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FINAL REPORT

Contract NAS 9-13647

Support of In-Flight Experiments

May 31, 1976

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Submitted to

National Aeronautics and Space Administration

by

Dr. Karl P. Kuchnow

Biology Department

Texas A&M University

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INTRODUCTION

The specimens of Fundulus heteroclitus used in this study were obtained from stock maintained at the National Aeronautics and Space Administration laboratories in Houston, Texas. These estuarine minnows were originally collected from the near shore water around Beaufort, North Carolina. In the laboratory fish were held in four LS-700 Living Stream Tanks.[1] The tanks were filled with approximately 150 gal. of 21‰ synthetic sea water.[2]

The water quality was monitored regularly for uniformity of salinity, temperature, pH, and ammonia concentration. For this study it was necessary to maintain the fishes in a state of continuous gamete production. This was accomplished by simulating the natural physical conditions which prevail in the Beaufort, North Carolina area during the spring and early summer breeding season. The 21‰ sea water was held at 20 $\pm$ 2C, and a light-dark cycle of 16 hours light and 8 hours dark was enforced. The fish were fed once daily (mid afternoon) with "Oregon Moist Pellets".

The fresh ova were stripped by holding the female in a damp paper towel with the ventral surface up and gently exerting pressure to force the ova out the ovipositor. Because of the relatively small quantity of sperm obtainable from males, it proved advantageous to

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[1] Frigid Units, Inc., Toledo, Ohio.

[2] Instant Ocean, Aquarium Systems, Inc., Eastlake, Ohio.

collect the sperm in a pipette. Males were held ventral surface up and the urogenital aperture was wiped clean. Pressure was applied to the sides of the abdomen and, using a Pasteur pipette (equipped with a small rubber suction bulb) placed over the spermatic opening, the sperm was drawn up as it exited. This collection method made it easy to harvest the small quantities of sperm and also prevented fecal matter and urine from contaminating the sperm sample.

The sections that follow outline the various techniques used and the results obtained of attempts to achieve satisfactory preservation of ova and sperm of Fundulus heteroclitus, in terms of the greatest amount of time that fertility could be retained, and also the retention of maximum fertility. Also included in this report are the results of tests on delayed embryogenesis, should the preservation of individual gametes not prove feasible, as well as preliminary treatment of data on the orientation of ASTP juveniles.

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II. OVA PRESERVATION TESTS

A. Ficoll-Dextran Solution.

Methods.

Stock solutions of Ficoll[1] (2.5 gm/100 ml) and Dextran[2] (5.0 gm/100 ml) were made up in $10^0/_{00}$, $20^0/_{00}$, and $35^0/_{00}$ Instant Ocean. These stock solutions were used to prepare 5 ml Ficoll-Dextran test solutions with the following composition.

a) Full strength Dextran

b) F:D = 1:1

c) F:D = 2:1

d) F:D = 5:1

e) F:D = 10:1

f) full strength Ficoll

Six batches of the above test solutions were made up from each of the stock solutions at $10^0/_{00}$, $20^0/_{00}$, $35^0/_{00}$, for 24, 48, and 72 hr tests to be run at both 4C and 20C. All control tests were carried out at the appropriate salinity.

Ova to be tested were stripped, pooled, and divided into four roughly equal lots and placed in 10 ml plastic petri dishes. One batch was used as a control group and was fertilized immediately. 5 ml quantities of one of the F:D test solutions were added to the other 3 dishes. These were placed in a temperature chamber at 20C and one of the dishes removed at 24, 48, and 72 hr intervals and the ova tested with fresh sperm. The above procedure was repeated at 4C.

Results.

The results of the tests are shown in Tables 1, 2, and 3. In no case tested did the presence of Ficoll or Dextran extend the fertilizable period of the ova.

Table 1: Effect of F:D solutions in $10^0/_{00}$ sea water on the fertilizability of Fundulus heteroclitus ova.

		20C		4C	
F:D Ratio	Time	# ova	# fertilized	# ova	# fertilized
F:D = 0:1	C	9	9	16	15
	24	14	0	10	0
	48	14	0	17	0
	72	9	0	13	0
F:D = 1:1	C	6	6	19	18
	24	15	0	20	0
	48	7	0	8	0
	72	9	1	14	0
F:D = 2:1	C	19	13	12	10
	24	9	0	24	0
	48	14	0	17	0
	72	42	0	17	0
F:D = 5:1	C	12	11	19	19
	24	13	0	16	0
	48	21	0	10	0
	72	10	0	12	0
F:D = 10:1	C	29	29	9	9
	24	28	0	8	0
	48	31	0	6	0
	72	16	0	6	0
F:D = 1:0	C	39	38	11	11
	24	21	0	18	0
	48	16	0	16	0
	72	17	0	20	0

Table 2: Effect of F:D solutions in 20$^0/_{00}$ sea water on the fertilizability of <u>Fundulus heteroclitus</u> ova.

		20C		4C	
F:D Ratio	Time	# ova	# fertilized	# ova	# fertilized
F:D = 0:1	C	12	11	12	11
	24	9	0	37	0
	48	10	0	22	0
	72	14	0	22	0
F:D = 1:1	C	16	16	16	16
	24	12	0	22	0
	48	18	0	23	0
	72	23	0	33	0
F:D = 2:1	C	16	16	16	16
	24	22	0	37	0
	48	24	0	38	0
	72	26	0	47	0
F:D = 5:1	C	16	16	16	16
	24	10	0	10	0
	48	12	0	8	0
	72	18	0	11	0
F:D = 10:1	C	13	13	13	13
	24	12	0	6	0
	48	10	0	6	0
	72	11	0	5	0
F:D = 1:0	C	17	17	17	17
	24	7	0	15	0
	48	11	0	12	0
	72	16	0	12	0

Table 3: Effect of F:D solutions in 35$^0/_{00}$ sea water on the fertilizability of Fundulus heteroclitus ova.

		20C		4C	
F:D Ratio	Time	# ova	# fertilized	# ova	# fertilized
F:D = 0:1	C	18	9	6	6
	24	13	0	13	0
	48	26	0	9	0
	72	28	0	10	0
F:D = 1:1	C	11	8	24	22
	24	11	0	25	0
	48	10	0	23	0
	72	10	0	26	0
F:D = 2:1	C	9	1	11	10
	24	6	0	10	0
	48	5	0	10	0
	72	5	0	10	0
F:D = 5:1	C	19	7	21	19
	24	23	0	18	0
	48	21	0	16	0
	72	18	0	15	0
F:D = 10:1	C	28	20	23	19
	24	25	0	19	0
	48	25	0	20	0
	72	29	0	19	0
F:D = 1:0	C	19	19	14	14
	24	26	0	14	0
	48	25	0	15	0
	72	28	0	9	0

B. Holtfreter's Solution.

Methods.

Stock solutions of 100%, 50%, 25%, and 10% Holtfreter's solution were prepared by dilution with distilled water. Freshly stripped ova were divided into 5 lots, one to act as control which was immediately fertilized, the other 4 lots placed in finger bowls with the test Holtfreter's solutions. The test lots were incubated at 22C for 48 hr and then tested with fresh sperm.

