C H A P T E R - VIII

EFFECT OF IONS (K^+ & Ca^{++}) IN EMBRYONIC STAGES OF CYPRINUS CARPIO

INTRODUCTION

Fertilized eggs are permeable to water and ions (Loeb and Wasteneys, 1915; Ikeda, 1937a,b; Yamamoto, 1954; Rudy and Potts, 1969; Kernan, 1980). Bachman and Runnström (1912) observed that osmotic pressure of eggs falls after fertilization, while Gray (1920) observed marked exosmosis of electrolytes following injury or death of eggs and leading to precipitation of globulin held in solution by neutral salts in living eggs. Na^+ and K^+ provide functional osmotic

and electrochemical gradient and are potential enzyme activators (Phipps, 1976). Inorganic cations are thus very important in several biological processes. The present author has studied (Chapter VII, p. 144) the levels of Na^+, K^+, Ca^{++}, Mg^{++} and Zn^{++} in the embryonic stages of development of Cyprinus carpio.

Abnormal concentrations (higher or lower) of inorganic cations including metallic ions adversely affect the rate of incubation, percentage mortality and hatching percentages. Ikeda (1937a), in his study on effect of Na^+ and K^+ on the rate of development in eggs of Oryzias latipes, observed a faster development in higher concentrations of Na^+ outside; K^+ depressed functional activity of embryo; early embryonic development was said to be accelerated both by Na^+ and K^+. Grande (1966) found small additions of Cu^{++} and Zn^{++} led to lower percentage of hatching in salmonid eggs, the latter particularly causing inbition of softening of capsules by affecting enzymic processes. Malone and Blaylock (1970) studied toxicity of chlorinated hydrocarbons, like DDT, chlordane, endrin, dieldrin and organophosphates, like diazinon and guthion to carp embryos reared in vitro and found that at 5 and 10 ppm concentrations, all insecticides caused significant mortality of embryos; however, at less than 1 ppm of chlordane,

there is slightly greater percentage of hatching, possibly due to its fungicidal and bactericidal properties. Kamler (1972) studied the effect of Fielik, a herbicide with active ingredient 2,4-D-Na (sodium salt of 2,4-dichlorophenoxy acetic acid) and found that while no changes occurred in embroys, mass mortality resulted in larvae affecting aerobic processes; embryonic development was said to be retarded at 50 mg A.I./litre concentration. Chojlakhyan and Shilyanskaya (1973) found blastoderm affected and gastrulation abnormal in isotonic Holtfreter solution of double concentration with high Na^{+} content. With larger incubation serious irreversible morphogenetic abnormalities were said to occur. Kounin et al (1976) studied the effect of Cd^{++} on eggs of Clupea pallasii and found that 10 ppm of Cd^{++} affects activity of all carbon dioxide fixing enzymes and further by binding with chorion makes it vulnerable producing earlier hatching and premature larvae. Esperanza et al (1977) observed that while the direct effect of Zn^{++} was known, nothing was known of its effect on spawning potential of fish. They found exposure to sublethal concentration of Zn^{++} for 3 days adversely affected egg production, egg viability and survival of embryos to hatching. Wright (1977) observed that adult

Salmo trutta were more sensitive to fluoride toxicity than eggs and fries of Salmo gairdneri. Blindness, exophthalmia, inhibition of respiration and death were described as a result of mercury poisoning in Anabas scandens (Panigrahi and Mishra, 1978). Mortality, retarded growth and inhibition of activities of all digestive enzymes due to mercury poisoning is also reported by Sastry and Agrawal (1977), Das et al (1980) and Jhingaran (1982). Pickering (1974); Blaylock and Griffith (1979) found developing eggs most sensitive to Ni^{+} toxicity and that 6 ppm concentration of Ni^{+} reduced hatchability by 50%. Eaton et al (1978) observed that 4-12 µg Cd^{++}/litre killed the eggs. However, Sauter (1976) considered them more resistant to Cd, Cu, Cr and Pb than fries. Subhedar and Rao (1980) found eggs of C. carpio to be far more sensitive than adults.

Ionic pollution of our inland waters, through intensification further and further of fertilizers and insecticides, needs no emphasis. It is evident, from the several citations above, that ionic effects are not always hazardous. Induced breeders would testify that there is extensive mortality at embryonic stages even under controlled conditions.

The present author has toiled with the idea of experimentally determining the accelerating/decelerating effects of concentrations in respect of certain ions, to start with K^+ and Ca^{++}, the two most important inorganic cations, whose importance in biological processes is unquestioned, on the embryonic stages of Cyprinus carpio, with a view to candidly assess the role of these ions under controlled conditions, keeping the ionic concentration as the only variable, at a stage of life where hormones or other defence/immune mechanisms have not developed. The present chapter includes an account of the effect of K^+ & Ca^{++} in the different embryonic stages of Cyprinus carpio.

