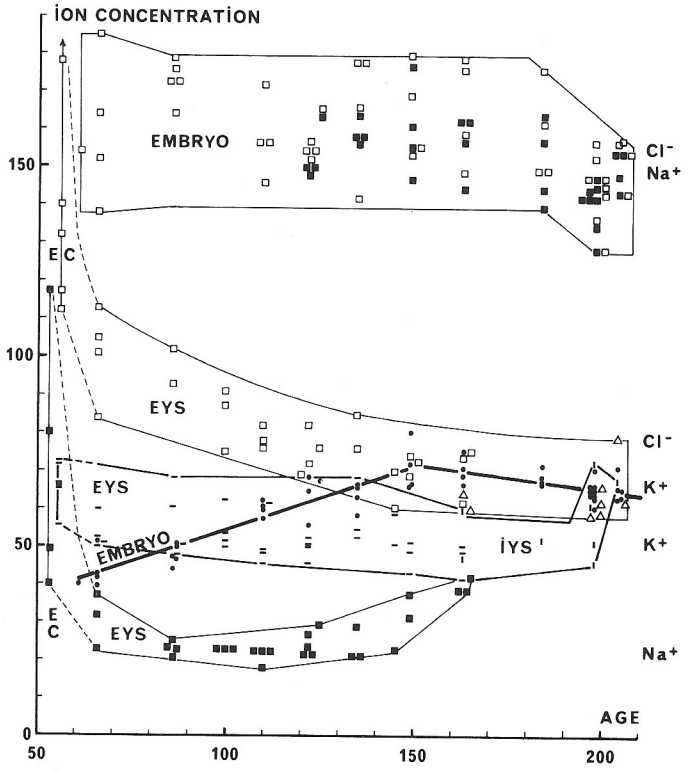

Fig. 6. Chloride, sodium and potassium ion concentrations (in mmol.l^{-1}) vs. time (age in days; same batches as in Figs. 3 and 5). EC, "egg cell" data, arranged along Y-axis (age unknown, but less than 30 days), and linked together in each set by one vertical bar (note the highest Cl^- datum was out of scale, 235 millimolar). Symbols: Cl^- in embryos, EYS and EC, open squares; Cl^- in IYS, open triangles; Na^+, solid squares; K^+ in embryos, dots; K^+ in EYS and EC, horizontal dashes; K^+ in IYS, vertical dashes. Each data distribution has been outlined, except K^+ data in embryos, which are fitted by two successive regression lines (thick lines): $Y = 0.3375\,X + 20.70$ ($n = 26$, $r = 0.91$, days 61–149), and $Y = -0.1184\,X + 89.43$ ($n = 26$, $r = -0.55$, days 149–205), both significant. All concentrations were expressed with reference to internal water, determined by freeze-drying. Note the constancy of Cl^- concentration (about 160 millimolar) in the embryo, except a small decrease at birth. Although Na^+ measurements are still lacking for the smaller embryos, Na^+ concentration appeared similar to Cl^-. Extracts from embryos corresponded to a mixture of extra- and intracellular media, both in a ratio roughly estimated to 1 : 3 with regard to various sources. Since Cl^- and Na^+ extracellular concentrations are generally close in chondrichthyans (about 280 millimolar), they must have been also equal in embryo cells (about 120 millimolar), but the actual ratio may vary widely according to cell types (Robin *et al.*, 1964). In this respect, Cl^- and Na^+ concentrations in EYS and IYS toward the end of development (lower, delineated relationships) best matched the intracellular ionic pattern, but K^+ was very low (50–60 instead of 100–200 millimolar which is generally found in animal cells). In some IYS, K^+ was close to embryo levels. The definite changes which seemed to occur in the embryo remain unexplained. Decrease of Cl^- and Na^+ in the yolk was very marked; this will be discussed in the text after viewing Fig. 7.