Results.

	# ova	# fertilized
Control	25	23
100% Holtfreter's	22	0
50% "	27	0
25% "	24	0
10% "	20	0

C. Sucrose Solution.

Methods.

A 2M sucrose solution was prepared in distilled water. Freshly stripped ova were placed in 5 ml aliquots of this solution and

stored at 20C and 4C. At 24, 48, 72 hr intervals batches of ova were tested for fertilizability with fresh sperm.

Results.

	Time	# ova	# fertilized
20C	24	26	0
	48	11	0
	72	-	-
4C	24	9	0
	48	15	0
	72	14	0

D. Dry Preservation.

Methods.

Freshly stipped ova were placed in small plastic capsules (Beem, Inc.) capable of holding appox. 150 ova. All tests were conducted with the ova in a dry state. No additional supportive media were added other than the ovarian fluid which accompanies the ova when stripped from the female.

The capsules were capped and placed in a Lauda-Brinkman K-2/R circulating water bath. The temperature range of 0-30C was tested at two degree intervals. Ova were tested for fertilizability at intervals of 6, 12, 24, 48, 72, 96 hr. at each temperature. For

testing, ova were removed from the water bath, placed in a Petri dish and fresh sperm was expressed onto them, after which they were covered with $20^0/_{00}$ sea water and allowed to incubate with the sperm for 5-10 minutes. The ova were then rinsed and checked 2-3 hr. later for fertilization, at which time they were at the 4-8 cell stage. Each batch was subsequently monitored until hatching, with abortions and abnormalities recorded. Each test was replicated at least 4 times.

Results.

Percent fertilization (with standard deviation) at the times and temperatures tested are shown in Table 4 and summarized in Fig. 1. Percent abnormality and abortion are given in Table 5.

E. Salinity.

Methods.

Stock solutions of Instant Ocean ranging from $0\text{-}35^0/_{00}$ at $5^0/_{00}$ increments were prepared. Ova were stripped into 100 ml Petri dishes containing about 20 ml of the test sea water. Batches were kept at 22C and tested at 1, 3, 6, 9, 12 and 15 hr intervals with fresh sperm.

Similar tests were also run at 12C. Fresh ova were placed in small plastic capsules which were then filled with water of a test salinity and stored in a water bath. Ova were checked for fertilizability at intervals of 6, 12, 24, 48, 72 hrs.

Table (4). Percent fertilization of temperature-treated ova after varying exposure times over the range of 0-30°C. The values represent the means.

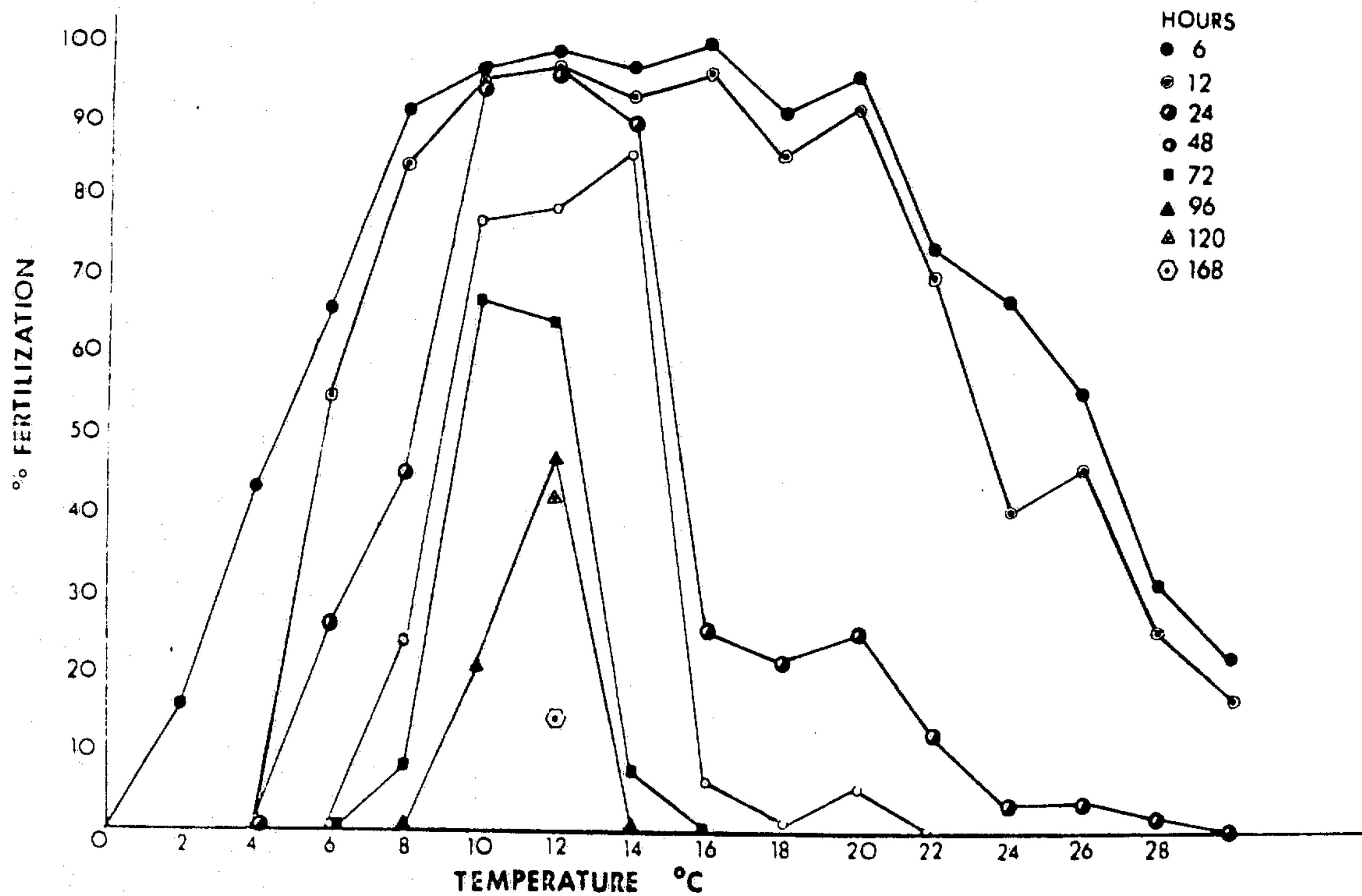
Temp. ^{o}C	Exposure Time (Hours)							
	6	12	24	48	72	96	120	168
0	0							
2	0							
4	42.9 ± 4 (a)	0	0	0	0	0		
6	65.9 ± 7	55.1 ± 11	26.7 ± 14	0	0	0		
8	90.6 ± 2	82.6 ± 4	44.5 ± 9	24.1 ±	8.4 ± 3	0		
10	95.0 ± 4	94.7 ± 5	94.3 ± 2	76.4 ± 13	67.4 ± 13	20.3 ± 7	0	0
12	98.0 ± 3	96.7 ± 3	95.9 ± 5	78.0 ± 4	64.1 ± 8	46.7 ± 2	42.2 ± 8	14.7 ± 9
14	96.3 ± 4	92.7 ± 5	88.7 ± 10	84.9 ± 11	7.8 ± 2	0		
16	98.6 ± 2	95.9 ± 4	25.3 ± 7	6.2 ± 4	0	0		
18	90.5 ± 3	84.3 ± 4	21.7 ± 12	1.2 ± 4	0	0		
20	94.6 ± 9	91.0 ± 3	24.3 ± 8	5.8 ± 3	0	0		
22	72.8 ± 4	69.0 ± 4	12.0 ± 8	0	0	0		
24	67.0 ± 5	40.0 ± 8	2.7 ± 3	0	0	0		

