Original Article

The hatching mechanism in Atlantic halibut *(Hippoglossus hippoglossus)*

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ABSTRACT In general fish larvae emerge from the protective egg after secreting a hatching enzyme (HE) from diffusely located hatching gland cells (HGCs). This proteolytic enzyme is distributed over the entire inner part of the eggshell *(zona radiata)*. In a marine flatfish halibut, *(Hippoglossus hippoglossus)*, we have found a more specialized hatching process. A strategic location of the HGCs in a narrow belt on the anterior part of the yolk sac leads to restricted degradation of the eggshell resulting in cleavage of the eggshell into two distinct rigid parts. This hatching process – termed «rim-hatching» – results in an empty eggshell with a lid approximately 1/4 the size of the bottom shell. During the hatching process the yolk sac is reshaped. The posterior part of the yolk sac contracts and the yolk mass is squeezed forward before hatching. This mechanism ensures close contact between the HGCs and the eggshell during the release of the hatching enzyme, which is a prerequisite for restricted degradation of the eggshell.

KEY WORDS: *eggs, hatching, fish, yolk sac, halibut*

Introduction

The teleostean eggshell is formed during growth and maturation of the fish oocyte. Large amounts of structural proteins accumulate between the follicle cells and the base of the oolemma (Kjesbu and Kryvi, 1989). These fibrous proteins are probably produced in an extra-ovarian tissue and transported to the growing oocyte (Oppen-Berntsen, 1990). After ovulation and a «hardening reaction», this structural protein envelope generates the protective eggshell around the embryo (Lønning *et al.*, 1984). In general oviparous fish eggshell is composed of a relatively thick and lamellated layer termed *zona radiata*. This innermost layer is covered by a relatively thin layer of homogeneous material constituting the diffusion barrier in the eggshell. The term *zona pellucida* is used for this layer together with the external jelly layer. The structure and thickness of the eggshell may reflect adaptation to different ecological conditions (Lønning, 1972).

At hatching the fish embryos leave this protective casing, but exit is not possible by muscular force alone (Ishida, 1985, Schoots *et al.*, 1982b). In order to diminish the mechanical strength of the eggshell, the larvae secrete an enzyme (Ishida, *op. cit.*) called hatching enzyme (HE), which dissolves the inner part of the eggshell *(zona radiata)*. Recently the hatching enzyme in *Oryzias latipes* was found to be an enzyme system consisting of two proteases acting cooperatively (Yasumasu *et al.* 1989 a,b) – a high choriolytic enzyme which causes swelling of the *zona radiata* and a low choriolytic enzyme which digests the *zona radiata*. The swelling effect has also been reported by Ohi and Ogawa (1970) and Schoots *et al.* (1983). When the eggshell has been weakened by proteolysis, the larvae are able to split the remaining eggshell structure apart.

Few studies describe the incident when the larva leaves the egg. Some anatomical studies have been performed on empty eggshells to describe the digestion status of the eggshell after natural hatching (Schoots *et al.*, 1982b). Most of the work is done *in vitro*, using hatching medium or purified hatching enzyme to artificially digest the eggshell (Yamamoto and Yamagami, 1975). These *in vitro* studies show that the hatching enzyme is able to digest the *zona radiata* completely, and that the HE does not fully digest the *zona pellucida*. The *in vivo* studies show that the digestion status of the eggshell after hatching is species dependent (Schoots *et al.*, 1982b). The *Nothobranchius korthausae* has a completely digested *zona radiata* after natural hatching while pike *(Esox lucius)* and zebrafish *(Brachydanio rerio)* have incomplete eggshell digestion. The eggshell of these fishes is probably equally digested throughout at hatching.

In halibut we found evidence that this fish is able to hatch after a restricted digestion of the eggshell (Helvik, 1988). The empty eggshell of halibut still maintains its original shape after hatching. This probably is due to limited digestion of the eggshell relating to strict localization of the hatching gland cells (HGCs) in a narrow belt on the frontal part of the yolk sac. The aim of this study was to investigate whether halibut possesses a specially adapted hatching mechanism and to see whether there is correspondence between the location of the HGCs and the digestion of the eggshell.

Abbreviations used in this paper: HE, hatching enzyme; HGCs, hatching gland cells.