METHODS

Two different procedures were adopted for study of effect of ions in the embryonic stages of C. carpio on the rate of incubation, percentage mortality at each embryonic stage, percentage of hatching and abnormalities, if noticeable in phenotype of embryos, namely, 1, embryos, gastrula onward only, were exposed to different concentrations of the ion, and 2, eggs, soon after fertilization, were exposed through out incubation period to different concentrations of the ion. However, since dead embryos could not be detected before gastrula stage,

records were kept of total percentage mortality only from gastrula stage onward.

Hatching programme in large buckets, involving management problems in relation to maintenance of definite ion concentration, handling of very large number of embryos and maintenance of optimum conditions, would have been very very cumbersome. Aside this, even with conditions satisfactorily monitored, with the limited facility available, assessment of effect of only one particular ion, and that too in a very limited range of different concentrations, would have been feasible in one instance. Since the success of any induced breeding depends on adequate environmental temperature and, since the suitable period is very limited, the present author had no option but to design a small hatchery unit, taking adequate precautions so that the basic conditions of adequate DO (dissolved oxygen) and water circulation were ensured (Figs. 3 & 4).

b) Tc[illegible]Y UNIT:

Transparent plastic jars of diameter 12 cm and length 20 cm with water holding capacity of about 1.5 litres were used for the incubation of eggs. Each jar was connected by a tube (diameter 0.6 cm) from its lower surface with an overhead

plastic bucket of diameter 90 cm, length 32 cm and water holding capacity of about 20 litres. A tap was provided to each bucket so as to be able to regulate flow of solution. A plastic tube of diameter 0.5 cm, covered with a net on inside surface, was attached on the upper half of plastic jar to act as an outlet for the flow of solution (Fig. 4).

PREPARATION OF IONIC SOLUTIONS:

(i) K^+ stock solution (1000 ppm):

1.53 g of anhydrous KCl was dissolved in 5 litres of tap water. 100 ml of this solution when diluted to 20 litres, gave a 5 ppm solution.

(ii) Ca^{++} stock solution (1000 ppm):

14.34 g of $CaCl_2.2H_2O$ dissolved in 5 litres of tap water. 100 ml of this solution, when diluted to 20 litres gave the concentration of 10 ppm.

Laboratory tap water was used through out the experiments. Results of analysis of tap water are as under:

Parameter	Mean values
pH	8.60
Dissolved oxygen	2 ppm
Na^+	20 ppm
K^+	2 ppm
Ca^{++}	29 ppm

Known numbers of fertilized eggs (after treatment with carbamide solution) were transferred either directly for ionic treatment (in case of K^+ and set II of Ca^{++}) or after incubation until blastula (in case of set II of Ca^{++}) in normal big hatchery buckets (as described in Chapter I, p.22, fig. 5).

Effect of K^+ was studied in different concentrations, viz. 5, 10, 20, 50 and 100 ppm; effect of Ca^{++}, however, was studied vis-a-vis tap water (which was found to contain 30 ppm of Ca^{++}) in concentrations respectively 10, 25, 50 and 100 ppm above that of normal tap water.

RESULTS

Effect of K^+

Data shown in Table 25 would reveal that with respect to the three different parameters, namely, rate of incubation, mortality at each stage of incubation and hatching percentage, chosen to study the effect of ions, tube well water emerged to be the most congenial in respect of normal development of the embryo. Concentrations higher than 10 ppm of K^+ induced greater mortality at different stages and, further, that at 100 ppm concentration about 12% of embryos hatched prematurely and died soon after. This could be plausibly due to the chorionic membrane having become affected.

Effect of Ca^{++}

In the first set of experiment, with approximately 500 embryos kept from fertilization stage itself in tap water and in different concentrations of Ca^{++}, removing the dead embryos (as soon as made out by their opaqueness) from gastrula onward at each subsequent stage, while the percentage of mortality at each stage and hatching time in the different concentrations of Ca^{++} were found more or less comparable with each other, hatching percentage was found much higher in Ca^{++} concentrations higher than in tap water (Table 26). In the second experimental set, when only 200 embryos were exposed from gastrula stage onward to corresponding concentrations of Ca^{++}, percentage mortality rate was much less and the hatching percentage much higher in higher Ca^{++} concentrations as compared from embryos kept in tap water. Most interesting observation, however, was the recovery of 3 abnormal four eyed tailless mutant embryos found in Ca^{++} concentration more than 100 ppm, quite different from the normal fry (Fig. 46).