Table 4 (continued)

Temp. ^{o}C	6	12	24	48	72	96	120	168
26	55.7± 6	46.0± 8	3.6± 5	0	0	0		
28	31.5± 6	25.4± 7	1.3± 2	0	0	0		
30	22.0± 3	16.9± 5	0	0	0	0		

(a) Standard deviations have been rounded to the nearest whole number.

FIGURE 1. The effect of temperature on the fertilizability of ova exposed over the range of 0-30C. Each fertilization percent represents the mean of four replicate tests.



HOURS
6
12
24
48
72
96
120
168
% FERTILIZATION
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
TEMPERATURE °C

Table 5 Percentage abortion of embryos from temperature-treated ova.

Temp. °C	Exposure Time (Hours) 6	12	24	48	72	96
4	87.2(a)	- (b)	-	-	-	-
6	65.0	90.0	56.0	-	-	-
8	20.4	42.7	77.5	71.4	87.0	-
10	12.0	23.0	19.6	50.4	63.0	74.0
12	10.0	7.3	8.0	37.7	22.8	50.0
14	6.8	4.1	14.0	22.0	22.2	-
16	5.0	3.0	26.6	42.8	-	-
18	14.8	12.0	7.6	0	-	-
20	0	2.0	57.9	40.0	-	-
22	8.3	11.1	46.1	-	-	-
24	37.5	44.2	100.0	-	-	-
26	60.0	54.5	77.0	-	-	-
28	10.3	38.0	65.0	-	-	-
30	33.3	50.0	-	-	-	-

(a) Each percent represents mortalities and those embryos which exhibited structural deformaties.

(b) Fertilization did not occur.

Percent fertility, abnormality and abortion were monitored as in Section D.

Results.

Percent fertilization at the salinities, times, and temperatures tested are shown in Tables 6, 7 and summarized in Figs. 2, 3. Abnormality and abortion rates are shown in Tables 8, 9.

F. pH

Methods.

Batches of ova were treated at 22C in sea water of two salinities, $10^{0}/_{00}$ and $22^{0}/_{00}$, the pH of which was adjusted to some value between 6.0 and 9.4. Standard buffers of pH 4.01 and pH 10.0 were used to adjust the pH of the test medium to the desired value. Ova were stripped into 100 ml Petri dishes containing 20 ml of the test solution, and tested at intervals of 1, 3, 6, 9, 12, 15 hr. exposure. Ova were also treated at 12C, in $10^{0}/_{00}$ salinity at various pH values.

Results.

Percent fertilization is shown in Tables 10-13 and summarized in Figs. 4-7 . Abnormality and abortion rates are shown in Tables 14-15.

Table 6. Percent fertilization of salinity-treated ova after varying exposure times over the range of 0-35 $^{o}/oo$ at ambient temperature (22-25 ^{o}C).

SALINITY ‰	Exposure Time (Hours) 1	3	6	9	12	15
0	0	0	0	0	0	0
5	78.2 ± 8(a)	67.3 ± 15	5.2 ± 2	1.2 ± 2	1.5 ± 2	0
10	96.0 ± 4	88.5 ± 4	6[illegible].9 ± 9	25.6 ± 8	14.0 ± 6	4.6 ± 1
15	49.7 ± 7	24.6 ± 6	25.0 ± 6	9.7 ± 2	3.6 ± 4	5.0 ± 1
20	35.7 ± 15	23.3 ± 12	5.1 ± 2	1.7 ± 2	1.2 ± 2	0
25	10.2 ± 3	4.5 ± 6	2.1 ± 2	0	0	0
30	2.3 ± 2	0	0	0	0	0
35	0	0	0	0	0	0

(a) Values represent the means. Standard deviation have been rounded to the nearest whole number.

Table 7. Fertilization percentages of ova exposed to a 0-35°/oo salinity range at 12°C over varying time periods. The pH of sea water was adjusted to 8.0-8.4 with pH 10.00 buffer.

SALINITY (°/oo)	Exposure Time (Hours) 6	12	24	48	72
0	0	0	0	0	0
5	88.7± 7(a)	70.5± 7	38.6± 9	4.1± 5	0
10	95.3± 2	88.4± 7	66.5± 7	10.4± 3	0
15	24.6± 6	20.0± 5	8.1± 4	1.0± 1	0
20	12.5± 3	1.7± 2	0	0	0
25	15.7± 5	0	0	0	
30	0	0	0	0	
35	0	0	0	0	

(a) Values represent the means. Standard deviations have been rounded to the nearest whole number.

FIGURE 2. The effect of salinity on the fertilizability of ova exposed over the range of 0-35 $^{0}/_{00}$ at ambient temperature (22-25C). Each fertilization percent represents the mean of four replicate tests.

