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Acid pH and Chorionase Activity of Atlantic Salmon (*Salmo salar*) Eggs

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Acid precipitation has become a subject of increasing concern in northwestern Europe and eastern North America. Low (4.0-6.0) pH has caused failure to spawn (BEAMISH et al. 1975) and mortality or delayed hatching of eggs (JOHANSSON et al. 1973, KWAIN 1975, MENENDEZ 1976, CARRICK 1979, PETERSON et al. 1980).

The hatching enzyme, chorionase, has been isolated from various teleosts. HAYES (1942) demonstrated the existence of such an enzyme in Salmo salar. Purified enzymes from Oryzias latipes (YAMAGAMI 1973), Salmo gairdneri (HAGENMAIER 1974a) and Fundulus heteroclitus (KAIGHN 1964) have been characterized as acting only on the inner layer of the chorion to initiate the hatching process. On the basis of inhibitory effects of the chelating agents, EDTA and EGTA, the chorionases of Salmo gairdneri (HAGENMAIER 1974b) and Onchorhyncus keta (BELL et al. 1969) have been classified as metalloenzymes.

Chorionase is secreted from cells in the head region and oral cavity. In the medaka, initial development of granular material is evident at the same time as development of eye pigment (YAMAGAMI 1973). While chorionase is essential to the progress of hatching by degrading the inner layer of the chorion, the final hatching action is a mechanical breakdown of the outer layer of the chorion (a mucopolysaccharide) by movement of the embryo.

In the present study some evidence was found to support the hypothesis that exposure to low pH during egg development causes a decrease in chorionase activity which can contribute to the delay in hatching observed by PETERSON et al. (1980).

MATERIALS AND METHODS

All salmon, Salmo salar, eggs were from Magaguadavic River stock and were stripped and fertilized on the same day (November 21, 1979). Fertilization was carried out in control water (Chamcook Lake water; for details see PETERSON et al. 1980) and the eggs hardened for at least 1 hour before transfer to the experimental containers.

Eggs were divided into two groups, one in which eggs were maintained at pH 4.5 (with H_2SO_4) and 6.5 at 8°C from time of fertilization, and a second in which eggs were raised under

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hatchery conditions (pH 6.5) at ambient temperature until eye pigmentation was developed, then transferred to pH 4.5 for varying time intervals (1-27 days). Details of the holding facilities were as described by DAYE (1980).

Collection of crude chorionase was carried out in a known quantity (1 mL/egg) of hatching medium. The four media tested were: water at pH 6.5 and 4.5, Tris HCl buffer (0.1M; pH 8.0), and ammonium bicarbonate buffer (0.05M; pH 8.0). Initially, nitrogen gas was used as described by HAGENMAIER (1974a) to stimulate hatching. An increase in "turning activity" was observed but there was no evidence of accelerated hatching. Depending on the stage of development, the hatching medium and incubation conditions, hatching was allowed to occur naturally or was aided by puncturing the chorion with forceps. Where hatching was carried out by applying gentle pressure with forceps the zona radiata was easily punctured; if this did not occur, those eggs were discarded. The embryos were removed and the crude enzyme preparation centrifuged at 15,000 g (IEC B-20A) and 6°C for 15 minutes. The supernatants were stored at -20°C.

Proteolytic activity in each crude preparation was determined in duplicate or triplicate by using casein as a substrate (HAGENMAIER 1974a). However, 0.1M Tris HCl buffer (pH 8.0) was used as the solvent for casein instead of phosphate buffer (pH 8.0) since phosphate inhibits chorionase activity (YAMAGAMI 1973).

Protein content of the crude extract was measured by using a modified Lowry method (HARTREE 1972). Expression of the results for enzyme activity in the normal format of enzyme activity per unit of protein was not valid in this case since the crude preparation contained not only chorionase but also variable amounts of digested chorion protein as well as perivitelline proteins. Activity was therefore calculated as proteolytic units (PU) per egg per hour, one PU being equivalent to an absorbance difference of 0.001 units at 280 nm (Beckman model 25 spectrophotometer).

Statistical analyses of the means of chorionase activity and protein content consisted of a one-way ANOVA for unequal sample sizes and Duncan's multiple range test (STEEL & TORRIE 1960).

