

Development of hatchability in halibut (*Hippoglossus hippoglossus*) embryos

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ABSTRACT Halibut (*Hippoglossus hippoglossus*) eggs raised in darkness hatched between days 14.5 and 16 after fertilization. Eggs incubated in white light ($3.2 \mu\text{E}/\text{sr}^1/\text{m}^2$) remained unhatched, so that time of *intra ovo* development could be doubled. Photo-arrest of hatching was non-diapausal since embryonic growth continued. Transfer of photo-arrested eggs to darkness induced rapid and synchronous hatching. This procedure allowed analysis of development of hatchability. Hatching was not observed prior to day 14. Nonsynchronous hatching over three days was seen when eggs were induced on day 14+1 h, or on day 14+9 h. However, darkness-induction on day 14+22 h produced synchronous hatching within 140 min. This high rate of inducibility persisted until day 18, before declining slowly. Hatching-induction was not observed beyond day 22. Low hatchability in long-term photo-arrested embryos apparently reflects a loss of the anatomical prerequisites for the rim-hatching mechanism. Altered hatchability and morphogenesis after prolonged *intra ovo* development indicate that hatching in halibut is possible only at an early, defined ontogenetic stage.

KEY WORDS: fish, halibut, hatching, development, light

The fish eggshell, or vitelline envelope (Dumont and Brummet, 1985), is a huge, cross-linked protein polymer (Oppen-Berntsen *et al.*, 1990). Hence exit from the egg at hatching is possible only after partial enzymatic degradation of the eggshell's *zona radiata*, before mechanical forces tear open the remaining eggshell (Yamagami, 1988). The halibut hatching enzyme (HE) is produced by an annular hatching gland (HG) on the anterior yolk sac (Helvik *et al.*, 1991b). The HG-cells first appear near the embryonic head as a disc-shaped structure, which is transformed to a belt during HG-cell migration. The HG-cells begin accumulating HE already during cell migration, and differentiated HG-cells arrive in their final position on day 12. Hence, halibut embryos seem ready for hatching well in advance of actual hatching on day 14 (at 6°C).

During hatching, the eggshell is cleaved into distinct lid and bottom parts, and the lid is pushed open by reshaping of the yolk sac and extension of the tail. This rim-hatching mechanism proceeds by direct contact between HG and eggshell, and requires that the HG-circumference be sufficiently large to create an opening compatible with the size of the yolk sac (Helvik *et al.*, 1991a). In halibut, exposure of eggs to light inhibits hatching, and return of such eggs to darkness results in synchronized hatching of all embryos within 90-140 min (Helvik and Walther, 1992). Light appears to control HE-secretion, which causes irreversible hatching. We have found few reports concerning the ontogenesis of hatchability of fish eggs. The rapid and photoregulated hatching mechanism in halibut facilitates studies of hatchability development.

The embryonic response to photo-induction of hatching changed during development (Fig. 1). When eggs were incubated in continuous darkness (control group) at 6°C, no hatching was seen prior to day 14. Such eggs hatched from the middle of day 14 until day 16, a period of about 1.5 days. Photo-induction of hatching early on day 14 (+1 h and +9 h) had no clear stimulating effect on hatching (Fig. 1A). The eggs hatched over a longer period (3 days) than the control group maintained in darkness. In contrast, induction of hatching 13 h later (day 14+22 h) resulted in 95% hatching within 140 min. The control group at this time had only 70% cumulative hatching. This acceleration of hatching from day 14+9 h to day 14+22 h is made evident by plotting the percent of hatched larvae against time after induction (Fig. 1B).

The development of hatchability is shown by plotting time elapsed before 50% hatching (Fig. 1C), and percent hatched larvae after 160 min post induction (Fig. 1D). The time of 50% hatching reached a minimum on day 16. This is almost two days after start of hatching in the control group and about one day after the control group had reached 50% hatching. At this stage all embryos hatched between 80 to 110 min after induction, or within a time window of only 30 min (Fig. 1B). The rapid hatching response in the majority of the embryos was observed from late on day 14 until day 18 (Fig.