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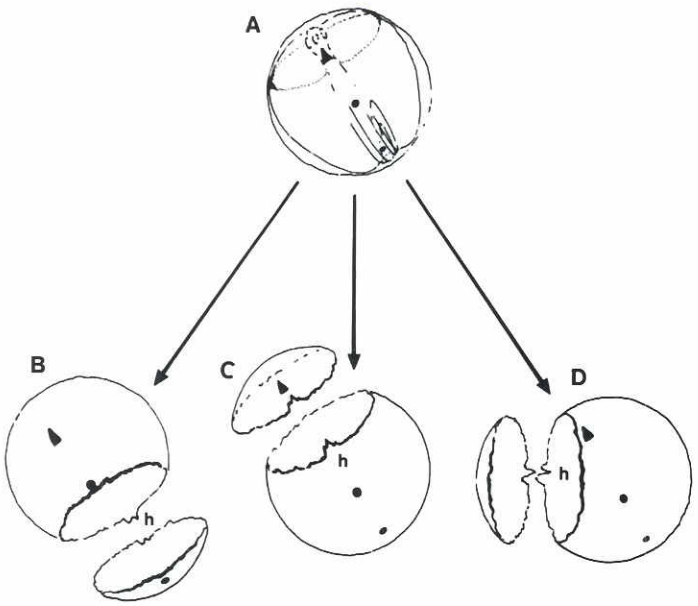


Fig. 1. Marking of unhatched eggs. *One spot is placed over the embryonic head and two spots are placed over the body axis, so that the HGC belt is located between the single (head) and the double (body) spots* **(A)**. *For the sake of clarity the spot over the head is symbolized by a triangle and the body spots with solid circles. If the larva does not rotate from the time of marking until hatching, the single spot will be located on the lid, and the double spots will be perpendicular to the opening* **(C)**. *If the larva has rotated inside the egg before hatching, several different possibilities exist: two examples are shown* **(B and D)**. *The letter «h» indicates hinge region.*

Results

Reshaping of the yolk sac

In a halibut egg on the verge of hatching, the first observable change is the reshaping of the yolk sac (Fig. 2A,B). The posterior part of the yolk sac contracts and the yolk mass is squeezed forward, resulting in closer contact between the frontal part of the yolk sac membrane and the eggshell. At the same time the perivitelline fluid in this area is squeezed backward over the contracting region. In eggs where the hatching process is controlled by light manipulation, this reshaping takes place about 30 minutes after removal of the inhibiting light conditions. Larvae maintain the contraction of the posterior yolk sac immediately after hatching, but they have regained a more rounded yolk sac when inspected several hours after hatching (Fig. 2C).

Opening and removal of the eggshell.

About 90 minutes after stimulation (transfer to darkness) the first larvae begin to split the eggshell apart (Fig. 3). The ventral area is the first to split open, and the shell continues to crack open on both sides back towards the neck, as the larva pushes itself out of the widening opening in the egg. The eggshell over the neck area is not digested, and the lid thus remains hinged to the bottom shell (Fig. 3). The time elapsed from the moment the crack is first observed until the larva exits from the egg is between 1 to 10 minutes depending on larval muscular activity.

The egg orientation in the field of gravity changes throughout the hatching process. Before hatching the body axis is positioned horizontally like a «keel» under the yolk sac. The belt of HGCs lies perpendicular to the body axis and is thus vertical relative to the water column. After rupture of the eggshell and protrusion of the yolk sac, the center of gravity shifts backwards and the larva tips 90 degrees upward so that its body axis comes to be vertical in the water. Accordingly the gland belt lies horizontally as the larva exits the heavy (proteinaceous) eggshell. This allows gravity to pull back the empty eggshell away from the larva. The hinge keeps the lid away from the head of the larva as it forces itself upward at hatching.

When eggs were marked two days prior to hatching along the larval body axis (Fig. 1), examination of the empty eggshells after completed hatching revealed that 80% of the larvae had not rotated during the process of hatching. Two spots were found on the bottom part of the eggshell aligned with the hinge area (Fig. 1C). The time of hatching was identical in marked and unmarked control eggs.