RESULTS

The chorion of eggs held at pH 4.5 from fertilization or after transfer from pH 6.5 to pH 4.5 at a later stage of development became opaque, hard, and appeared to be swollen. There was also a delay in hatching. Daily observations of the holding containers indicated that embryos at pH 4.5 had difficulties in completing the hatching process. An initial split of the zona radiata was accomplished and the tail emerged but alevins did not break out further. A similar effect has been reported by DAYE & GARSIDE (1979). If at this point mechanical assistance was provided, the emerging alevin appeared normal.

During collection of chorionase, regardless of the incubation regime, alevins hatched almost immediately and with 100% success when placed in Tris-HCl buffer (pH 8.0). This was most noticeable for eggs held at pH 4.5; the chorion lost its opaque appearance and broke down easily. After centrifugation the pellet resembled those from control eggs consisting of clear orange membrane fragments. This reversal was not apparent when eggs incubated at pH 4.5 were placed in the other hatching media.

An initial attempt to use a pH 4.5 buffer (citrate-phosphate (GOMORI 1955)) as a hatching medium caused the eggs to become opaque, extremely hard and hatching could not be induced mechanically. Similarly 0.1M ammonium bicarbonate buffer (pH 8.0, HAGENMAIER 1974a) caused death of the embryos either before or immediately after hatching. Changing to 0.05M buffer prevented death of the embryos but still turned the chorion opaque and natural hatching did not occur in eggs incubated at pH 6.5.

Eggs incubated at pH 4.5 and hatched in pH 4.5 or 6.5 water had the lowest values for extractable protein levels (300 and 510 µg protein/egg (Table 1)). On the other hand, eggs incubated at pH 6.5 and hatched in water at either pH had the highest extractable protein levels (1210 and 1400 µg protein/egg). The amount of extractable protein was significantly different ($p < .01$) between the two incubation regimes (Table 1). The differences in extractable protein between the two incubation regimes were not apparent when Tris or ammonium bicarbonate buffer (pH 8.0) were used. However, the amount of protein extracted by using Tris buffer (pH 8.0) was less than the amount extracted by using ammonium bicarbonate buffer, regardless of the incubation pH regime. The differences were significant at the $p < .05$ level but not significant at the $p < .01$ level.

Statistical analysis of the chorionase activity indicates again that both the incubation regime and choice of hatching medium contribute to the observed differences (Table 2). The chorionase activity obtained by hatching the eggs in Tris buffer pH 8.0 was greater than that from hatching in ammonium bicarbonate buffer. In fact, Tris buffer appears to give the highest chorionase activity which was unexpected since the amount of extractable protein (Table 1) was approximately 50 and 67% of that obtained by using water at pH 6.5 and ammonium bicarbonate buffer, respectively.

The eggs incubated at pH 4.5 and hatched in water at pH 4.5 or 6.5 had 49% of the chorionase activity (210 and 270 PU/egg/hour (Table 2)) of the eggs incubated at pH 6.5 (480 and 500 PU/egg/hour) and hatched in water at either pH. Statistical analysis of the chorionase activity between only the groups using water at pH 4.5 or 6.5 as the hatching media showed no significant differences in the chorionase activity for eggs from the same incubation regime regardless of the pH of the water used for hatching. However, there was a significant difference ($p < .01$) in chorionase activity between eggs incubated at pH 4.5 and 6.5.

TABLE 1. Mean values of μg protein/egg for the treatments used. Results of Duncan's multiple range test are presented for $p < .01$ and $p < .05$ (lines indicate values between which there is no significant difference).

Incubation pH	4.5	4.5	6.5	4.5	6.5	4.5	6.5	6.5
Hatching medium	H_2O pH 4.5	H_2O pH 6.5	0.1M Tris	0.1M Tris	.05M NH_4HCO_3	.05M NH_4HCO_3	H_2O pH 4.5	H_2O pH 6.5
Mean (n*)	300 (24)	510 (11)	660 (13)	690 (18)	970 (11)	1040 (13)	1210 (4)	1400 (20)
$p < .01$								
$p < .05$								

*n represents the number of extracts collected; each extract was from 3-20 eggs at one egg/mL.