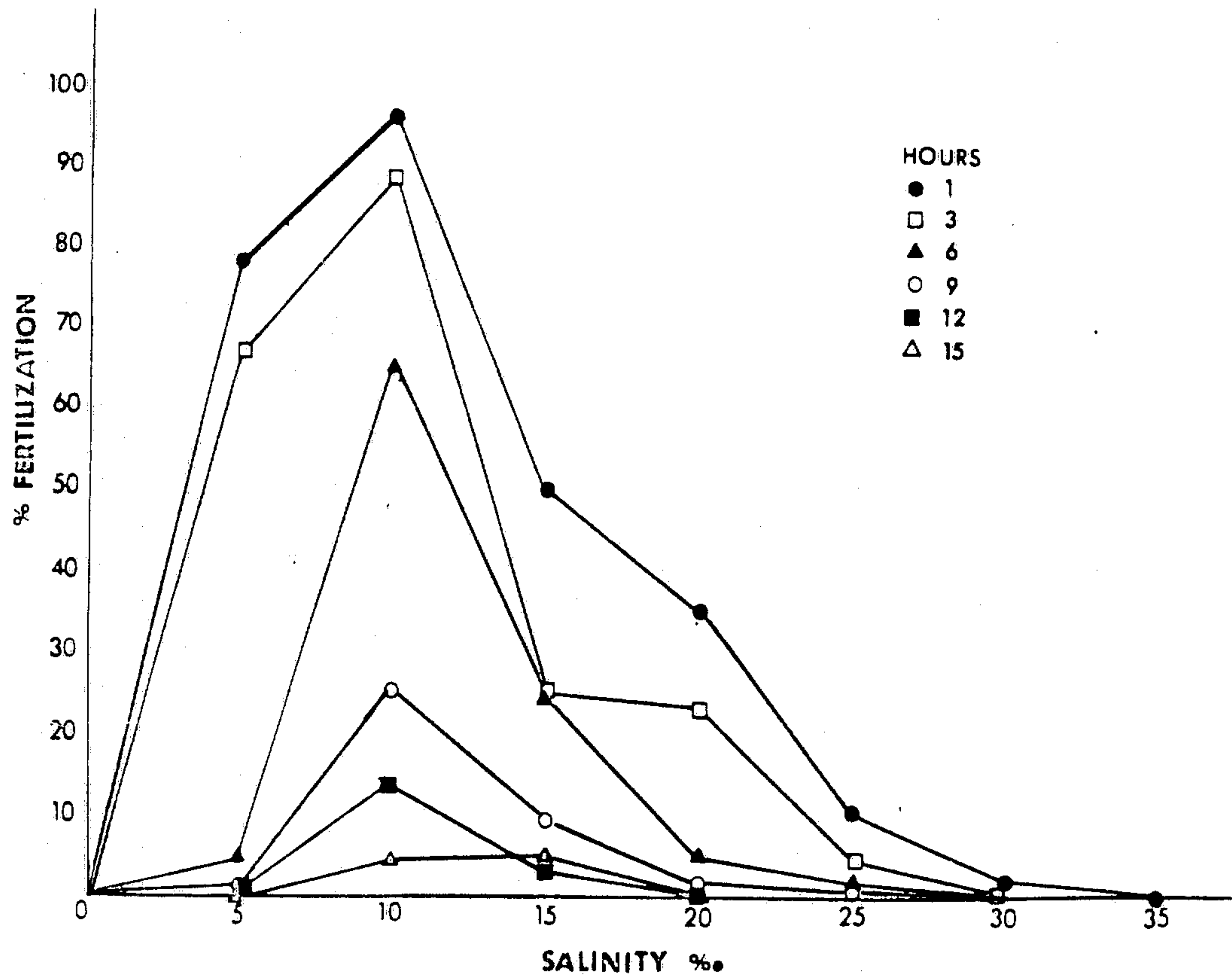
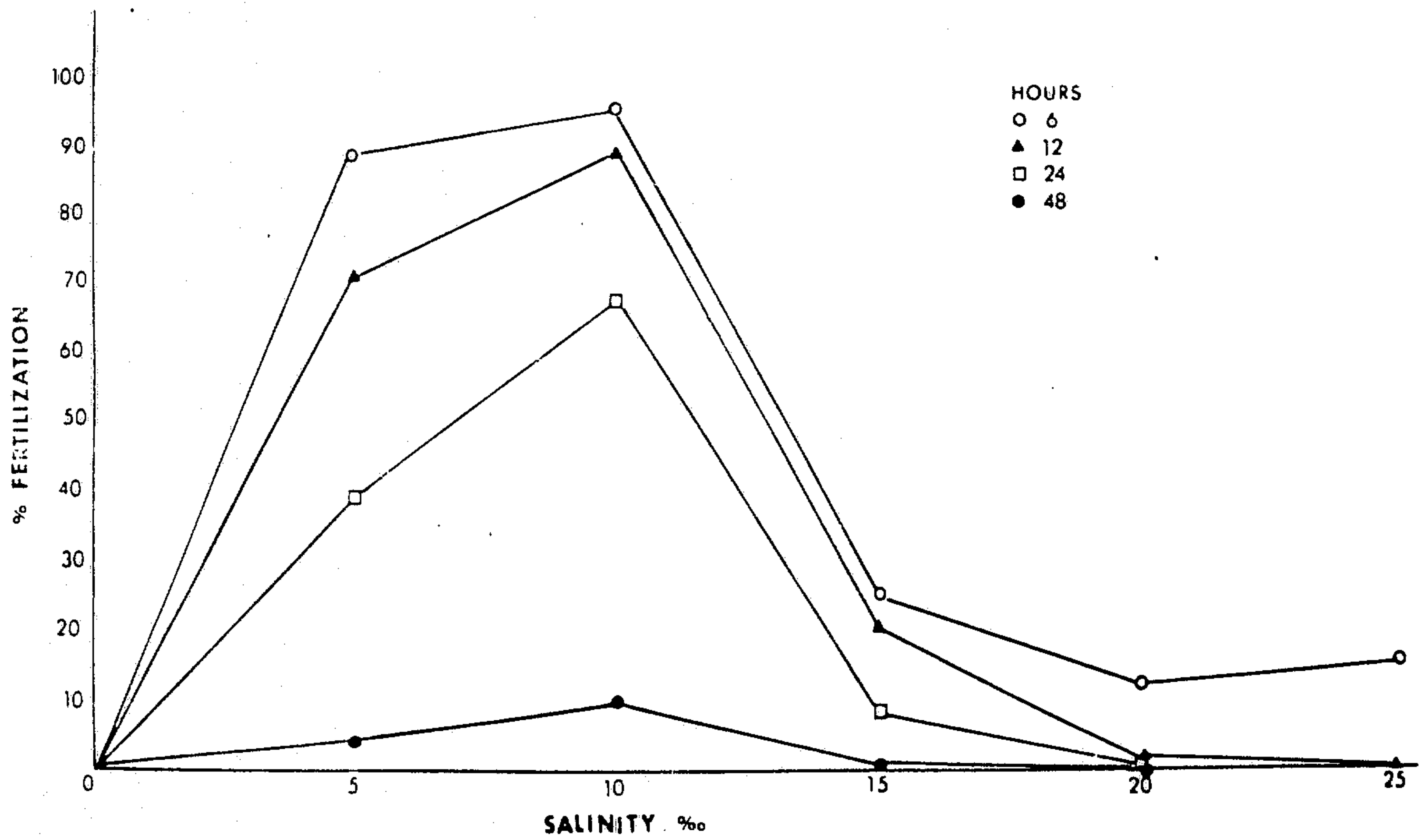

% FERTILIZATION
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
SALINITY ‰
HOURS
1
3
6
9
12
15

FIGURE 3. Percent fertilization of ova exposed to the salinity range of 0-25 $^{0}/_{00}$ at 12C. pH of artificial sea water was adjusted to 8.0-8.4.



HOURS
6
12
24
48
% FERTILIZATION
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
SALINITY ‰

Table 8 Percentage abortion of embryos resulting from salinity exposed ova maintained at ambient temperature, 22-25°C.

Salinity o/oo	Exposure Time (Minutes) 1	3	6	9	12	15
5	0	4.2(a)	0	0	0	-(b)
10	0	0	8.7	4.7	0	0
15	2.7	0	3.8	14.0	0	0
20	2.0	0	12.4	0	0	-
25	3.4	0	0	-	-	-
30	0	0	-	-	-	-

Table 9 Percentage abortion of embryos resulting from salinity exposed ova maintained at 12°C.