In eggs where the embryo had rotated prior to hatching, the eggs also opened with a smaller lid on top of a larger bottom section of the egg. In some of these eggs all three spots could be observed on the bottom egg section (Fig. 1D).

Ultrastructure of the eggshell

TEM analysis of the empty eggshell shows complete digestion of the eggshell only in the area which was located over the HGC belt. TEM of different positions on the lid is shown in Fig. 4A. Only at the cracking edge are all the structural characteristics of the *zona radiata* dissolved with no lamellae remaining. Closer to the midpoint of the lid the lamellar structure becomes increasingly prominent and less affected by the hatching enzyme. In this area the *zona radiata* resembles an intact eggshell with 17 electron dense lamellae.

The *zona radiata* swells as the digestion takes place. At the cracking edge the completely unstructured *zona radiata* is about 30% thicker than it was in the intact eggshell. The empty eggshell (Fig. 3D) still retains its original shape and there is no tendency to collapse.

The *zona pellucida* is much less affected during hatching. When hatching has occurred, it appears that its outermost part is hardly altered, while its plane of contact to the underlying *z. radiata* is much looser, more disintegrated and expanded in width.

Discussion

We have found that hatching in halibut is different from earlier reported fish hatching mechanisms. The strategic location of the HGCs in a belt over the yolk sac causes a restricted degradation of the eggshell and results in splitting the eggshell into two solid and undigested parts. We have named this mode of hatching «rim-hatching».

Mechanism of «rim-hatching»

The hatching process in halibut starts with a reshaping of the yolk sac which forces the anterior portion of the yolk sac into very close contact with the eggshell and squeezes the perivitelline fluid backwards (Fig. 2B). In halibut the HGCs are located in a belt on the frontal part of the yolk sac (Helvik, 1988; Figs. 2,3). Thus yolk sac contraction and the ensuing juxtaposition of HGCs to the eggshell guarantees a direct deposit of the HE above the HGC area. After reshaping, the elongated yolk sac is pinned against the eggshell in both the front and back parts. This probably prevents the larva from rotating inside the egg. An embryonic rotation during HE release

