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The effects of hypo-salinity on embryos and larvae of olive flounder *(Paralichthys olivaceus)*

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The hypo-salinity effects on fertilized eggs, embryos and larvae were investigated in olive flounder *(Paralichthys olivaceus)* obtained from hatcheries in Cheju-Island, Yeosu and Chungnam. Those were treated to eight concentration; 0, 3.4, 6.7, 10.1, 13.4, 20.2, 27.4 and 33.6 ‰. It was not discrepancy in the survival rate and hatching success rate of fertilized eggs obtained from different regions. Also, in the larvae, the regional difference was not appeared. The survival rate and hatching ability of embryos significantly diminished in the lower groups than 13.4 ‰ compared to 33.6 ‰. After fertilization, namely embryos are tolerant of a wide range of salinity (13.4 - 33.6 ‰). Reduced salinity induced an increase of the malformed embryo and larvae including various deformities; irregular embryo membrane (or yolk sac depression), fin erosion and swim bladder inflation in the flounder embryo. The hatching success of embryos was significantly reduced in lower salinity than 13.4 ‰. Notably, the reduction of larval survival rate significantly was observed in ≤ 10.1 ‰ treated groups with the same manner of survival rates of the embryos. Additionally, olive flounder was found to be adequate model for measuring external impulses because there are no the regional differences.

Key words: Hypo-salinity, Olive flounder, Early-life stages, Malformation

Introduction

The life-cycle toxicity test is considered by most aquatic toxicologists to be the ultimate test in risk assessment and establishes long-term "safe" environment concentrations of external threat or toxic chemicals for both vertebrate and invertebrate aquatic populations. The early life stage (ELS) of a fish species is treated during the established schedule through ova fertilization embryonic, larval to early juvenile development. Development fish biology including survival and growth is reviewed in ASTM (1980). The ELS is sensitive parts of fish life cycle because of the many critical events that take place in a very short span of time (Von Westernhagen, 1988).

Many marine fish species spawn in offshore areas, relying on ocean currents to distribute their offspring. This scatter mechanism often brings embryos and larvae into areas of varying salinity. These alterable effects are important considerations during fertilization and incubation of marine fish embryos. Many papers have reported an influence of water salinity on fish development and growth. In many species, embryo fertilization and incubation, yolk sac re-sorption, early embryogenesis, swim bladder inflation and larval growth are dependent on salinity (Perrott *et al.*, 1992; Carroll *et al.*, 1994; Fuentes and Eddy, 1997). Fertilization rates vary with salinity but this is complicated by the fact

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that the salinity in which the brood-stock are held appears to alter the salinity tolerance of the embryos (Fonds, 1979; Liu *et al*., 1991).

The main purpose of the present study was to investigate the effects of hypo-, namely low-salinity on survival, hatching success and deformities in olive flounder (*Paralichthys olivaceus*) embryo and larvae. Generally previous studies have investigated the eco-toxicological responses of fish from a single location. Such studies cannot account for geographical or physiological patterns of adaptation. In Korea, different fish stocks are used in aquaculture facilities and the present study compared the sensitivity of embryos and larvae derived from fish that originated from 3 regions with varied environmental anthropogenic inputs.

Table 1. Chemical components of seawater used in the experiment. Values indicate mean ±SEM.

Parameter	Value
Temperature (℃)	20.00±1.0
pH	8.10±0.20
NH_4-N ($\mu g\ L^{-1}$)	12.66±1.25
NO_2-N ($\mu g\ L^{-1}$)	1.37±0.28
NO_3-N ($\mu g\ L^{-1}$)	9.62±1.01
PO_4-P ($\mu g\ L^{-1}$)	5.05±0.96
Suspended solids ($mg\ L^{-1}$)	5.62±0.20
Dissolved oxygen ($mg\ L^{-1}$)	6.74±0.84
COD ($mg\ L^{-1}$)	1.52±0.08
Fe ($\mu g\ L^{-1}$)	5.02±0.87
Cu ($\mu g\ L^{-1}$)	2.32±0.12

Materials and Methods

Experimental animals and water conditions

Fertilized eggs of olive flounder *(P. olivaceus)* were obtained form the hatcheries in Cheju Island, Yeosu and Chungnam, South Korea (Fig. 1). They were transported in aerated insulated containers to the Fisheries Science Research Institute laboratory. Prior to experimentation, fertilized eggs were acclimated in aerated aquaria at 20±0.5 ℃ for 3 hours (Table 1). The test conditions are shown in Table 2. The used salinity groups were 0, 3.4, 6.7, 10.1, 13.4, 20.2, 27.4 and 33.6 ‰. All experiments carried out separately for each region.