H_2O. Thus, we must take into account a possible 7 per cent reduction of internal osmolyte concentrations in the samples from batch C, on which the majority of results were obtained. Despite the lack of prior measurements of sea water osmolality and ionic composition, the constancy of Cl^- and Na^+ concentrations was achieved by frequent restoring of the water

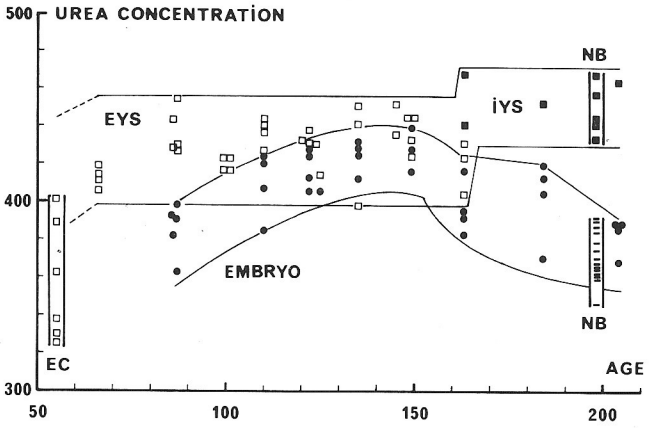

Fig. 7. Urea concentration (mmol.l^{-1}) vs. time (age in days refers to batch C as in Figs. 3–6, with additional data for comparison). Urea nitrogen was determined after deproteinization according to the user's instructions of the kit (see Methods), using a 4-fold further dilution since original urea N concentration was too high. Results were then expressed in mmoles urea per liter water space. Results from the smallest embryos are omitted, since they have been obtained by a different method and seemed too low. Data are the means of two independent measurements for each extract. EC, egg cells; NB, newborns; dots, embryos; dashes, newborns; open squares, EC and EYS; solid squares, IYS. Discrepancies between embryo, EYS, and IYS urea levels are discussed in section "Osmolyte Concentrations" of Results.

level in the tank, using less than 1 per cent volumes of tap water each time. Constant Cl^- and Na^+ concentrations were actually found in the jelly samples, collected from the 61st to the 100th day. For these reasons, changes in osmolyte concentrations described in Figs. 6 and 7 can hardly be considered as artifacts. They reveal the complexity of osmolyte control by the embryo and its EYS.

Comparing Figs. 6 and 7, it is noticeable that Cl^- and Na^+ levels first decreased in the EYS and thereafter became more accurately regulated than in egg cells, while urea level behaved exactly opposite. Urea concentrations were within the limits expected for a marine chondrichthyan fish. It may be supposed that the increase of concentration observed during the first third of development compensated for Cl^- plus Na^+ decrease, and that these ions were excreted into sea water by active transport.

The higher concentration of urea observed in the IYS compared to EYS, the declining concentration in the embryo after pre-hatching until birth, and the relatively constant urea concentration in the EYS after day 80, were all reminiscent of previous results on Mediterranean dogfish eggs (Mellinger and Wrisez, 1983). These first urea measurements were performed on supernatants of 9 : 1 diluted homogenates in a similar way, but without any preliminary freeze-drying, and results were only expressed with reference to the fresh weight or volume of the embryo, EYS or IYS.

Urea supposedly diffuses only in internal water, since its partition coefficient between olive oil and water is 0.00015. A strict equilibrium of concentrations was expected in the three parts of the embryonic system, but Fig. 7 shows this was not observed. This result for the IYS would be understandable, if its high concentration reflected the real concentration in the embryo, which admittedly was the source of urea (liver ureogenesis) and would have maintained a small concentration gradient against EYS. This supposes that the lower concentration in embryos and newborns was actually underestimated because part of the internal water did not contain urea at all. Indeed, the digestive tract may be responsible for

that discrepancy, despite thorough blotting dry of the pharynx sea water contents. This led us to examine the status of gut compartment(s) in the embryo.

Transitory closures of the digestive tract

Balfour (1878) first described "the solid oesophagus" of the dogfish embryo. This closure occurred "at a stage slightly younger than K". He was unable "to state the exact period at which the lumen reappears", since he still found it solid "by the close of stage Q". Another closed section of the digestive tract was described by Forssner (1907) in embryos and pups of *S. acanthias,* from stage 11 mm until birth: the rectal closure. In a similar way to the esophagus, it tightly closes the section behind the posterior intestine (which may be called *spiral gut,* since it is characterized by possessing the spiral valve), and ends anterior to the opening of the rectal gland.