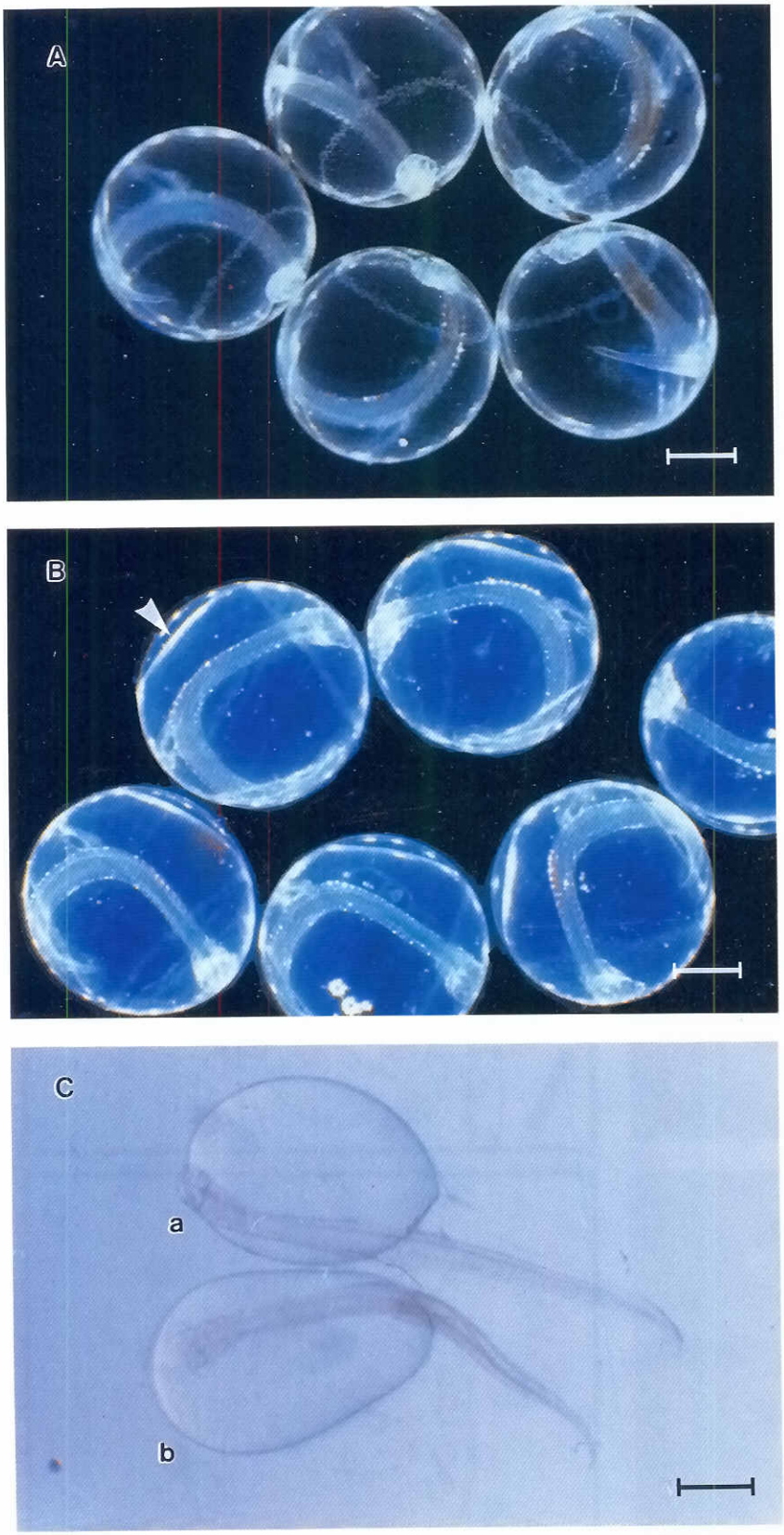
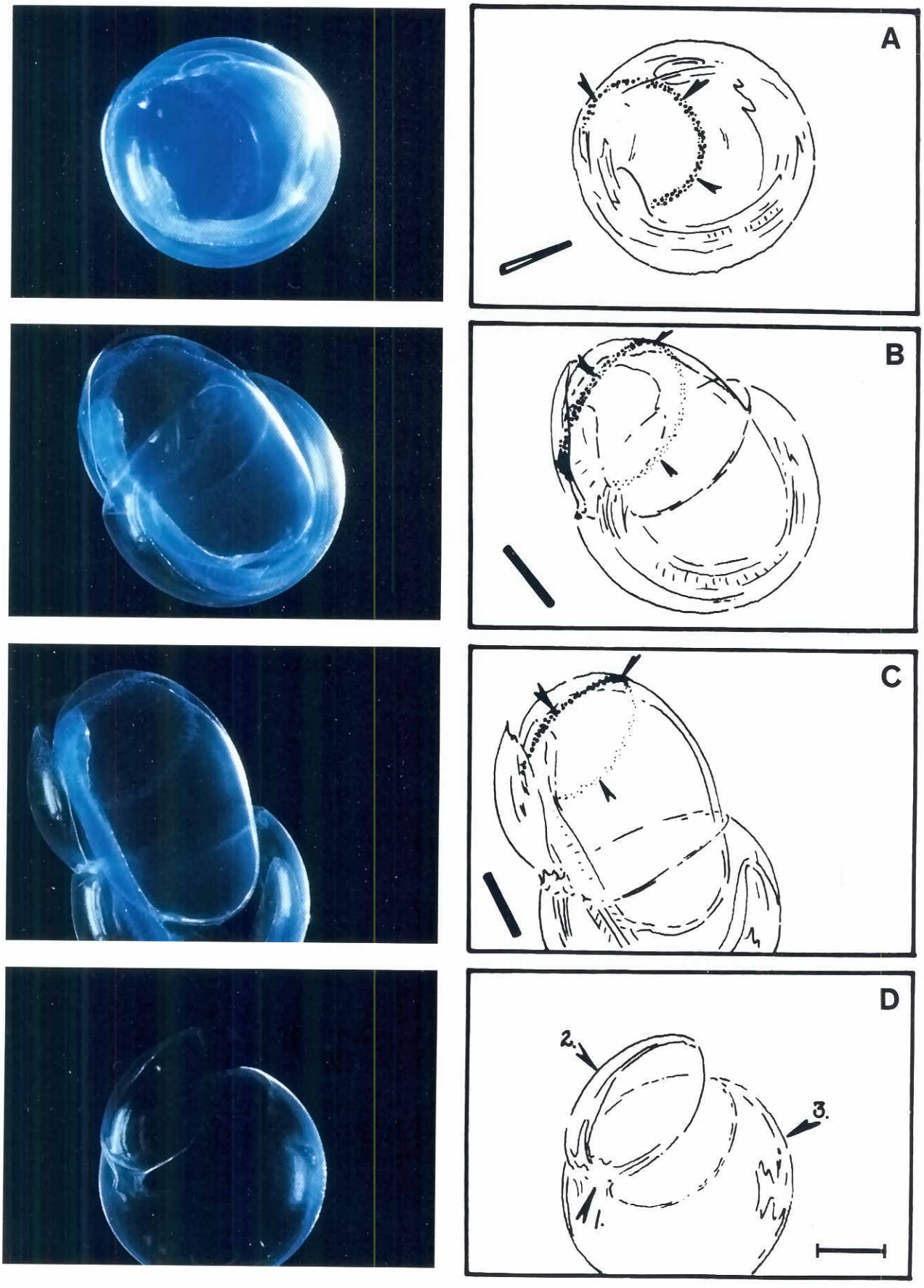