Embryo test

The fertilized eggs obtained from each region

Table 2. Test conditions for the definitive exposure test with *P. olivaceus* embryo and larvae.

Test type	static renewal
Duration	36hrs / 72hrs
End points	survival rates, hatching rates, malformation feature
Temperature	20±1℃
Light quality and photoperiod	ambient laboratory light (12L : 12D)
Dilution water	filtered and UV-treated
Test chamber size	500 mL
Test solution volume	300 mL
Renewal of test solution	every 24hrs
Age of test animals	less than 10hrs after fertilization
Number of embryos per chamber	50
Number of replicates per concentration	4
Feeding regime	not required
Test concentrations	8
Test acceptability criterion	90% or greater hatching in control

Fig.1. Geographic location of the hatchery (●) offering fertilized eggs of *P. olivaceus*

were collected and rinsed several times with artificial seawater and around 7 h post-fertilization (hpf) the fertilized eggs (blastula stage) were transferred directly into test solutions in 500 mL glass beakers. For each test concentration and the control there were 3 replicates. Fifty fertilized eggs per chamber were kept in the beaker containing various salinity levels. The development of embryos were monitored every 3 h up to an endpoint of 40 h or until hatching was complete. At each time interval, embryo mortality, hatching successes and any abnormalities were noted using a microscope connected to a camera device (×400). Embryos were considered dead when part of the embryo turned opaque or white and these were removed immediately to avoid fungal infection of other embryos.

Preliminary experiment had identified potential malformations in the embryos after test as, an irregular fertilized egg membrane, atrophy of yolk sac and abnormal tail flexure. Any individual showing one or more of these characteristic abnormalities were noted.

Larvae test

The newly-hatched larvae were treated to the same test concentration and control as the embryo test. There were 3 replicates for each experimental condition, each containing thirty embryo-larvae held in aerated 500 mL glass beakers. The larva were monitored every 6 h for up to 72 h after exposure. Larvae were considered dead when there was no visible heart beat or body movement. These were removed every 6 h. The survival rate was recorded throughout the experiment and deformities were monitored at the end of the experiment. In preparation experiment, the physical abnormalities in larvae included the yolk sac edema, craniofacial malformation and inhibition of swim bladder inflation. The individual having one or more characteristic of these malformations was regarded as abnormal and dysfunction individual. Representative larva from both normal condition (33.6 ‰) and treated groups were described and photographed with a photo-microscope (×400)

Statistical analysis

In all graphs the results were expressed as means ± standard error (S.E). Statistics were using one-way analysis of variance (ANOVA) followed by Duncan's multiple comparisons test of mean values if significant differences were found (P<0.05).

Results

No discrepancy in the survival rate, hatching success and deformity of fertilized embryos and larvae collected from different regions. Then, all data were indicated as a mean value of the 3 regions.

Effects on embryo development

Survival of embryo at 13.4, 20.2, 27.4 and 33.6 ‰ was not affected after 36 hours. Survival was above 90 % in 33.6‰. Embryo mortality was firstly noted after 6 h of exposure. In other hands, signifi-

cant decreases of survival were observed in 6.7 ‰ of 32.7±4.7 % and 10.1 ‰ of 62.1±3.7 %, respectively (Fig. 2). At 0 and 3.4 ‰, treated embryos were rapidly died within an hour exposure and embryos had died perfectly after 2 hours. And hatching ability trials were not conducted at these salinity groups.

Hatching rate of *P. olivaceus* fertilized eggs are shown in Fig. 3. Hatching was started at 26 h and was completed 28 h post-incubation in all trials. In embryo experiment, when the salinities were maintained, hatchability was no significant difference against 33.6 ‰ in higher than 13.4 ‰ groups. The hatchability in 33.6 ‰ was ranked highest with 93.2±2.4%, then test salinity of 27.4, 20.2 and 13.4 ‰ were registered 91.5±2.8, 85.1±3.7 and 79.1 ±4.6%, respectively (P>0.05). However, 10.1 and 6.7 ‰ treatments were registered 60.2±4.6 and 27.2±4.0%, respectively.