Salinity o/oo	Exposure Time (Hours) 6	12	24	48
5	8.3(a)	7	16	100
10	5.0	3.1	20.4	84
15	2.1	6.3	34	100
20	20.4	0	0	0
25	10	0	0	0

(a) Values represent total mortalities and structurally deformed embryos.

(b) Fertilization did not occur.

Table (10) Percentage fertilization of ova exposed to 10‰ sea water adjusted to the pH range 6.0-9.5. Tests were conducted at ambient temperature (22-25°C). Values represent the mean.

	Exposure Time (Hours)					
pH	3	6	9	12	15	24
6.0	28.5 ± 6(a)	1.25 ± 2	0	0	0	0
6.5	23.8 ± 4	2.4 ± 2	0	0	0	0
6.8	42.7 ± 17	15.9 ± 6	5.5 ± 4	2.3 ± 3	0	0
7.0	61.4 ± 25	19.2 ± 8	4.6 ± 3	0	0	0
7.4	96.5 ± 3	76.0 ± 6	29.7 ± 10	11.3 ± 4	0	0
7.8	96.5 ± 4	73.9 ± 3	27.4 ± 8	7.9 ± 3	3.7 ± 3	0
8.2	97.5 ± 5	92.1 ± 6	50.8 ± 16	42.6 ± 15	8.3 ± 3	0
8.6	96.9 ± 4	83.6 ± 11	46.6 ± 11	12.5 ± 3	0	1.4 ± 2
9.0	94.1 ± 4	53.9 ± 14	37.3 ± 7	0	0	0
9.2	92.6 ± 6	45.4 ± 7	25.1 ± 6	0	0	0
9.5	0	0	0	0	0	0

(a) Standard deviations have been rounded to the nearest whole number.

Table (11) Percent Fertilization of ova exposed to a salinity of 10 $^{o}/oo$ at 12 ^{o}C over the pH range 6.0-9.5. Values represent the means.

Exposure Time (Hours)

pH	24	48	72
6.0	10.6 ± 2(a)	0	0
6.5	32.0 ± 4	11.8 ± 8	0
6.8	31.3 ± 6	6.6 ± 2	0
7.0	47.5 ± 7	11.3 ± 8	0
7.4	72.0 ± 6	9.8 ± 5	0
7.8	66.7 ± 6	10.7 ± 4	0
8.2	51.5 ± 8	12.2 ± 9	1.6 ± 1
8.6	39.1 ± 13	5.4 ± 4	1.1 ± 1
9.0	57.8 ± 15	9.8 ± 3	0
9.2	54.1 ± 9	11.5 ± 9	0
9.5	0		

(a) Standard deviations have been rounded to nearest whole number.

Table (12) Percent fertilization of ova exposed to 22°/oo sea water at 12°C over the pH range 6.5-9.5. Values represent the means.

	Exposure Time (Hours)		
pH	6	12	24
6.5	0	0	0
6.8	0	0	0
7.0	3.3 $\pm$ 1 (a)	0	0
7.4	9.7 $\pm$ 2	8.9 $\pm$ 4	0
7.8	11.9 $\pm$ 2	7.8 $\pm$ 3	0
8.2	13.1 $\pm$ 3	7.7 $\pm$ 3	0
8.6	8.1 $\pm$ 2	5.0 $\pm$ 2	0
9.0	8.2 $\pm$ 2	2.6 $\pm$ 2	0
9.2	3.1 $\pm$ 2	0	0
9.5	0	0	0

(a) Standard deviations have been rounded to nearest whole number.

Table (13) Percentage fertilization of ova exposed to 22°/oo sea water adjusted to the pH range 6.0-9.5. Tests were conducted at ambient temperature (22-25°C). Values represent the mean.

		Exposure Time (Hours)			
		1	3	6	9
	6.0	0	0		0
	6.5	3.5 ± 1(a)	0	0	0
	6.8	0	0	0	0
	7.0	5.9 ± 1	1.7 ± 2	0	0
	7.4	16.2 ± 5	3.9 ± 2	0.6 ± 1	0
pH	7.8	21.3 ± 1	8.0 ± 2	0	0
	8.2	24.3 ± 4	16.6 ± 2	2.0 ± 1	0
	8.6	17.5 ± 3	8.5 ± 2	0	0
	9.0	10.6 ± 2	8.6 ± 4	0	0
	9.2	2.6 ± 1	0	0	0
	9.5	0	0	0	

(a) Standard deviations have been rounded to the nearest whole number.

FIGURE 4. Percent fertilization of ova treated at $10^0/_{00}$ over the pH range of 6.0-9.5 at 22-25C.