Our own histological observations, done with several dozen *S. canicula* embryos, showed that the esophagus closed at a definite time in development: between stages I1 and I2. The lumen remained fully open during stage H (one embryo, 2×28 myotomes) and stage I1 (one embryo, 2×33 myotomes, 6.3 mm length), but closure was complete at stage I2 (one embryo, 2×39 myotomes, 6.7 mm body length, 16 μm for the length of the closed section) and in all embryos from stage K1 (length of closed section: 180–200 μm) to stage Q1 (body length: 57 mm or less). Three larger embryos (59, 88, and 90 mm) still showed a tight but short occlusion at the boundary between esophagus and stomach, where the end of the Leydig organ produced an epithelial folding into the lumen. In two embryos at stage Q3 (lengths: 95 and 99 mm), a partial closure still remained present at this level. It had completely disappeared in the newborns. The stomach secreted a mucous plug during stages Q2 and Q3.

In Mediterranean embryos, the same closure occurred. During stage I2, a very narrow lumen persisted in one embryo (about 2×40 myotomes), but closure was complete in two others (37 myotomes, length of closed section 77 μm, and 41 myotomes, 125 μm closed). All more developed embryos always showed the closed esophagus.

As described by Balfour, the epithelium first retains its cylindrical shape, closing the lumen only by increasing its thickness, then it becomes broad and flat. Closure was checked on semithin and ultrathin sections (electron microscopy) in one embryo (stage O). The arrangement of cells was reminiscent of a parenchyme.

In every case, the closure preceded the appearance of the first holes in the buccal membrane and second pair of visceral pouches, which were pierced at stage K1a. The opening of the pharynx allowed entry of perivitelline fluid, the innermost, liquid part of the jelly. The alimentary canal, i.e., the future stomach and gut, had a very broad ventral opening to the yolk at this stage. Together with the neural tube, it constituted the main fluid compartment of the embryo. Both were connected by the neurenteric canal, and the yolk itself was only separated from this fluid by a subtle cytoplasmic layer. If a barrier did not prevent diffusion of salt into the embryo and urea diffusion outside, osmoregulation would not have been possible. The esophageal closure thus clearly provides this barrier. It is an osmoregulatory device, which probably becomes less necessary when the muscular layers of the digestive tract are functional, and when the stomach's mucous plug completes the barrier (Fig. 8).

A cloacal opening was lacking before pre-hatching. After pre-hatching, as the kidneys already seemed functional and the rectal gland certainly also was, urine and rectal fluid might have been excreted through the cloacal slit, which appeared, like in Balfour's description, opening at the shallow hind end of the ectodermal involution and slowly stretching forward.

The embryo differed from the adult by its deeply embedded cloacal chamber, opening to the exterior only by a very narrow slit. This part of the hindgut was readily distinguished from the rectum by histological structure, anatomical relationships, and function. Extending from,

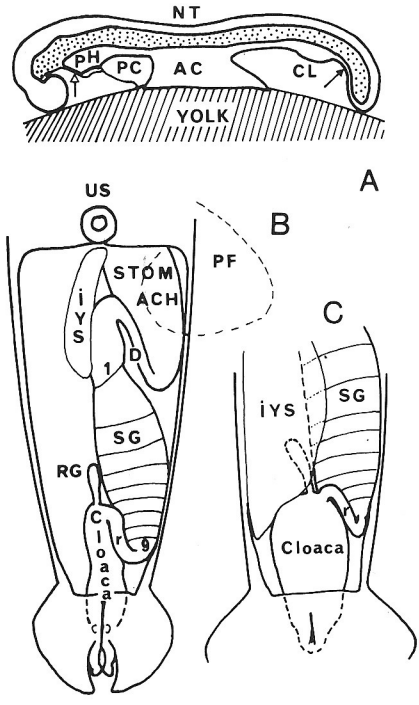

Fig. 8. Anatomical sketches. A) Main compartments of the embryo body before the piercing of the buccal membrane (open arrow, on the left); stage I1, sagittal section; besides the heart, pericardial cavities (PC), and the dorsal cord surrounded by other axial mesoderm derivatives (stippled area), this diagram shows the continuity of pharynx (PH), esophagus, "alimentary canal" (Balfour, 1878; AC), hindgut with cloaca (CL), neurenteric canal (solid arrow), and neural tube (NT).