Developmental events of 33.6 ‰ were observed that the blastoderm began to expand (epiboly, about 1/4, 1/3 and 1/2 of the yolk sphere) over the surface of the yolk sphere, and the presumptive region of the embryonic shield arisen as a thickened margin (dorsal lip) of the blastoderm. The oil-droplet (O.D) were visible in 33.6 ‰ (Fig. 4A). But embryo treated hypo-salinity was unidentified because the form is strange (Fig. 4B). In Fig. 4B, the small size picture was described that embryo just before died by hypo-salinity. Fig. 4C is the neurula stage that the head part (rudimentary brain) was distinctly recognized in an anterior embryonic body and a small vacuole (Kupffer's vesicle) appeared at the underside of the caudal end of the body. Also, in the stage, the brain and nerve cord in the arrow-shaped embryonic body developed as a solid rod of cells. A solid optic bud (rudimentary eye vesicle) appeared on each side of the cephalic end. However, in the hypo-salinity groups, there was observed that abnormal embryo had not a solid optic bud, the brain and nerve cord. Especially the yolk sac of treated embryo was depressed (Fig. 4D). Fig. 4E is somite stages that the optic vesicles was differentiated into optic cups and lenses, the tubular heart (heart anlage) was appeared underneath head, and the cuvierian ducts (blood vessels) and the vitellocaudal vein was appeared on the yolk sphere. However, a treated embryo developed abnormally by the effect of hypo-salinity. (Fig. 4F). As well as the size of yolk sac was smaller than normal, caudal

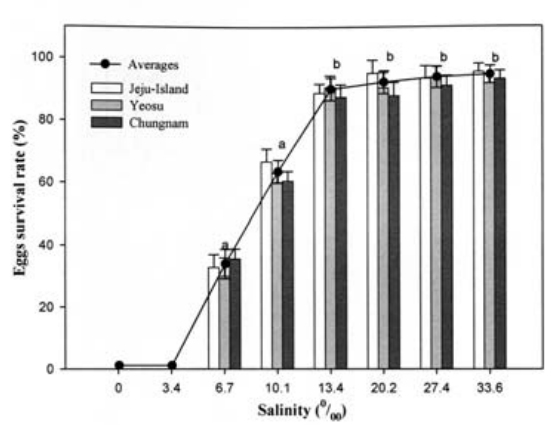

Fig. 2. Survival rate of *P. olivaceus* fertilized eggs treated to different salinity during 40 hours. Superscripts on the bars are not significantly different (P>0.05).

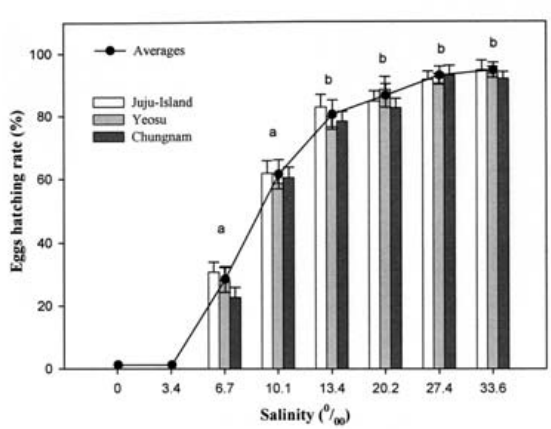

Fig. 3. Hatching rate of *P. olivaceus* fertilized eggs treated to different salinity during 40 hours. Superscripts on the bars are not significantly different (P>0.05).