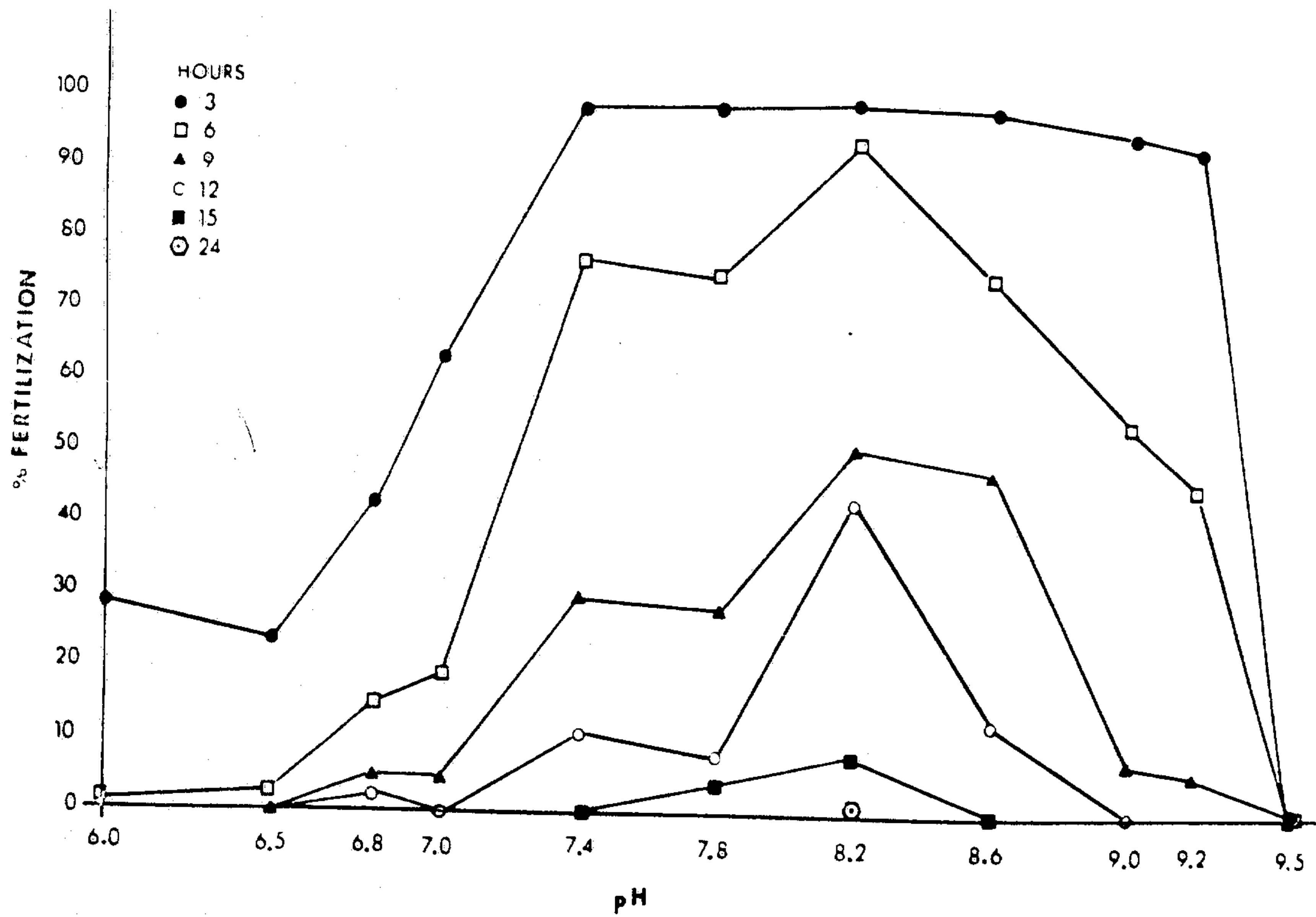

HOURS
3
6
9
12
15
24
% FERTILIZATION
0
10
20
30
40
50
60
70
80
90
100
6.0
6.5
6.8
7.0
7.4
7.8
8.2
8.6
9.0
9.2
9.5
pH

FIGURE 5. Percent fertilization of ova treated at $22^0/_{00}$ over the pH range 6.0-9.4, at 22-25C.

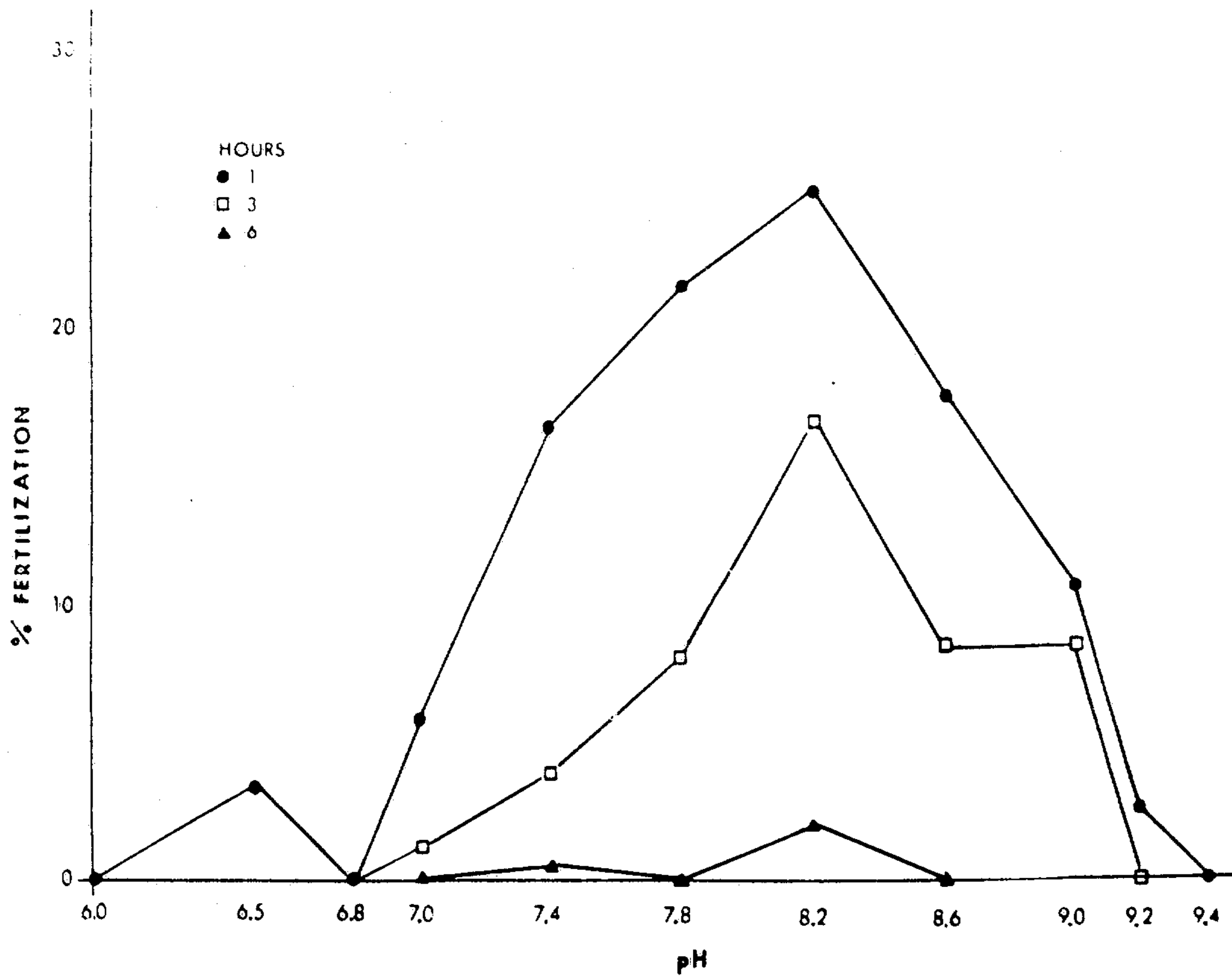

HOURS
1
3
6
% FERTILIZATION
20
10
0
6.0
6.5
6.8
7.0
7.4
7.8
8.2
8.6
9.0
9.2
9.4
pH

FIGURE 6. Percent fertilization of ova exposed to $10^0/_{00}$ at 12C over the pH range of 6.0-9.5.