B), C) Ventral aspect of the digestive tract (abdominal cavity cut open), before (B) and after (C) the loading of IYS, which finally abuts on the swollen cloaca and masks the rectal gland (RG) and part of the spiral gut (SG, with its nine spiral valve septae, 1–9). The rectum (r), from gut to cloaca, was closed. D, duodenum; US, umbilical stalk (sectioned); PF, pectoral fin. Note also cloacal slit, pelvic fins with pterygopods (in B).

or slightly anterior to, the rectal gland opening, it was often found very distended and seen in live embryos as being able to expel its fluid by a sudden contraction. The rectal gland stood straight up on the anterior end of this genuine bladder, and it may be more truly called the "cloacal gland", for the narrow rectum of the embryo was merely a connection between the cloaca and the spiral gut (Fig. 8).

The rectal closure, which was confined to the rectum *s. str.*, was found in all embryos from stage N (about 22 mm) to stage Q2 (90 mm body length). The lumen began to narrow at the very beginning of rectal gland morphogenesis, during the end of stage M2 or at stage N, and this simultaneous differentiation was also observed by Scammon (1911) in *Squalus*. Like in the esophagus, epithelial cells were tightly packed and the epithelial cylinder soon looked parenchymatous. Several basal buds then gave it an irregular outline in transverse sections, during stage Q. Lacunae formation indicated the reorganization of the epithelium into several longitudinal folds, which were first fused at their tips. During stage Q3, seven embryos had their rectum reopened, while eight kept the folds fused. Of 14 newborns, nine still showed their rectums more or less closed.

When newborns were put into a small aquarium with still water, undigested yolk pellets sometimes came to the surface. Thus, the primary role of the rectal closure might have been to prevent the yolk escaping from the spiral gut. But later this could perhaps also be achieved by the sinuous course of the rectum, and by muscular contraction. Rectal closure probably was not absolutely necessary after stage Q1.

Osmoregulatory effectors

Chondrichthyans are isosmotic or slightly hyperosmotic to sea water due to high Cl^- and Na^+ plasma concentrations, supplemented with urea and trimethylamine oxide, which are massively retained. Ion exchange rates are very low, compared to teleosts (1% for Na^+, according to Evans, 1980). The different functions played in Cl^- and Na^+ excretion

by the rectal gland, kidneys and gills are not well understood.

Both the gill epithelium and the rectal gland of *S. canicula* were studied by light microscopy in more than 30 embryos, using paraffin sections stained by Herlant's tetrachrome, or prior impregnation by Zn iodide (Champy-Maillet's method, as described by Garcia-Romeu and Masoni, 1970). Twenty-eight embryos and newborns of 17 mm to 107 mm body length were used for scanning electron microscopy (SEM) of the gills after fixation in 5 per cent glutaraldehyde, followed by post-fixation in 1 per cent osmium tetroxide, both cacodylate buffered.

By light microscopy, with the tetrachrome staining, large pink cells covered by flat surface cells, and sometimes showing crypts, were observed located along the primary lamellae, where they were building up an almost continuous layer in newborns. Metallic impregnation varied from black to grey according to cells. This cell type was already present in the differentiating gill lamellae of pre-hatching embryos. No mucous cells were found, either by tetrachrome nor by toluidine blue staining. This avoided mistaking chloride cells and mucous cells in SEM.