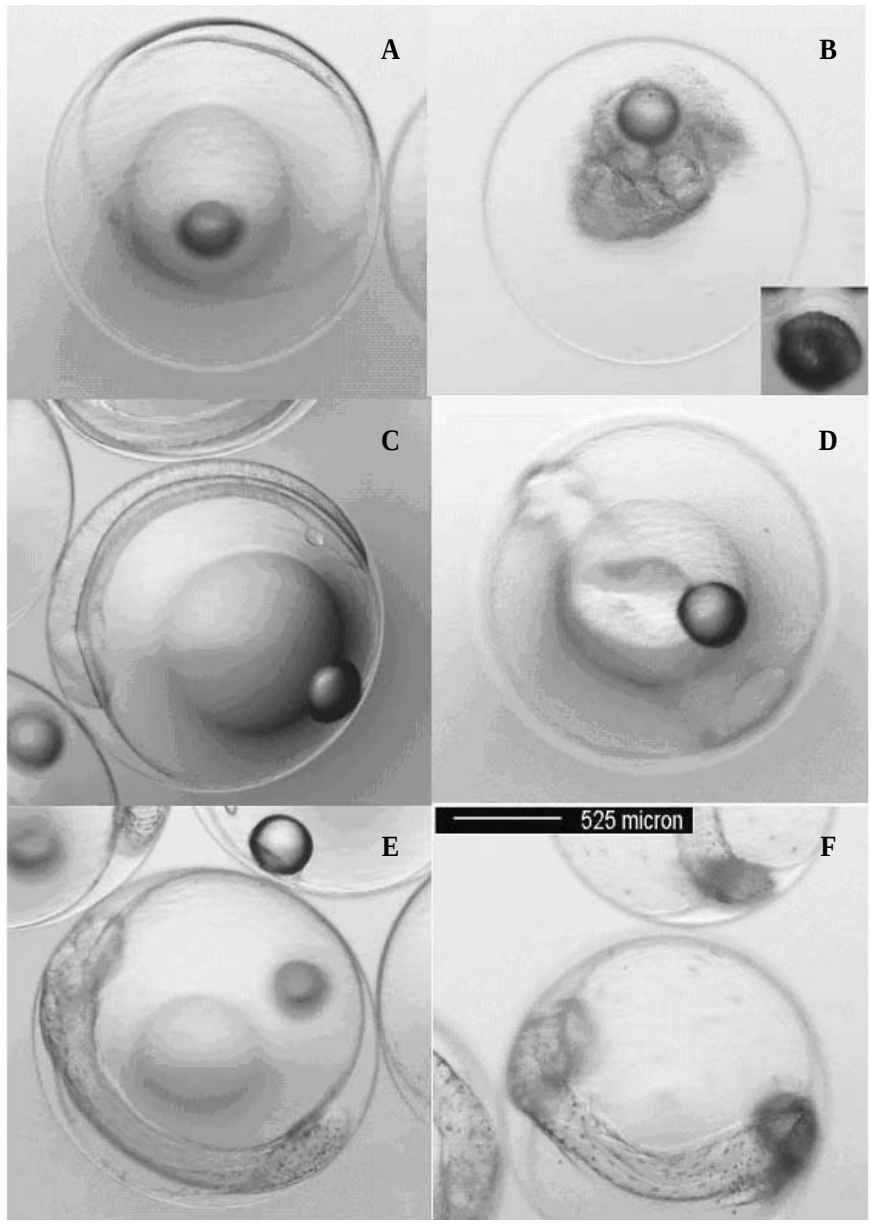

Fig. 4. *P. olivaceus* fertilized eggs treated to different salinity. A, C and E: Fertilized egg or embryos of 33.6 ‰ group (normal individual). B: Fertilized egg has absurd form, because fertilized egg division did not consist normally. Small picture is fertilized egg that just before died by the extreme hypo-salinity (6.7 ‰). D: Abnormal formation of embryo, especially the yolk sac was depressed (10.1 ‰). F: Embryo has deformed tail region, morphologically irregular yolk sac and oil globule detachment (13.4 ‰).

region of embryo was saw as rot.

Effects on larva development

Larvae began hatching from 50 hours post fertilization in the indoor tanks. A mouth and anus were not yet open. Within a few hours, larvae of 6.7 and 10.1 ‰ groups had high mortalities; the survival rate of each group is 30.2±1.5 and 69.8±1.6%. Sur-

vival rates of other groups (13.4, 20.2, 27.4 ‰) were not significant difference compared to 33.6 ‰.