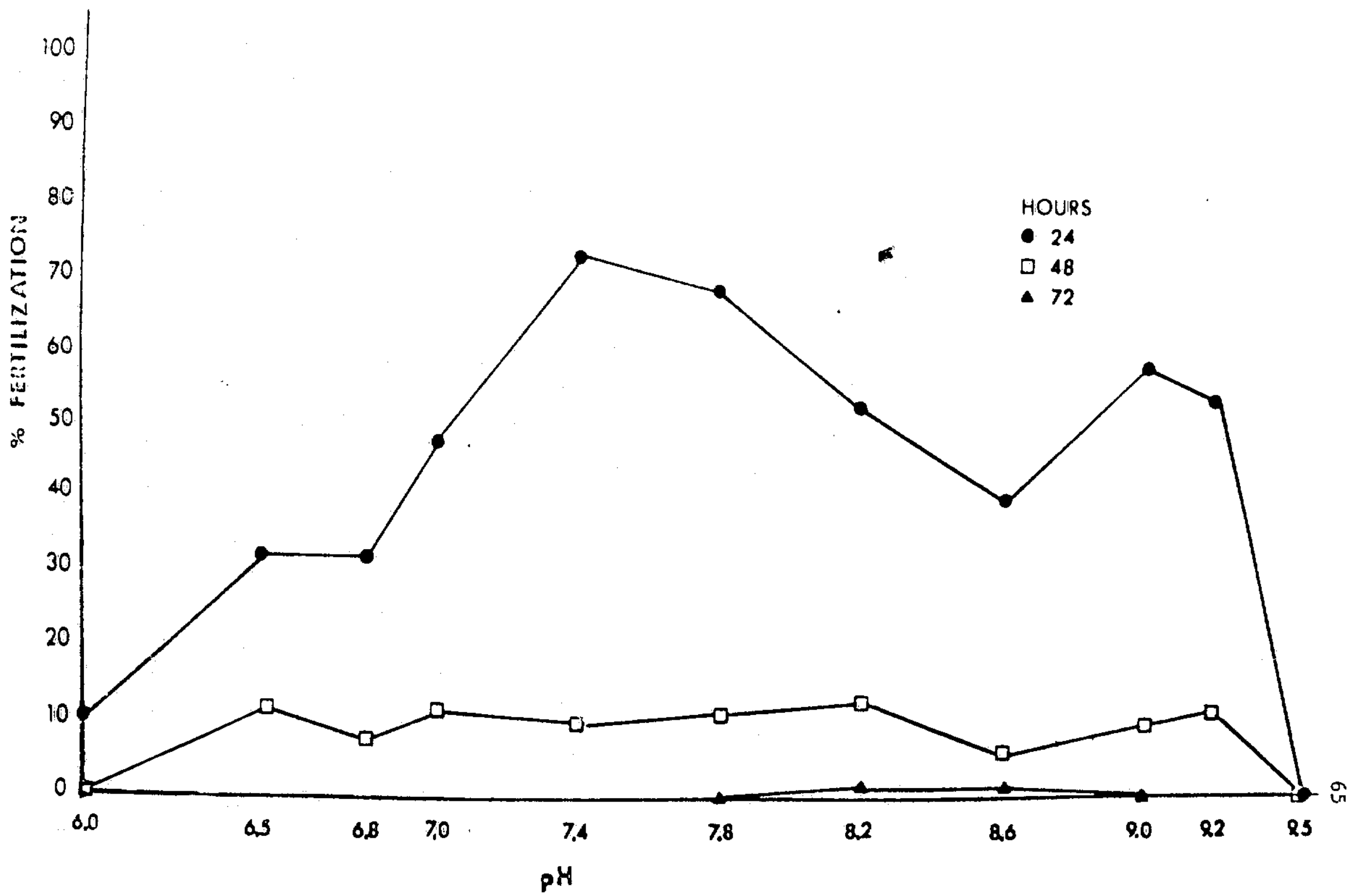
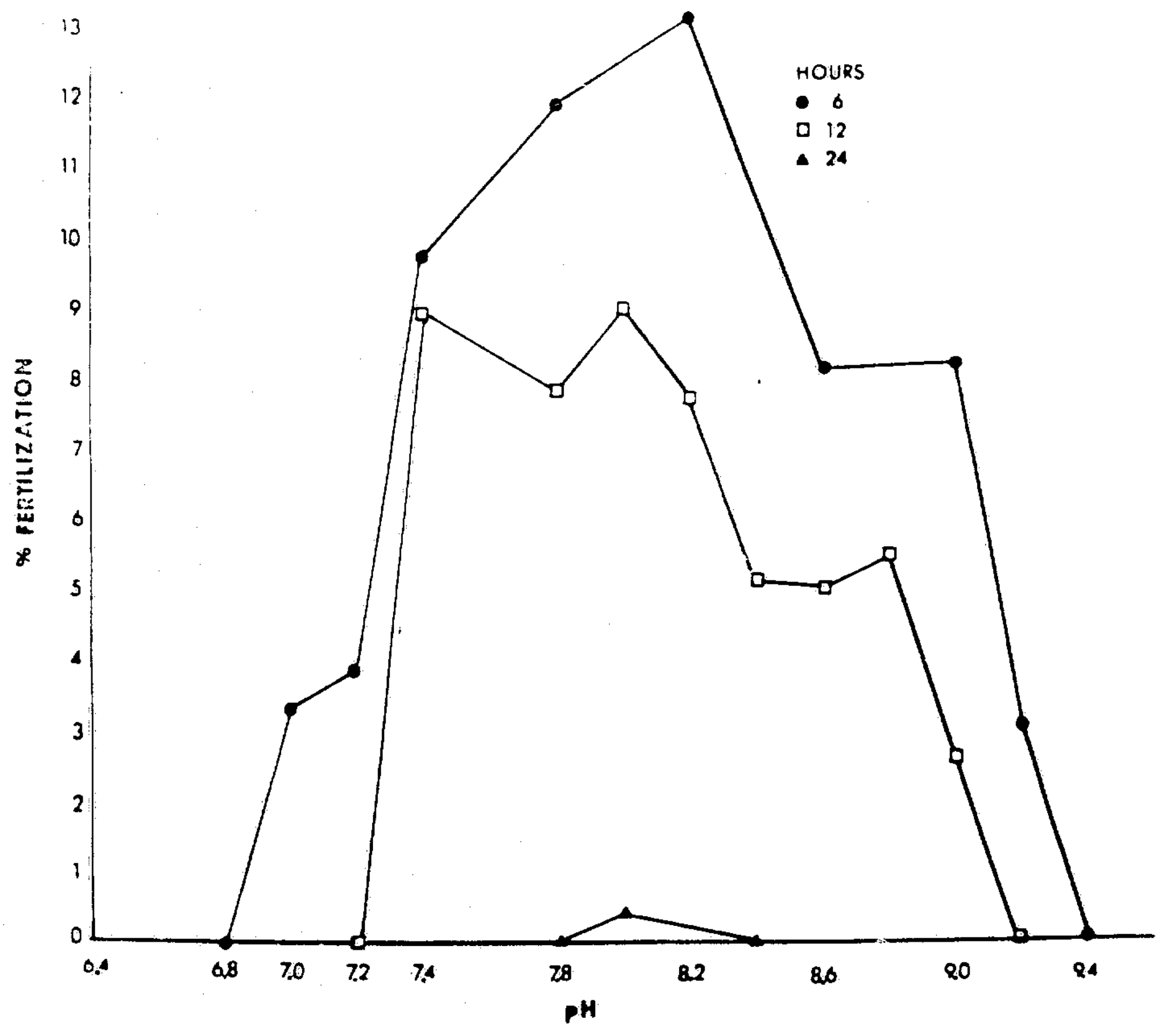

100
90
80
70
60
50
40
30
20
10
0
% FERTILIZATION
HOURS
24
48
72
6.0
6.5
6.8
7.0
7.4
7.8
8.2
8.6
9.0
9.2
9.5
pH

FIGURE 7. Percent fertilization of ova exposed to $22^0/_{00}$ at 12C over the pH range 6.4-9.4.