Fig. 2. Reshaping of the yolk sac. (A) *Halibut eggs before the hatching process has been induced. The bar represents 1 mm.* **(B)** *About 30 minutes after stimulation of hatching, the posterior part of the yolk sac has contracted, resulting in closer contact between the HGCs and the eggshell. The perivitelline fluid is squeezed backwards relative to body axis. Arrow indicates the contracting parts of the yolk sac of a representative embryo. The bar represents 1 mm.* **(C)** *Halibut larvae photographed immediately after hatching (b) and 6 hours after hatching (a). The bar represents 1 mm.*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would cause diffuse deposition of the HE in the perivitelline space. However, such embryonic rotations do not occur in most embryos during hatching. Even in the 20% of embryos which do rotate prior to hatching, «rim-hatching» and not general eggshell degradation is observed.

Comparing the localization of the digestion area with the morphology of the HGC belt (Fig. 3) confirms that the belt structure consists of HE-producing cells, since the dissolving area lies exactly over the belt structure. The hinge between the lid and bottom of the eggshell is formed as a result of an absence of HGCs in the neck area (Helvik, 1988).

Is there a «zipper» in the eggshell ?

The occurrence of «rim-hatching» (Fig. 3) is consistent with either a localized deposit of hatching enzyme onto the *zona radiata*, or alternatively, with a preexisting hatching enzyme target in *zona radiata* (a «zipper»). However, a «zipper» mechanism is inconsistent with our observations of hatching from eggs in which the embryos had rotated. We observed that all eggs «rim-hatched» whether or not embryonic rotations had taken place. With embryos that rotated prior to hatching (20%), the placement of markings on the eggshells was random (see Fig. 1). If a fixed «zipper» existed in the eggshell, the location of markings on the eggshell should be constant, with spots invariantly aligned with the hinge region (Fig. 1C).

A more complicated version of the «zipper» theory may be envisioned: embryos could rotate freely during development but align with the «zipper» during hatching. According to this model embryos would need a system of orientation to find the «zipper». Additionally, the biogenesis of a «zipper» in the eggshell would require complicated regulation processes during oogenesis. Hence, we interpret «rim-hatching» in favor of a localized deposit of hatching enzyme onto the *zona radiata.*

Preconditions for «rim-hatching»

Several essential conditions must be met in order for a larva to be able to «rim-hatch». The HGCs must have a ring-shaped morphology with a ring-diameter resulting in a hole sufficiently large for the larva to escape. A close distance between the eggshell and the HGCs is very important in preventing the HE from diffusing into the entire perivitelline space. Movement of the HGCs relative to the eggshell when HE is released will also lead to deposition of the HE over a bigger area of the *zona radiata.* In halibut the gland belt is forced against the eggshell by the large reshaped yolk sac, thereby guaranteeing that the HE will be deposited directly onto the eggshell. The reshaping also prevents the larva from rotating inside the egg at the moment of hatching.