From Fig. 6A to 6G, in 33.6 ‰, the yolk sac was absorbed according to grow, and the head region was developed and mouth was occurred. Also, the intestine is prolonged according to body length grow and distribution of veins was observed. Unlike the embryos in 33.6 ‰ group, hypo-salinity treated embryo could not be hatched and the malformation of embryo was appeared (Fig. 6B). Also, developmental speed was quite late than normal individual. As be looked in Fig 6D and F, alterations in all hypo-salinity treatment groups included irregular enlargement of fins; dorsal, ventral and caudal regions. Additionally, darkened body, vertebral deformity (torsion, kyphosis), eye deformity and yolk sac unevenness (edema or atrophy) were rarely occurred (Fig. 6H). The Observed typical effects were pericardial and yolk sac edema, craniofacial malformation and inhibition of swim bladder inflation in hypo-salinity groups (Fig. 6F and H).

Discussion

The results from present study confirm that early life stages of the olive flounder *(P. olivaceus)* are sensitive to hypo-salinity, reducing the survival of embryos and larva and negatively affecting their development. However, the responses were generally similar in fish raised from stock cultures from different areas in Korea.

Among the ecological factors, salinity is specific to the aquatic environment. Many authors have demonstrated the influence of external salinity on growth capacities in fish (Bucket *et al.*, 1995; Gaumet *et al.*, 1995; Likongwe *et al.*, 1996; Jonassen *et al.*, 1997; Alava, 1998; Swanson, 1998; Peterson *et al.*, 1999). This is true for a lot of species, including both marine and freshwater fish. In fact, species not influenced by salinity changes during their development and growth are rare. Also, many authors have reported on the influence of water salinity on fish development. In sea bream *(Sparus aurata)* larvae, growth was estimated at 15 to 40 ‰ (Tandler *et al.*, 1995). In greenback flounder *(Rhombosolea tapirina)* embryo fertilization, incubation and yolk sac resorption are dependent on salinity, with 28 ‰ resulting in the best overall performance (Hart and Purser, 1995). In other flatfish, as Southern flounder *(P. lethostigma)* or the summer flounder *(P. dentatus)* early development and larval growth were also affected by salinity, optimal conditions being 5-30 and 8-14 ‰, respectively (Smith *et al.*, 1999; Specker *et al.*, 1999). In general, embryos of teleost keep near internal osmotic pressure of body cavity such as adult's body fluids osmotic pressure (300-400 mosm) and adjustment of osmosis and existence of chloride cells in gastrula formative period in embryo of some of species (Guggino, 1980; Hwang and Hirano, 1985; Alderdice, 1988). This fact could be difference between species, but could analogize that fertilized embryos have saturation adjustment ability during early life stages (Alderdice, 1988).

Hatching successes at salinity of 6.7 and 10.1 ‰ were significantly lower than those of 33.6 ‰ (P<0.05) in the present study. And no embryos survived at 0 and 3.4 ‰ and only 60.2% embryos survived by day 1 at 10.1 ‰. From 13.4 to 33.6 ‰, percentage of hatched larvae was high (>80%) and unaffected by salinity. Our main finding was that hypo-salinity has detrimental effect on survival, hatching success and development of embryo and larvae of olive flounder below 13.4 ‰. However, Specker *et al.* (1999) have reported that hypo-salinity has no detrimental effect on survival, growth or development of larval summer flounder *(P. dentatus)*. And they provided evidence that larvae raised

in hypo-salinity (8 ppt) can grow better and develop more quickly than their cohorts in seawater (30 ppt) or high salinity (38 ppt).

On the other hand, Chun and Rho (1991) was evaluated that olive founder embryo died all, had treated at 7.6 and 14.3 ‰. In this study, the survival rate at 13.4 ‰ was no significant difference comparing with 33.6 ‰.

Previous studies have revealed that hatching success was normally more correlated to the rate of abnormal blastomeres (early cell development) than to the fertilization rate in marine fishes such as wolffish (*Anarhichthys lupus* L.) (Pavlov and Moksness, 1994). Likewise, the viability of cod *(Gadus morhua)* yolk sac larvae in high salinity stress was shown to be strongly correlated to the rate of abnormal blastomeres in the embryo batch (Kjorsvik *et al.*, 1995). With their study finding, various kinds of malformation phenomenon appeared in embryos that treated to the change of salinity groups in our research (Fig. 4 and 6). The salinity of embryos as decided by salinity of broodstock tank may be optimal for fertilization and this should be taken into account if broodstock was to be maintained at salinity other than those in which the embryos will be fertilized (Hart and Purser, 1995). The hatching failure, as described in previous studies, may be due to various mechanisms that include (1) the diminished activity of the embryo and abnormal distribution of the hatching enzyme (Rosenthal and Alderdice, 1976), (2) the inability of the emerging larvae to break through the non-digestible outer part of the embryo shell (Sinha and Kanamadi, 2000).