SEM observations showed polygonal surface cells (Figs. 9, 10) on primary and secondary lamellae, and also on external gill filaments where present. Their surface had very short microvilli. Chloride cells were only detectable by their narrow distal ends, appearing between several pavement-like cells, either embedded in a hole (crypt) or protruding above the epithelial surface (tufts). Both aspects occurred in some lamellae (Fig. 10), and possible transitional forms existed. In every case, chloride cells had longer microvilli than pavement-like cells. Surface morphology of the gill epithelium in P–Q stage embryos looked like the adult condition, described by Crespo (1982), but we did not observe microridges. There was also a third cell type, covered with thicker microvilli. Each of these disseminated cells was generally found lying at the edge of a crypt. Perhaps they were merely desquamating pavement-like cells, as they were shrunken in appearance (Fig. 12).

The surface distribution of chloride cells appeared very restricted by SEM studies. Crypts and tufts of chloride cells were only seen on the primary lamellae near their naked bases and in the adjacent space between the secondary lamellae. This corresponded to a similar distribution in teleosts, on the afferent side with regard to blood flow. There were no chloride cells on the secondary lamellae and external gill filaments, which are specialized respiratory areas. Close inspection of the skin surface in several embryos did not show any chloride cells. From this preliminary study, we conclude that chloride cells are present on the embryo's gills at pre-hatching and then increase in number.

The relationship between the development of the cloaca, rectal gland and kidneys has already been dealt with in the preceeding section. The rectal gland was fully differentiated at the beginning of stage P, according to preliminary transmission electron microscopic observations: membrane stacks of the latero-basal system which carries the Na^+, K^+-ATPase responsible for Cl^- and Na^+ transport were already present, and mitochondria appeared as numerous as in the adult. Moreover, the relative volume of the gland to body was considerably higher during stage P than at any other time in the dogfish's life. Vascularization developed very early, and vessels form a dense network during stage P. For these reasons, the rectal gland could have been the principal source of fluid that swells up the cloaca like a bladder during stages Q2–3 and in newborns.

Birth

Development lasted 5 1/2 to 6 1/2 months at 16°C. In newborns, body length reached 101–110 mm, body weight 3.0–3.8 g (one exception, not included in Table 1: 92 mm, 2.2 g). Toward the end of development, the embryo completely filled its egg-case, and therefore