Yolk sac reshaping

Contraction of the yolk sac before hatching was reported in *Fundulus heteroclitus* by Milkman (1954). The mechanism that causes the yolk sac in halibut to alter its form is not clear, and requires further studies of the anatomy of the yolk sac membrane.

We have observed stripes on the rear part of the yolk sac in darkfield microscopy (data not shown) which might indicate the presence of an extensive cytoskeletal network. Whether the forces opening the eggshell rely on cytoskeletal elements in the yolk sac or on somatic muscles remains to be determined. The relaxation of yolk sac contraction in larvae after hatching emphasizes the active nature of this process. The exact contributions to hatching itself of chemical lysis and muscular contractions have not been adequately estimated.

Ultrastructure of the eggshell

Few studies have been presented on *in vivo* analysis of the degradation status of the eggshell at the moment of hatching. The *Nothobranchius korthausae*, whose eggshell has double the thickness (18 µm) of halibut eggs, has a completely digested *zona radiata* at hatching (Schoots *et al.*, 1982b), while the zebrafish *(Brachydanio rerio)* and pike *(Esox lucius L.)* both hatch with an incompletely digested eggshell (Schoots *et al.*, 1982b).

It is difficult to compare such findings with digestion in halibut, because the species that Schoots and co-workers studied showed a general degradation of the eggshell. The splitting edges of empty halibut eggs have the same morphology as the *Nothobranchius korthause, i.e.* a completely digested *zona radiata* (Fig. 4). The ultrastructural changes during digestion of the *zona radiata* of halibut are similar to what Schoots *et al.* (1982b) found in pike, and what Yamamoto and Yamagami (1975) reported in *Oryzias latipes*. The digestion proceeds in the front, starting with the most internal lamellae, and moves outwards to the *zona pellucida*. This differs from the zebrafish, where digestion expands out along pores in the shell. The swelling of the halibut *zona radiata* during hatching indicates the presence of a similar enzyme system of proteases as described by Yasumasu *et al.* (1989 a,b).

Halibut hatching gland

The hatching gland in halibut has recently been shown to consist of about 2000 cells with each cell containing around 20-30 vesicles (Helvik *et al.*, 1991). It is natural to compare halibut with pike since the egg diameter (d) and eggshell thickness (t) in these two species are about the same (halibut: d is about 3.0 mm and t is 9 µm, Lønning *et al.*, 1982; pike: d is 2.8 mm and t is 7.3 µm, Schoots *et al.*, 1982b). In pike Schoots *et al.* (1982a) found 1200 HGCs with 30 vesicles, a lower number of cells than reported in halibut. It would appear more logical that halibut with restricted digestion should need fewer HGCs than pike, which has general digestion of the eggshell. Alternatively, hatching enzyme may be present in excess in both species.

Speculations on the evolution of «rim-hatching»

Teleostean hatching is a biological process in which enzymatic lysis works together in concert with muscular forces. Breaking through an incompletely digested, deformable covering requires strong participation of muscular forces. The halibut larva leaves the

Fig. 3. Photo sequence of a hatching halibut larva. (A) *The larva has just begun to split open the digested zone of the eggshell, and a crack has appeared ventrally on the eggshell. The orientation bar indicates tilt of the embryonic axis. Arrows indicate HGCs.* **(B)** *The crack has widened continuously towards the two sides of the neck as the larva forces itself out. The frontal part of the yolk sac is out of the egg, and the lid is apparent. The picture shows that the lid is formed from the eggshell which is anterior to the ring-formed HGC belt. Arrows indicate HGCs.* **(C)** *The lid is fully opened, and gravity helps to pull away the eggshell. Arrows indicate the HGCs.* **(D)** *The empty eggshell showing the two parts: the lid (2) and the bottom (3), hinged together in an undigested area (1) which was positioned over the larval neck. Scale bar represents 1 mm.*