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

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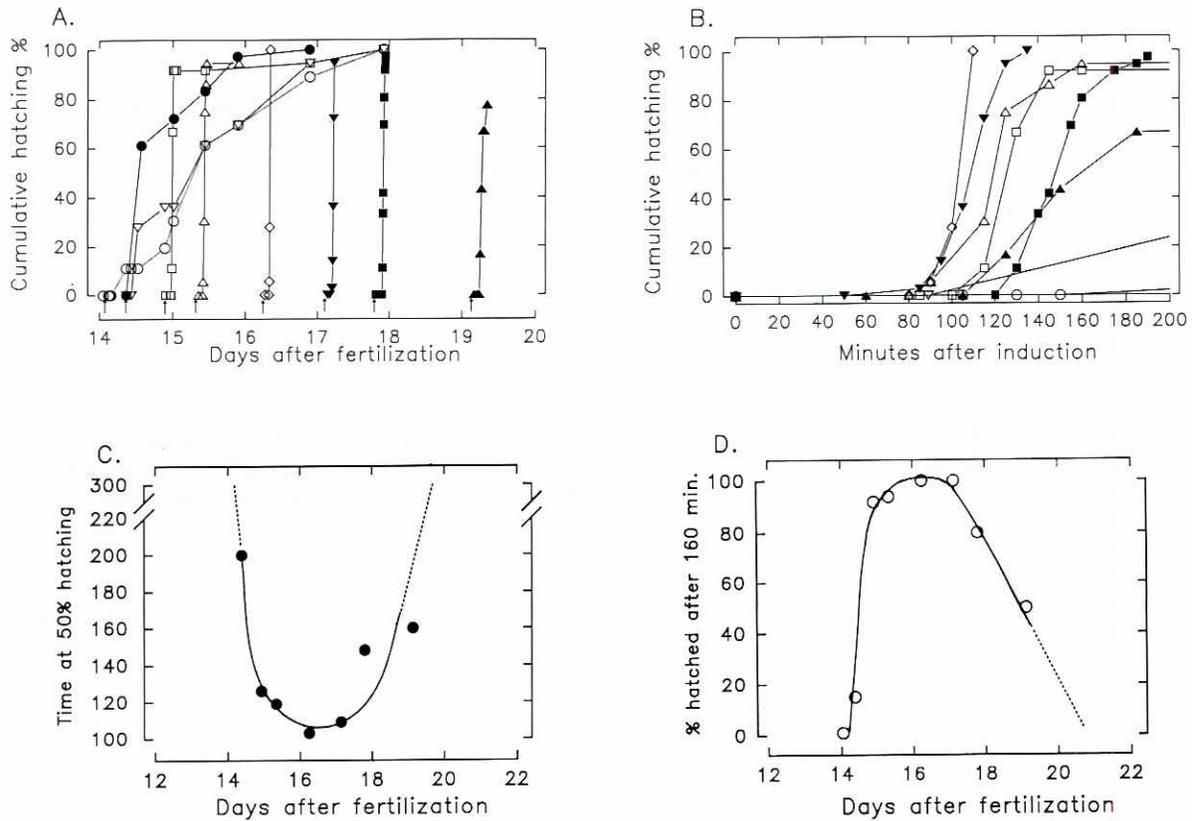