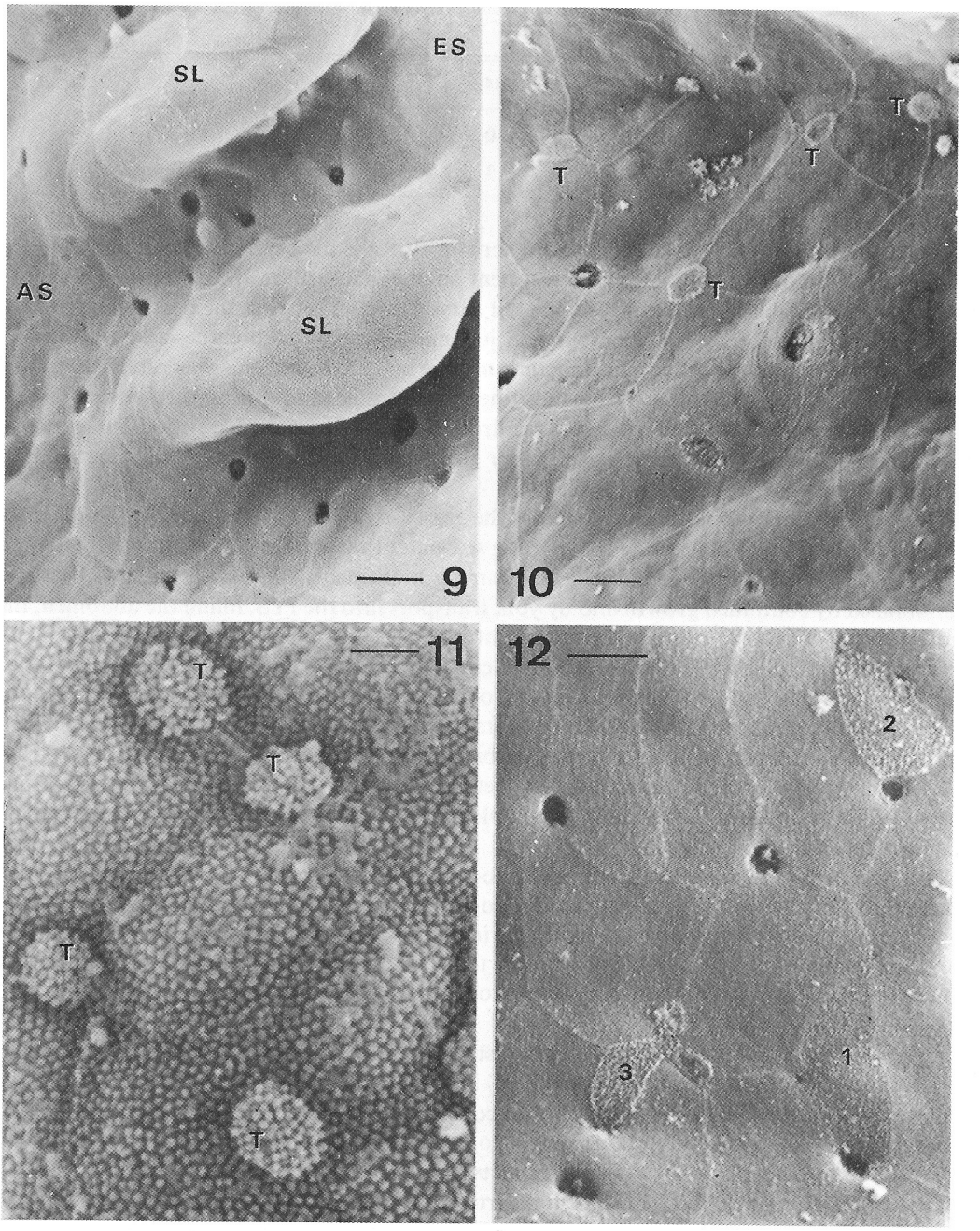

Fig. 9. Distribution of chloride cells near the afferent side (AS) of the primary gill lamella and in adjacent interlamellary space (SL: two adjacent secondary lamellae; ES, efferent side). Chloride cells are only detectable by their crypts (holes), opening between the polygonal pavement-like cells. Stage Q3, SEM, bar $=5\,\mu$m.

Fig. 10. Area of gill epithelium showing mixed types of chloride cells: T, tufts of microvilli; holes, ordinary crypts. Stage Q3, SEM, bar $=5\,\mu$m.

Fig. 11. Higher magnification of a primary lamella in a 33 mm-long embryo, showing the first appearance of chloride cells as protruding microvilli tufts (T), among the shorter microvilli of pavement-like cells. Stage P (beginning), SEM, bar $=1\,\mu$m.

Fig. 12. The third cell type of primary lamella surface (1, 2, 3: three cells, with supposed shrinkage sequence). Cells 1 and 2 were at the edges of crypts (holes), cell 3 was not. Stage Q2, SEM, bar $=5\,\mu$m.

Table 5. Number of births during three consecutive days in two different samples, O (obscurity) and L (light).

Day:	183rd	184th	185th	
Sample O:	7	0	0	($n_1=7$)
Sample L:	2	3	3	($n_2=8$)

became almost immobilized in a very characteristic attitude: the head was positioned into the flat anterior extremity, the trunk region was bent on one side to allow the tail base to loop into the bulging caudal half of the egg-case, the tail curved inward between the dorsal fins, and the caudal fin lay askew over the snout. It had just enough room to breathe, and was able to turn from one side to the other by rolling around the body axis, but this was rarely seen. This orientation was adopted whatever position the egg was placed in during incubation: several eggs were suspended by their posterior tendrils, hence a downward position for embryos before birth, although they were sitting over their EYS until stage Q1. Only one embryo adopted the reverse position, and it died.

In stage Q2, body length exceeds that of the egg-case. The EYS first hangs in the center of the caudal loop. It may contribute to pumping sea water through the slits by its passive to and fro motion accompanying the embryo's swimming movements. When the embryo becomes immobilized by continued growth and by yolk transport into the IYS, filling the abdomen, the almost empty EYS still remains in that location for a few days, with its umbilical stalk stretched under the belly. The stalk then shortens and turns at a right angle, before the rest of EYS becomes resorbed. It takes 8–18 days from stalk rotation to birth (stage Q3).