After fertilization, flounder embryos are tolerant of a wide range of salinity, 15-45 ‰ (Fig. 5). Total mortality of Baltic Sea turbot embryos occurred at salinity of less than 5 ‰ (Kuhlmann and Quantz, 1980). Fonds (1979) found that the optimal salinity range was 20-40 ‰. Holliday and Jones (1967) showed that plaice *(Pleuronectes platessa)* embryos were able to osmoregulate directly after fertilization. However, a high mortality was recorded at salinity below 10.1 ‰ (Fig. 5). Besides, similar with our results, Haddy and Pankhurst (2000) demonstrated that in black bream *(Acanthopagrus butcheri)* no embryos incubated at 0 ‰ survived and 54% of embryos survived to day 1 when incubated at 5 ‰ and the survival rate of hatched larvae was high and unaffected by salinity from 15 to 35 ‰. Also, fertilized embryos of Australian bass *(Macquaria novemaculeata)* cease developing within 2-3 h post transfer to fresh water, and at 5 and 10 ‰, only a small percentage of larvae hatch, but hatching success increase to above 75% when embryos are incubated at salinity of 15-35 ‰ (Van Der Wal, 1985). Tsuzuki et al. (2000) showed that the Atherinid species, *P. odontesthes* and *O. hatchery* are euryhaline, although the highest hatching rates were consistently obtained at intermediate salinity (5 to 20 ‰). It has been suggested that failure to successfully hatch at hypo-salinity results form poorly developed tail musculature and/ or larvae finding it difficult to free themselves from the chorion (Holliday, 1969; Young and Duenas, 1993).

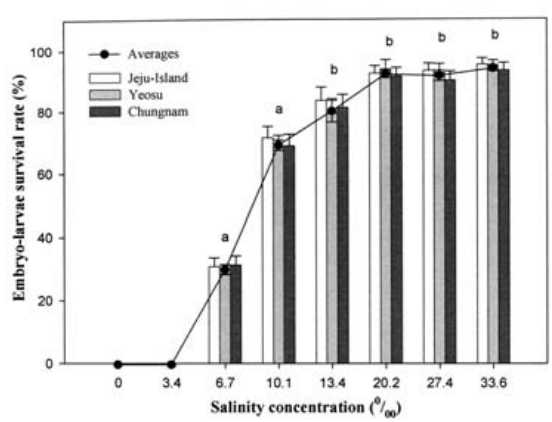

Fig.5. Survival rate of *P. olivaceus* larvae treated to different salinity during 72 hours. Superscripts on the bars are not significantly different (P>0.05).