When the total incubation period of the first and second experimental set ups was compared, complete hatching was found to occur about 6 hours earlier in the second set, when only about 200 embryos were exposed from gastrula onwards to different concentrations of Ca^{++}.

TABLE - 25

Effect of K^+ in the different embryonic stages of Cyprinus carpio

Stages	Time in hrs. (after fertilization)	Experimental concentrations of K^+																											
		Control					5 ppm					10 ppm					20 ppm					50 ppm					100 ppm				
		*1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Gastrula	5 to 8	465	248	0	53	0	355	195	0	54	0	334	147	0	44	0	426	233	0	54	0	340	170	0	50	0	416	226	0	54	0
Closing of blastopore	13 to 14	193	12	0	6	0	141	4	0	3	0	164	9	0	6	0	170	19	0	11	0	195	38	0	19	0	195	15	0	8	0
Comma (eye up formation)	27 to 28	174	11	2	6	0	123	4	3	3	2	118	4	4	3	3	130	7	3	5	2	162	8	3	5	2	156	3 (+20*)	6	25	4
Prior to hatching (about 50% hatching)	41 to 42	136	4	60	3	44	114	0	9	0	8	107	0	9	0	8	121	7	10	6	8	180	4	63	2	35	110	2	17	2	15
Complete hatching (nearly 100%)	52 to 53	108	4	72	4	67	97	7	69	7	71	92	4	67	4	73	102	7	92	7	90	62	21	41	34	66	53	6	24	7	54
Total Mortality and hatching:		465	279	134	60	29	355	210	81	59	23	334	164	80	49	24	426	273	105	64	24	340	241	107	71	31	416	272	47	55	11

* = No. of total eggs; 2 = No. of dead eggs; 3 = No. of hatchling; 4 = % mortality; 5 = % Hatching; *hatchling.

TABLE - 26

Effect of Ca^{+} in the different embryonic stages of Cyprinus carpio

Stages	Time in hrs (after fertilization)	About 500 eggs kept soon after fertilization																								
		Control					10 ppm					25 ppm					50 ppm					100 ppm				
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Gastrula	6.40	500	196	0	39	0	562	215	0	38	0	557	207	0	37	0	529	197	0	37	0	449	199	0	44	0
Closing of blastopore	12.40	502	15	0	3	0	506	8	0	2	0	568	17	0	4	0	330	31	0	9	0	246	14	0	6	0
Comma	15.40	287	9	0	3	0	293	15	0	5	0	547	7	0	2	0	299	28	0	9	0	236	14	0	5	0
Post Comma (twitching)	22.10	278	5	0	2	0	283	6	0	2	0	540	3	0	1	0	271	13	0	5	0	220	6	0	3	0
Post Comma (eyecup formation)	27.10	273	9	3	3	1	277	8	4	3	1	533	9	8	1	2	258	3	6	1	2	214	1	3	0	1
Eye stage	31.10	261	3	3	1	1	265	0	5	0	2	522	3	8	1	2	249	1	2	0	1	210	1	6	0	3
Prior to hatching	38.10	255	1	3	0	1	260	2	42	0	16	511	2	45	0	14	246	3	5	1	2	203	0	63	0	31
Complete hatching	52.40	231	0	209	0	90	208	0	203	0	97	247	0	246	0	99	226	22*	197	9	87	135	5*	129	4	95
Total mortality and hatching:		500	238	218	47	43	562	254	254	45	45	557	246	307	44	55	529	298	210	56	40	449	240	201	53	44

1 = No. of total eggs; 2 = No. of dead eggs; 3 = No. of hatchlings; 4 = % Mortality; 5 = % Hatching; * hatchlings.

Table - 26 (contd)

About 200 eggs kept at Gastrula stage

Stages	Time in hrs. (after fertilization)	Control					10 ppm					25 ppm					50 ppm					100 ppm				
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Gastrula	0.40																									
Closing of blastopore	14.40	197	12	0	6	0	177	17	0	10	0	196	14	0	7	1	193	1	0	4	0	195	7	0	7	0
Young	20.40	185	7	0	4	0	154	13	0	8	0	182	9	0	5	0	181	5	0	5	0	188	10	0	5	1
Post Comma (tail budding)	23.10	175	5	0	3	0	141	4 +5*	0	3	0	173	3	0	2	0	172	2 +2*	0	2	0	178	3 +6*	0	5	0
Post Comma (eye cup formation)	27.10	173	12	0	7	0	132	13	1	10	1	170	5	4	3	2	168	1	4	0	2	169	1	4	0	2
Eye stage	31.10	161	3	0	2	0	118	0	1	0	1	161	0	4	0	2	160	0	4	0	2	163	2	5	3	3
Prior to hatching	38.10	158	5	2	3	1	117	0	2	0	2	157	0	9	0	6	156	0	7	0	4	156	0	51	1	3
Complete hatching	47.40	108	2	97	2	90	113	1	109	1	96	130	1	111	1	33	149	0	138	0	93	102	2 +35	95	2	1
Total mortality & hatching		197	46	99	23	50	171	53	113	31	66	196	32	128	16	65	169	22	153	11	81	195	34	153	17	78