Extracted from the egg-case at this final stage, embryos were slippery and lacked erupted ordinary scales and teeth, possessing only embryonic scales, except one 183 day embryo that was found prickly by SEM. It seems that teeth and ordinary scales eruption occurs a few hours before hatching, as it was always complete in newborns. The latter were rough to the touch.

Continuous observation during multiple-hour sessions never allowed witnessing the natural hatching behavior, which is probably delayed by light. In past studies newborns were obtained on several occasions in our aquarium, but almost exclusively during night, as artificial illumination was applied from 9:00 to 17:00. During the present study, light was limited to diurnal visits. After the appearance of the first four newborns of batch E (day 181), an experiment was tried (Table 5).

The maximum difference between both distributions, observed on the 183rd day (first day of experiment), would be significant ($p=0.05$) in the Kolmogorov-Smirnov one-tailed test if both samples had been equal. To reach a firm conclusion, new experiments are required, but it is assumed that hatching is subject to a physiological control that favours newborn survival by delaying birth until complete darkness protects it against predators.

Hatching could be elicited in some fully grown embryos by squeezing the posterior end of the egg-case. In one glimpse, the newborn straightened his tail and pushed with its head through the egg-case aperture until the trunk protruded and then escaped by swimming. Some embryos were unable to hatch and got stuck at their pelvis in the opening, until they were freed by hand. This never occurred during natural birth.

Both egg-case slits adjacent to the aperture were examined after natural birth in batch E, to see if breakage of the 1–3 mm long wall that separates them from the latter would help hatching. Both were broken in about half of the sample (9/16), one egg-case remained intact, and others had a unilateral break. In most instances, a preformed breakage line was followed.

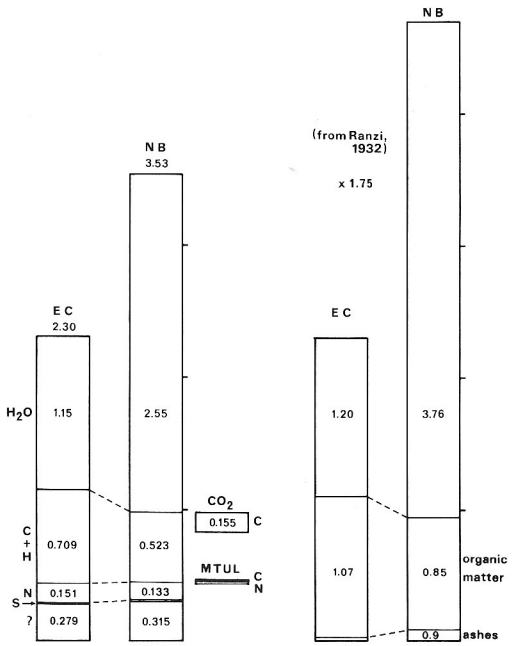

Fig. 13. Chart of the chemical balance between egg cell (EC) and newborn (NB, with IYS), computed from Table 3 (left) and from Ranzi's (1932) data (right), multiplied by 1.75 to equalize ECs fresh weights (larger water contents, 3.76 vs. 2.55, in the two NBs used by Ranzi were unexplained). All results in grams. Nitrogen (N) and carbon (C) losses are shown as urea (MTUL, maximum total urea loss) and carbon dioxide (CO_2).

Thus, these longer anterior slits play a role in hatching.

Discussion

Unlike birds (Romanoff, 1960), chondrichthyans have seldom been subjected to a physiological approach by embryologists. Some early investigators showed an interest in the sequential appearance of the main functions in the dogfish embryo, like digestion (Beard, 1878) and breathing (Polimanti, 1911), while others confined themselves to the study of organogenesis. In this respect, the brilliant synthesis worked out by Ranzi (1932, 1934) was a breakthrough, despite of the lack of statistical treatment which weakened some of his conclusions. Ranzi based his quantitative analysis of maternal-fetal relationships for viviparous selachians on his prior knowledge of dogfish egg and newborn chemical composition. He determined water content by heat dessication, ashes by burning, and then he calculated the amount of organic matter by the difference. There was a loss of 20.6 per cent organic matter during development, a minimum figure for oviparous and lecithotrophic viviparous species.