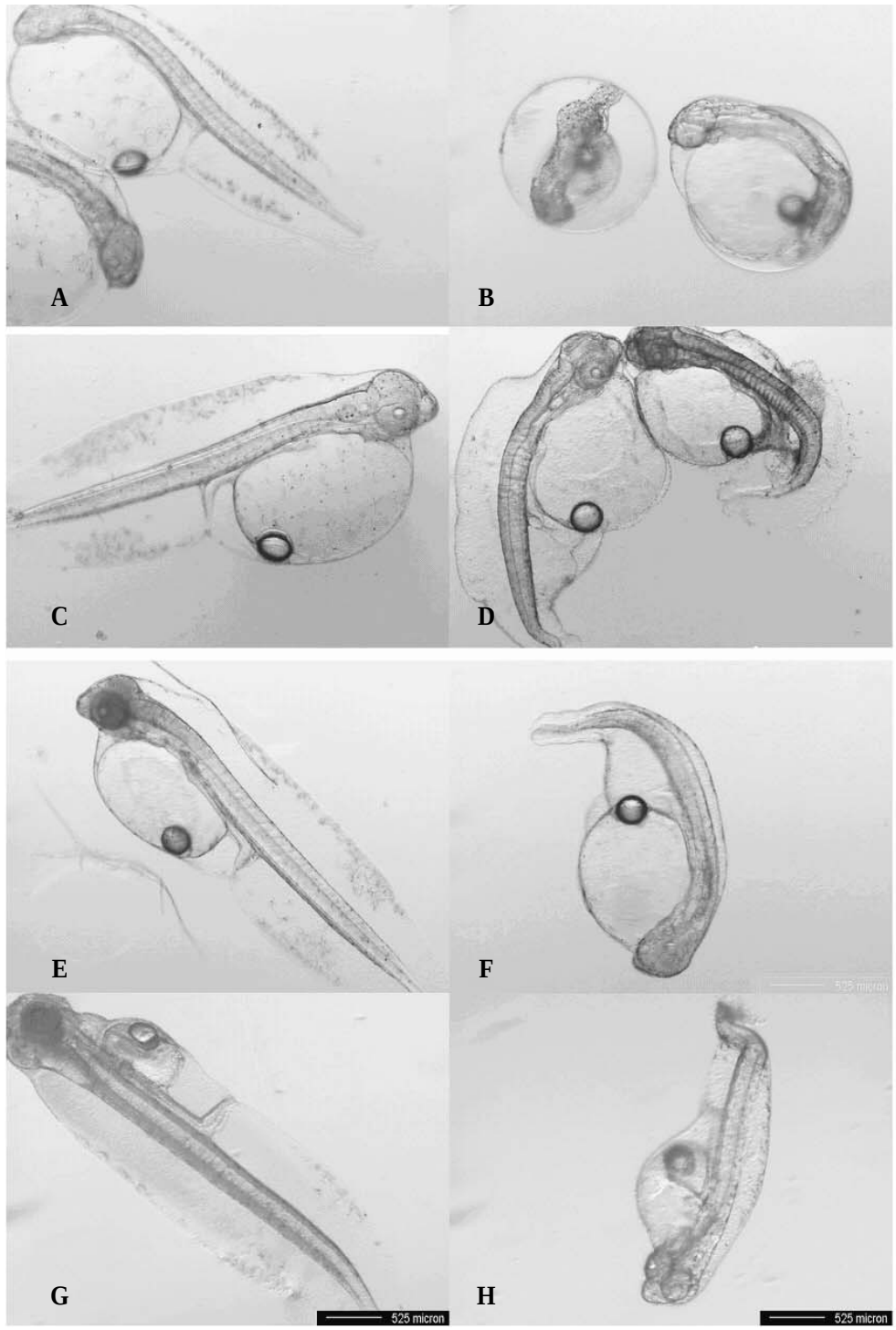

Fig.6. *P. olivaceus* larvae treated to different salinity. A, C, E and G: newly hatched larvae of 33.6 ‰ group. B: larvae that failed to hatch because couldn't break fertilized egg-shell (20.2 ‰). D: deformed larvae that have extended fins (13.4 ‰). F: eye deformities, undeveloped or abnormal development of intestine and vertebral malformation (10.1 ‰). H: stunted development, the spine of caudal region extremely curved (6.7 ‰).

Results in this study support this suggestion, as the hatching success of larvae decreased at hypo-salinity and, in some cause, the larvae died in a partly emerged state (Fig. 6B).

The observed malformations in this study can be classified as relatively unspecific response to a number of pollutants (Strmac and Braunbeck, 1999). Namely, the edema (or atrophy) is the general morphological malformation in response to hypo-salinity, although it has also been found in response to other inorganic or organic pollutants (Fent and Meier, 1992; Guiney *et al.*, 1990). It is characterized by leakiness of endothelial vessels supplying the yolk sac a result of cardio-vascular dysfunction (Guiney *et al.*, 1990), or it could be interpreted as an indicator of metabolic or osmotic disruptions possibly caused by mitochondrial malfunction due to the chemical (Sinha and Kanamadi, 2000).

In summary, this study has shown that olive flounder adapt relatively to hypo-salinity, under these conditions, development proceeds normally and is unchanged over a salinity range of 13.4-33.6 ‰. However, survival and hatching rate of embryos and larvae were significantly reduced at below 10.1 ‰. The data from the present study suggests that early life stages of olive flounder are sensitive to the low-salt content (hypo-salinity). Additionally, early life stage of olive flounder could be recommended to be adequate model for measuring about environmental changes because of no discrepancy in the survival rate, hatching success and deformities of fertilized embryos and larvae collected from different regions.

Acknowledgments

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