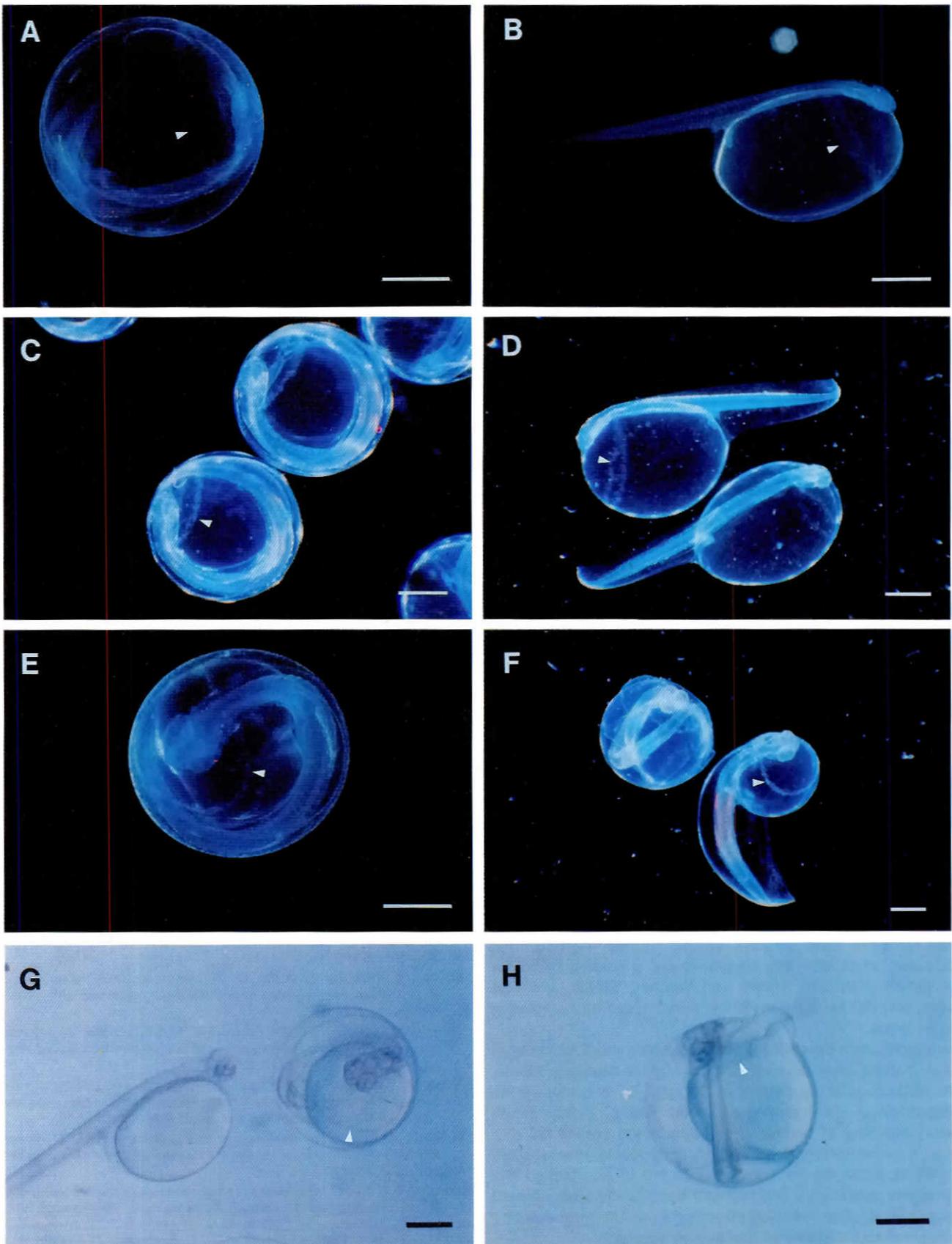
Fig. 1. Hatchability of halibut eggs after photo-induction of hatching at various developmental stages. (A) Hatching is plotted as cumulative hatching percent. One egg group developed in continuous darkness (●). Other egg groups were developed in light and transferred to darkness (induction) at different developmental stages: 14 d+1 h (○); 14 d+9 h (▽); 14 d+22 h (□); 15 d+8 h (△); 16 d+6 h (◇); 17 d+3 h (▽); 17 d+19 h (■); 19 d+3 h (▲). (B) The hatching results from Fig. 1A are plotted against min after induction of hatching. The expanded scale shows the progress of hatching. Symbols as in Fig. 1A. (C) The hatching data presented as time elapsed from induction until 50% of the eggs had hatched, against the developmental stage when induction was performed. (D) Percent hatching larvae scored at 160 min after induction, plotted against the developmental stage when induction was carried out. Dashed lines are approximations.

1D). After this stage, hatchability decreased substantially. Six days beyond «normal» hatching time (after day 20), synchronized hatching by transfer of eggs to darkness was no longer observed, although individual embryos were occasionally seen to hatch.

The halibut embryo gained the capability to hatch at a specific developmental stage. Since the HG had already arrived on day 12

in its final location with HE content apparent, or two days prior to normal hatching (Helvik *et al.*, 1991b), the development of a control system for inducing secretion of HE may be the limiting step in generating physiological hatchability in halibut. The developmental stage of fish larvae at hatching exhibits considerable interspecies variation (Blaxter, 1988). Several environmental parameters trigger