TABLE 2. Mean values of PU/egg/hour for the treatments used. Results of Duncan's multiple range test are presented for $p < .01$ (lines indicate values between which there is no significant difference).

Incubation pH	4.5	4.5	6.5	4.5	6.5	6.5	4.5	6.5
Hatching medium	H_2O pH 4.5	H_2O pH 6.5	.05M NH_4HCO_3	.05M NH_4HCO_3	H_2O pH 4.5	H_2O pH 6.5	0.1M Tris	0.1M Tris
Mean (n*)	210 (24)	270 (11)	400 (11)	430 (13)	480 (4)	500 (20)	510 (18)	590 (13)

*n represents the number of extracts collected; each extract was from 3-20 eggs at one egg/mL.

DISCUSSION

The different levels of chorionase activity observed when the eggs were hatched in water, Tris buffer or ammonium bicarbonate buffer present some interesting observations. Ammonium bicarbonate buffer was used in order to duplicate the conditions of HAGENMAIER (1974a) with rainbow trout eggs. There was no significant difference in either protein level or enzyme activity between eggs incubated at pH 4.5 or 6.5 when hatched in the ammonium bicarbonate buffer. This was also true for eggs hatched in Tris buffer, pH 8.0. The two buffers seem to enhance the level of enzyme activity for eggs incubated at pH 4.5 to that of control eggs (incubated and hatched in water at pH 6.5). For eggs incubated at pH 6.5, on the other hand, the individual buffers did not significantly alter the levels of enzyme activity from the level of the controls but there was a significant decrease in activity in eggs incubated at pH 6.5 and hatched in ammonium bicarbonate when compared to eggs raised at pH 6.5 and hatched in Tris buffer. When considered in conjunction with the levels of protein (Table 1) present in the treatment groups (control eggs hatched in water > eggs hatched in ammonium bicarbonate > eggs hatched in Tris > pH 4.5 eggs hatched in water), it would seem that the use of Tris-HCl buffer as a hatching medium reverses the decrease in enzyme activity produced by incubation at pH 4.5. Two possible mechanisms for this reversal may be that the buffers stimulate the release of all the chorionase from the rostrum of the emerging alevin which does not occur during the incubation of the egg at pH 4.5, or induce favorable changes in the conformation of enzyme. The latter seems less likely since all the proteolytic determinations are performed in identical Tris buffer.

The significant decrease in both proteolytic activity (Table 2) and the amount of protein present for eggs incubated at pH 4.5 from those of eggs incubated at pH 6.5 (Table 1) supports the hypothesis of PETERSON et al. (1980) that chorionase is inhibited at low pH.

HAGENMAIER (1974a) and YAMAGAMI (1973) found that the pH optima for chorionase of trout and medaka were pH 8.5 and pH 7.9, respectively. The chorionase activity in trout decreased to 10% at pH 5.2. The increase in hatching time required by Salmo salar eggs at pH 4.5 may be a reflection of a similar pH-activity profile since the pH of the perivitelline fluid readily nears that of the incubation media (PETERSON et al. 1980). However, our results indicate that there are other factors involved in lowering enzyme activity such as a possible decreased amount of enzyme release at low pH to inhibit the hatching process. The decrease in proteolytic rate may possibly be counteracted by a longer hatching period to allow sufficient breakdown of the inner layer of the chorion for hatching to occur. Embryos held at pH 4.5 were still able to hatch or at least break the chorion.

The effect of the varied times of exposure to low pH on chorionase activity was inconclusive and further studies on this aspect are

required. However, since the eggs had varied times of exposure to low pH and still exhibited low enzyme activity, the effects of sudden changes in the environment, such as spring runoff, may be of great importance for the evaluation of hatching success.

It would seem that there are at least two reasons for the reduced hatching success of salmon eggs exposed to low pH: first, a decrease in chorionase activity and second, a change in the physical structure of the outer mucopolysaccharide layer of the chorion as evidenced by the hardening and opaque appearance; this latter effect makes it more difficult for the alevin to physically break out of the chorion once the inner layer is digested by chorionase. Similar effects were attributed to the delay of hatching in perch eggs at pH 4.5 (RUNN et al. 1977).

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