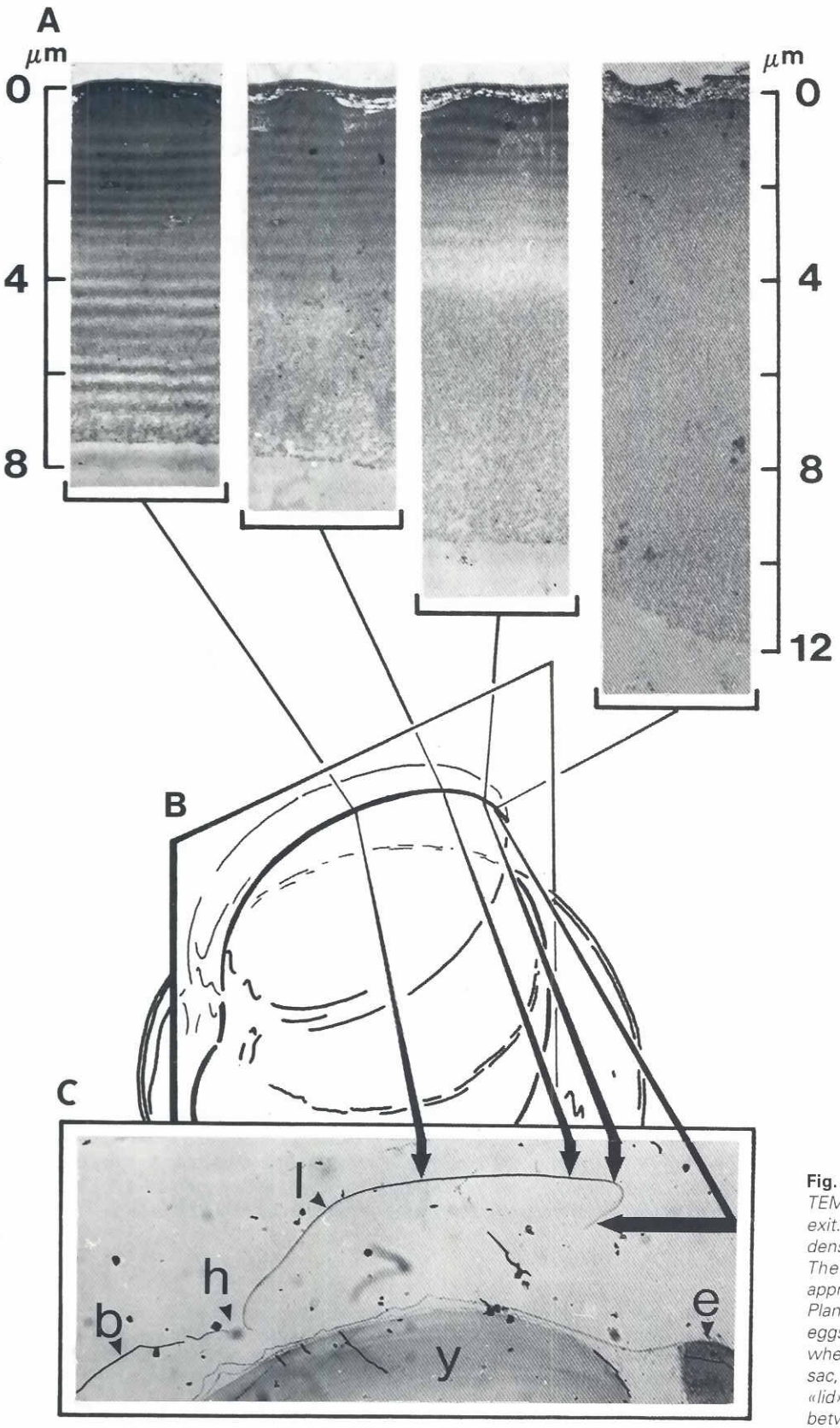

Fig. 4. Ultrastructure of an empty eggshell. (A) *TEM of different parts of the lid during the larval exit. The zona radiata consists of (17) electron-dense lamellae, and an external thin zona pellucida. The number of lamellae decreases as one approaches the ruptured part of the eggshell.* **(B)** *Plane of section through the «lid» part of the eggshell.* **(C)** *Section through the hatching larva when it leaves the egg. Abbreviations are y: yolk sac, e: embryo, b: «bottom» part of the eggshell, l: «lid» part of the eggshell, and h: hinge region between «lid» and «bottom» (see Methods).*