Fig. 2. Micrographs of halibut eggs and larvae. (A) Halibut egg at the time of normal hatching, showing the inconspicuous embryonic axis below a large yolk sac (on day 15 after fertilization). The yolk sac with the annular HG faces upwards in the water. (B) Newly hatched halibut larva on day 15 after fertilization, showing the larval body axis on top of the yolk sac with a straight tail. Annular HG may be discerned. (C) Side-view of halibut embryos which have developed intra ovo for 10 days past normal hatching time (until 25 days after fertilization). The embryonic body constitutes a larger part of the egg volume. HG is clearly visible, but width is reduced. (D) Hatched larvae after 4 extra days of development intra ovo (until day 19 after fertilization), showing a beginning curve at both tail-tips, but in opposite directions. A smaller annular HG may be discerned. (E) Top-view of halibut embryo which has developed intra ovo for 15 days past normal hatching time (to day 30 after fertilization). The embryo is now much enlarged (cf. Fig. 2A). HG is visible. (F) Intra ovo halibut and a dissected larva, both of which have developed intra ovo for 15 days past normal hatching time (until day 30 after fertilization). HG is visible. In the unhatched embryo, the hooked tail-tip is seen, where the tail touches the head. The dissected larva is clearly abnormal. (G) Halibut larva after normal hatching, and embryo which developed intra ovo, both on day 25 after fertilization (10 days past normal hatching). Note that the annular HG in the hatching-arrested embryo is prominent, while it is almost not discernable in the normally hatched larva. (H) Halibut larva which is about to hatch after 10 extra days of intra ovo development (day 25 after fertilization). Note that the eggshell is only opened where the yolk sac contacts the eggshell. The yolk sac is small relative to egg diameter, and the tail is positioned between the eggshell and the annular HG, so that most of the HG does not touch the eggshell. Arrows point to the hatching gland (HG); Bars represent 1 mm in all photos.



hatching in fish embryos, suggesting a complex and probably a neural mode of hatching regulation (Yamagami, 1988). In halibut the primary environmental factor controlling the induction of hatching is light. Our data do not suggest whether inducibility of hatching resides in the HE secretory apparatus, or in the control of this secretion.

While embryonic responsiveness to photo-induced hatching changed during embryogenesis, altered morphology also influenced the prerequisites for rim-hatching. Photo-inhibited hatching prolonged the period of halibut development *intra ovo* (Fig. 2). At normal hatching time the halibut embryo constituted a small part of the egg. After additional growth *intra ovo*, the embryonic body became more prominent and occupied more of the *intra ovo* space (Fig. 2C). Upon shrinkage of the yolk sac, the width of the HG-ring gradually decreased, and also became distanced from the eggshell by the straightening of the embryonic neck (Fig. 2B,G). Direct contact between the HG and the eggshell was partly blocked by day 24 after fertilization when the tail was inserted between the eggshell and the HG (Fig. 2E). All these morphological changes limited HG-contact with the eggshell to a small area, producing a diminutive hole in the eggshell. These events may account for the decline in the hatchability observed in Fig. 1.

During prolonged *intra ovo* growth the tail developed a kink, which did not straighten when the eggshell was removed (Fig. 2D,F). In many fish larvae, e.g. herring, the tail grows completely around the yolk sac before hatching, but straightens during hatching (Klinkhardt, 1984). While the halibut tail straightens after normal hatching, unknown teratological changes prevent this after prolonged periods *in ovo*. These larvae swam only in circles, since the tail was shaped like a scoop. In photo-arrested halibut larvae, the annular HG was more distinct, and remained visible at later developmental stages compared to hatched larvae (Fig. 2G), where the HG had almost vanished 4 days after hatching. HG-cytolysis occurs in many species after normal hatching (Schoots *et al.*, 1983; Yamagami, 1988; Helvik *et al.*, 1991b), but does not seem to take place in non-hatched halibut larvae (Fig. 2F and G).

Annual fishes arrest development (diapause) at different developmental stages (Wourms, 1972), one being hatching. The embryo nearly ceases development at a pre-hatching stage until a suitable environment for hatching materializes. In halibut pre-hatching diapause was not observed since the embryonic development continued inside the egg in a manner resembling of hatched larvae.

Exposing halibut eggs to light allowed a doubling of the time of *intra ovo* development. The fact that halibut larvae survive inside the eggshell for more than 10 days beyond normal hatching time supports our conclusion that oxygen is not a limiting factor for halibut growth *intra ovo* (Helvik and Walther, 1993), and that darkness, and not low oxygen, is the main trigger for hatching in halibut embryos.

The present data bear on earlier speculations that hatching of immature halibut larvae is an artefact of surface rearing systems. Atlantic halibut spawn at depths of 300-700 m, and there is no information on *in situ* hatching of halibut (Haug, 1990). In the laboratory hatching of halibut eggs takes place around 80 day-degrees (°C), when embryos are at a very early developmental stage compared to other marine species (Lønning *et al.*, 1982). The abnormal morphogenesis and reduced hatchability after delayed hatching indicate that hatching observed in our rearing systems accurately reflects the time of hatching in nature.

Experimental Procedures

One female halibut about to spawn was manually stripped of eggs. Fertilized eggs were incubated in 250 l tanks until day 7, at which time epiboly was complete. From day 7 to day 13 (one day before normal hatching time), the eggs were incubated in 6 l beakers with stagnant UV-treated seawater; salinity 34‰. Each beaker contained about 200 eggs. Dead eggs were removed daily to avoid bacterial growth. All experiments were carried out in a climate-controlled room at 6°C.