Far more accurate results have been obtained by freeze-drying in our experience, and microanalysis (which needs to be completed by oxygen and phosphorus determinations). These studies have several advantages over the traditional method used by Ranzi (1932). Once accurate and reproducible percentages of various elements have been obtained on small samples (Table 3B), we only need to multiply them by the mean dry weights of larger samples (Table 3A) to get accurate figures of mean amounts in egg cells and newborns. This gives more precision to the estimates of the chemical balance of development (Fig. 13). The maximum total urea loss (MTUL) can be computed from nitrogen loss, under the assumption that no other nitrogenous compounds are being excreted. Thus, the remaining carbon loss can be attributed to CO_2 production. MTUL was 0.66 mmol and CO_2 loss 13 mmol during the entire development (about 6 months). These figures can be compared to urea pools in EC (about 0.4 mmol) and newborn (about 1 mmol), which are of the same order of magnitude as MTUL.

Thus, urea appears as a conserved molecule.

Ranzi (1932) and Read (1968) did not realize the existence of definite changes in water content during development. Whereas a slow decrease of water percentage in embryos is common to all vertebrates, that it occurs parallel in the yolk seems relevant to water transport mechanisms from this compartment into the embryo. Water amounts gained by the embryo were probably overestimated by Ranzi, and inorganic matter (ashes) underestimated (Fig. 13). Our figure for C + H loss is 26 per cent. No definitive conclusions can be drawn about the meaning of water and osmolyte distribution patterns in the ES, particularly ions, until the dynamic state of each constituent has been characterized by tracer studies. It is however evident that the embryo has to absorb a large volume of external water without being invaded by Cl^- and Na^+ ions contained in this water. Since this penetration starts only at mid development, osmoregulatory effectors must be functional by this time. This was shown to be the case with the rectal gland, cloacal bladder and branchial chloride cells. Osmoregulatory needs may also grow because of surface increase by the elongated external gill filaments at stage P. Thus, rectal gland function may be more vital to the embryo than to the adult.

Other osmoregulatory effectors may exist during younger stages, particularly in the EYS wall. On the other hand, a high impermeability of ES membranes to urea (and probably also to ions) has been advocated (Read, 1968). This is indirectly supported by our observation of esophagus closure occurring just before pharynx opening. Around the yolk mass, four different membranes could play a role: first, the vitelline membrane and the cytoplasmic membrane of the egg cell, then the avascular EYS (only closed on day 40), and finally the vascularized EYS. In *S. canicula,* as in *S. stellaris* (Hochstetter, 1905), the EYS grows much faster than in a viviparous species like *Torpedo marmorata,* and this would explain why vascularization does not keep pace with EYS spreading in the former (Rückert, 1921). We measured 445 $mmol.l^{-1}$ urea in the uterine fluid of one gravid *T. marmorata* female. It is well-known that uterine fluid resembles plasma in most viviparous selachians. If the difference in EYS development rate mentioned by Rückert (1921) is a distinctive feature of oviparous vs. viviparous selachians, this would be an argument in favour of the EYS contribution to ES impermeability.

The wall of the egg-case is unable to retain urea in the jelly (Read, 1968; Hornsey, 1978; Foulley and Mellinger, 1980b). Contrary statements (Evans, 1981) should be reconsidered. The ionic composition of jelly is similar to sea water, but a Donnan equilibrium may be present.

The role of the EYS in osmoregulation, digestion and respiration needs further study. Owing to the presence of cysteine lyase, its endoderm is also able to synthetize cysteine using sulfate ions, which abound in sea water (Fischer *et al.,* 1983). We found a constant amount of sulfur in the ES, and a net gain was absent (Table 3; Fig. 13). The role and turnover of cysteine in EYS needs further study before this function can be fully understood.

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