egg at a primitive developmental stage (Lønning *et al.*, 1982) and therefore has poorly developed muscular apparatus available for participation in the hatching process. The halibut may have developed «rim-hatching» to compensate for the lack of developed muscles, since splitting apart two *solid* structures («opening a lid in the egg») probably requires less participation of the muscular apparatus than would the breaking of a soft elastic cover. The yolk sac of the halibut larva almost fills the entire egg volume at the time of hatching. A hatching mechanism that will distribute the HE uniformly in the entire perivitelline space to perform general degradation of the eggshell would be difficult in the halibut because of large yolk sac volume.

We may ask why the halibut does not hatch at a later stage of development, when the perivitelline space is larger and larval muscles are more developed so that general hatching may occur. It is not readily apparent what advantages there might be for the halibut larva hatching at such a primitive stage. Future studies will probably give the answer to whether larvae of other teleosts that hatch at very early developmental stages also use the «rim-hatching» mechanism.

Materials and Methods

Eggs

Eggs were obtained from a stock of mature halibut kept at Austevoll Marine Aquaculture Station, Institute of Marine Research, Norway. One female was stripped of eggs and fertilized by the addition of sperm from two males. Excess sperm and ovarian fluid was removed by rinsing the eggs in sea water before transport to the laboratory. About 100-200 eggs were incubated in one-liter beakers at 6°C. Dead eggs were removed daily during the incubation period. Every second day 90% of the water was exchanged with UV-treated 34 ppt. seawater under minimally turbulent condition.

Marking of eggs

In order to detect embryo rotation inside the egg during development and hatching, the localization of the body axis was indicated on the eggshell. A felt pen (Staedtler lumocolor 318) was used to apply three spots on the eggshell. One spot was placed on the eggshell over the head (Fig. 1) and two spots were placed close together over the body axis so that the HGC belt area was located between the single and the double spots. The spots were carefully applied on the eggshell by lifting the egg with forceps until the area to be marked broke the water surface. The pen was carefully brought into contact with the eggshell using a stereo microscope. A group of 40 eggs was marked two days before hatching and incubated in continuous darkness. Empty eggshells were removed several times each day during the hatching period, and the position of the spots relative to the opening in the eggshell was determined as shown in Fig. 1. A control group of 40 unmarked eggs was incubated under the same conditions to see if the markings affected the time of hatching.

Photography

Hatching of halibut larvae was photographed using an Olympus OM4 camera with Olympus 20 mm macro lens. When photographed the eggs were placed in a transparent plastic container (10 x 10 x 3 cm) with sea water. The pictures were taken through the front glass of the container. Two electronic flash lamps controlled by the camera were mounted so that one shed light on the top view, and the other on the side view. The hatching time was synchronized by light manipulation according to Helvik *et al.* (1990). Synchronized hatching was achieved by inhibition of the hatching process by incubating the eggs under light condition (100 lux). Transfer to darkness stimulates the hatching process, and the larvae hatch in synchrony after 90 minutes.

Microscopy

Live eggs were photographed using a darkfield stereo microscope (Wild). In order to avoid movements, the eggs and larvae were placed in seawater in a Petri dish coated with a thin sticky film of Ficoll-solution (1:1 = Ficoll:seawater).

Preparation for transmission electron microscopy (TEM)

The halibut eggs were rapidly fixed at the moment of hatching. When the larva exits from the egg, the shape of the egg appears to change from round to oval. This change is readily observable to the eye, and such eggs were fixed immediately. A solution of 2.5% formaldehyde, 0.5% glutaraldehyde, 20 mM cacodylate buffer pH 7.2 with 4 mM calcium chloride was used as fixative, followed by a postfixation in 1% OsO_4,dehydration through an ethanol series and finally embedded in paraplast according to conventional histological procedures. Serial cross sections (1 µm) of the yolk sac were stained with 1% Toluidine blue. Sections were examined in a Leitz inverted microscope.

Specimens for TEM studies were obtained and fixed as described above. Serial sections (0,5 µm) of the yolk sac were mounted on grids and stained with 1% uranyl acetate and lead acetate and examined in a JEOL JEM 100CX transmission electron microscope.

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