Intra ovo development was prolonged by incubating about 200 eggs in two 6 l beakers and placing them under 3.2 $\mu\text{E}/\text{sr}^1/\text{m}^2$ white light (about 50 lx) from day 13 after fertilization. From these storage beakers 36 eggs were transferred to darkness on each subsequent day until day 20 (6 eggs each in a Nunc tray with 6 wells). The number of hatched larvae was counted every 10 min during the active hatching period, using a weak flashlight covered with a red film. A control group was incubated in continuous darkness in Nunc trays from day 13.

The light source was two double fluorescent tubes (Osram 20 W/30 Yellow white de luxe), installed in a cubic black plastic tent (1x1x1 m) about 80 cm above the water surface. For spectral composition of the white light, see Helvik and Walther (1992). Light intensity was measured using a LI-1000 photometer (LI-COR; Lincoln, Nebraska, USA). A Wild-Heerbrugg microscope equipped with automatic camera was used to record eggs and larvae Kodak ET 160 film, after larvae were tranquilized with 10 ppm Hypnodil (methomidat; Janssen Pharmaca, Belgium).

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References

- BLAXTER, J.H.S. (1988). Pattern and variety in development. In *Fish Physiology*, Vol. XI, part A (Eds. W.S. Hoar and D.J. Randall). Academic Press Inc., London, pp. 1-58.
- DUMONT, J.E. and BRUMMET, A.R. (1985). Egg envelopes in vertebrates. In *Developmental Biology: A Comprehensive Synthesis* Vol. 1 (Ed. R.W. Browder). Plenum Press, New York, pp. 235-288.
- HAUG, T. (1990). Biology of the Atlantic halibut, *Hippoglossus hippoglossus* (L., 1758). *Adv. Mar. Biol.* 26: 1-70.
- HELVIK, J.V. and WALTHER, B.T. (1992). Photo-regulation of the hatching process of halibut (*Hippoglossus hippoglossus*) eggs. *J. Exp. Zool.* 263: 204-209.
- HELVIK, J.V. and WALTHER, B.T. (1993). Environmental parameters affecting hatching of Atlantic halibut (*Hippoglossus hippoglossus*) eggs. *Mar. Biol.* 116: 39-45.
- HELVIK, J.V., OPPEN-BERTSEN, D.O. and WALTHER, B.T. (1991a). The hatching mechanism in Atlantic halibut (*Hippoglossus hippoglossus*). *Int. J. Dev. Biol.* 35: 9-16.
- HELVIK, J.V., OPPEN-BERTSEN, D.O., FLOOD, P.R. and WALTHER, B.T. (1991b). Morphogenesis of the hatching gland of Atlantic halibut (*Hippoglossus hippoglossus*). *Roux Arch. Dev. Biol.* 200: 180-187.
- KLINKHARDT, M. (1984). Zum Einfluss des Salzgehaltes auf die Befruchtungsfähigkeit des Laiches der Rügenschon Frühjahrsheeringe. *Fisch.-Forsch. Wissenschaftl. Schriftenr.* 22: 73-75.
- LØNNING, S., KJØRSVIK, E., HAUG, T. and GULLIKSEN, B. (1982). The early development of the halibut, *Hippoglossus hippoglossus* (L.), compared with other marine teleosts. *Sarsia* 67: 85-91.
- OPPEN-BERTSEN, D.O., HELVIK, J.V. and WALTHER, B.T. (1990). The major structural proteins of cod (*Gadus morhua*) eggshells and protein crosslinking during teleost egg hardening. *Dev. Biol.* 137: 258-265.
- SCHOOTS, A.F.M., EVERTSE, P.A.C.M. and DENUCÉ, J.M. (1983). Ultrastructural changes in hatching-gland cells of pike embryos (*Esox lucius* L.) and evidence for their degeneration by apoptosis. *Cell Tissue Res.* 229: 573-589.
- WOURMS, J.P. (1972). The developmental biology of annual fishes. III. Pre-embryonic and embryonic diapause of variable duration in the eggs of annual fishes. *J. Exp. Zool.* 182: 389-414.
- YAMAGAMI, K. (1988). Mechanisms of hatching in fish. In *Fish Physiology* Vol. XI part A (Eds. W.S. Hoar and D.J. Randall). Academic Press, New York, pp. 447-499.