1 = No. of total eggs; 2 = No. of dead eggs; 3 = No. of hatchlings; 4 = % Mortality; 5 = % Hatching; * hatchlings; $ abnormal ecdyon

DISCUSSION

Fresh water teleost eggs are known to have hyperosmotic osmoregulation. K^+, along with Na^+, provides functional osmotic and electrochemical gradient and, further, K^+, along with Na^+, is important structure promoter and stabilizes a particular protein or nucleic acid conformation by binding at anionic sites on macromolecules. K^+ is also an important enzyme activator (Phipps, 1976; Hernan, 1980).

Ikeda (1937a), however, pointed out that K^+, at higher concentrations, is an inhibitor for embryonic development and the rate of development is accelerated when K^+ is removed from the interior in exchange with Ca^{++} ions. When embryos of Oryzias latipes were exposed to higher concentration of K^+ in M/5 KCl, embryos ceased to develop and became opaque; in M/50 KCl, toxic effects began to be seen, while in M/500 KCl, although the embryo differentiation was seen most on the 5th day, the hatching of embryos was affected. Ikeda (1937a) summed up stating that a certain concentration of K^+, say M/500 KCl, may appear to accelerate the functional activity of embryo and the development became arrested sooner or later.

The present author's observations, that tube well water (with utmost 2-3 ppm K^+ concentration) appeared to have the most beneficial effect on development and, further, all other concentrations from 5 to 100 ppm, as experimented upon during the present study, retarded development in one way or another, even causing lysis of chorionic membrane, resulting in premature emergence of embryos at comma stage, are entirely in tune with Ikeda's (1937a) work on Oryzias latipes.

Ca^{++} is undoubtedly known to be intimately involved in the activation of eggs following fertilization (Terrill, 1974). Ca^{++} plays a fundamental role (in cytoplasmic gelation; activation of ATPase activity) and is the main phenomenon at fertilization (Brachet, 1960). However, its role in later embryonic development has not been assessed as well.

This author has found higher concentration of Ca^{++} conducive to lower mortality rates, increased rate of incubation and a larger percentage of hatching. Even concentration as much as 100 ppm more than normal tube well water was not found to have deleterious effect on development, in fact, it only accelerated development. An interesting feature, however, was the recovery of 3 embryos at just before hatching stage, which had very pronounced neural differentiation (much more than in normal

embryos at this stage) and had two pairs of fully differentiated eyes, each including an optic vesicle (of outer pigmented and inner retinal layer) and a lens rudiment along with adequate pigmentation of each eye. The eyes looked thoroughly normal, only a little smaller than in the normal embryos (Figs. 46 & 48). The most perplexing feature, however, was that the mesenteron and the tail structure, including both chorda and muscles, remained entirely undifferentiated (Fig. 47). It is obvious that at this concentration of Ca^{++} (100 ppm + tap water concentration), in some embryos, the animalizing factors came into full play while the vegetalizing factors were completely inhibited. With only neuralizing factor operating, naturally greater differentiation of the neural structure and mutation in respect of number of differentiated eyes occurred. Mg^{++} has been said to cause cyclopial development of a median eye in place of paired eyes in a fish embryo (Lopemann, 1933; Herrill, 1974). Toivonen and Saxen (1958) and Yamada (1961) suggested that two factors were involved in primary induction, one neuralizing principle and the other mesodermalizing principle; anteriorly, the neuralizing principle was said to act alone causing neural differentiation. Posteriorly, however, the mesodermal principle gave effect to spinocaudal structure. In the middle, the two together differentiated to form midbrain, hindbrain, etc.

The recovery of 3 embryos, with two pairs of eyes fully formed and without the spino-caudal differentiation at all, is a very interesting manifestation of the effect of Ca^{++}. The author is obliged to speculate that at high concentrations Ca^{++} can inhibit the mesodermalizing principle resulting in accelerated manifestation of neuralizing factor. Subjection of Fundulus eggs to cold or reduced oxygen tension for a certain period, shortly before embryonic shield formation begins, results in a percentage of double monsters (Trunnett, 1953). Further, exposure to UV does the same. In all cases, the embryonic axes is said to duplicate and the embryos remain conjoined (Perrill, 1974). The effect of high concentrations of Ca^{++}, as observed by the present author, in duplicating eye structure is most interesting.