HOURS
6
12
24
% FERTILIZATION
0
1
2
3
4
5
6
7
8
9
10
11
12
13
6.4
6.8
7.0
7.2
7.4
7.8
8.2
8.6
9.0
9.4
pH

Table 14 Percent abortion of embryos from pH-treated ova.
A. Ova exposed to 10‰ sea water at 22-25°C.
B. Ova exposed to 22‰ sea water at 22-25°C.

Exposure Time (Hours)

A. pH	3	6	9	12	15
6.0	21.8(a)	100	-(b)	-	-
6.5	38.4	40	-	-	-
6.8	9.2	19.0	25	50	-
7.0	10.5	21.0	45.4	-	-
7.4	4.3	3.5	9.0	21	33
7.8	4.0	3.5	4.3	14.2	-
8.2	3.2	3.3	15.5	8.1	50
8.6	6.1	5.7	10.4	11.1	33
9.0	7.3	10.4	18.1	-	-
9.2	14.5	20.0	10.0	-	-

Exposure Time (Hours)

B. pH	1	3	6
6.5	100(a)	-(b)	-
7.0	37	50	100
7.4	31.3	100	-
7.8	27	66	-
8.2	24	81.2	66.6
8.6	26	81.8	-
9.0	85.7	33.3	-

(a) Values represent total mortalities and embryos exhibiting structual deformities.

(b) Fertilization did not occur.

Table 15 Percentage abortion of embryos from ova exposed to two salinities at the same temperature and pH range.

A. Ova exposed to 10°/oo at 12C.
B. Ova exposed to 22°/oo at 12C.

Exposure Time (Hours)

A.

pH	24	48	72
6.0	70.5 (a)	- (b)	-
6.5	30.4	75.0	-
6.8	24.0	56.0	-
7.0	27.7	30.5	-
7.4	9.7	50.0	-
7.8	12.5	22.7	-
8.2	21.8	16.6	66.0
8.6	17.0	28.5	0
9.0	27.0	49.0	-
9.2	48.0	42.0	-

B.

pH	6	12	24
7.4	10.0 (a)	30.7	- (b)
7.8	0	30.0	-
8.2	9.3	11.1	-
8.6	21.0	28.5	-
9.0	29.0	66.0	-
9.2	39.0	-	-

(a) Values represent total abnormalities and embryos which exhibited structural deformities.
(b) No fertilization occurred.

G. Analysis of Ovarian Fluids

1. pH.

Methods.

a) Ovarian fluid accompanying stripped ova was drawn into 50μl capillary tubes.

b) The tubes were sealed at both ends to prevent further changes in pH due to absorbance of CO_2 from the air.

c) The fluid was analyzed using a BMS3 MK2 Blood Microsystem Apparatus.

d) The fluid from 10 females was measured and a mean value obtained.

Results.

a) The ovarian fluid averaged pH 8.0 ± 0.4.

2. Elemental Composition.

Methods.

a) Ovarian fluid accompanying stripped ova was pooled and stored in a frozen state until about 0.5 ml had accumulated.

b) Blood was also collected from the caudal artery for comparison with ovarian fluid.

Results.

Table 16.

μgm/gm sample

	Na	Cl	K	Ca	Mg
Blood	2480 (3.2%)*	3800 (6.1%)	592 (4%)	821 (25%)	200 (10%)
Ovarian Fluid	3860 (3.0%)	5350 (6.0%)	374 (11%)	217 (25%)	116 (10%)

* Values in parentheses indicate the estimated error within the measurement. Na, Cl, K analyses were performed by instrumental neutron activation analysis (i.e. reactor neutron irradiation followed by gamma ray spectrometry). Ca, Mg results obtained by atomic absorption flame photometry.

III. SPERM PRESERVATION TESTS

A. Ficoll[1]-Dextran[2] Solution

Methods.

1) Stock solutions of Ficoll and Dextran were prepared as in II-A.
2) The same F:D ratios were used as in II-A.
3) Quantities of pooled sperm were incubated with the various F/D solutions at both 4C and 20C for 48 and 72 hr and then tested with fresh ova.

Results.

Table 17 : Fertility of _Fundulus heteroclitus_ sperm incubated in various Ficoll-Dextran solutions in $35^0/_{00}$ sea water, at 4C for 48 hr.

F:D Ratio	# ova	# fertilized
0:1	16	0
1:1	16	0
2:1	19	0
5:1	17	0
10:1	13	0
1:0	10	0

---

[1]Ficoll: Pharmacia Chemicals - Lot No. 2300

[2]Dextran: General Biochemicals - Lot No. 41923B

Table 18: Fertility of Fundulus heteroclitus sperm incubated in various Ficoll-Dextran solutions in $20^{0}/_{00}$ sea water, at 4C for 72 hr.

F:D Ratio	# ova	# fertilized
0:1	21	0
1:1	27	0
2:1	21	0
5:1	18	0
10:1	24	0
1:0	19	0

Table 19: Fertility of Fundulus heteroclitus sperm incubated in various Ficoll-Dextran solutions in $35^{0}/_{00}$ sea water at 20C for 48 hr.

F:D Ratio	# ova	# fertilized
0:1	6	0
1:1	4	0
2:1	5	0
5:1	5	0
10:1	17	0
1:0	17	0

B. Preliminary Tests on Sperm, Temperature, pH, Salinity, Extender Solution Interactions.

Methods.

A large number of single tests were performed to investigate the role of various parameters on sperm preservation in an attempt to find possible favorable areas for more definitive experimentation.

Results.

Table 20: Role of Various Parameters on Preservation of Sperm Fertility.

	Conditions	Time (hr)	Temp. (°C)	# ova	# fert.	% fert.
1)	dry-mixed w/20‰ SW prior to exposing to ova	24	4	28	0	0
2)	dry-mixed w/stock pH7 buffer prior to exposing to ova	24	4	33	7	21
3)	dry-mixed w/pH7 buffer diluted 10 X prior to exposing to ova	24	4	49	19	39
4)	as in (3) - diluted 100 X	24	4	39	6	15
5)	incubated in pH7 solution	24	4	43	29	67
6)	incubated in pH7 solution	24	8	54	46	85
7)	dry-add pH7 prior to exposure to ova	24	8	65	55	85
8)	incubated in pH7 solution	48	8	103	0	0
9)	" " " "	48	4	79	48	61
10)	" " " "	72	8	42	18	43
11)	" " " "	72	4	102	9	9

	Conditions	Time (hr)	Temp. (^oC)	# ova	# fert.	% fert.
12)	incubated in pH7 solution	24	8	64	51	80
13)	incubated in 0.05 M glycine	24	8	112	0	0
14)	incubated in pH7 – $\frac{\text{sperm}}{\text{buffer}} = \frac{1}{1}$	72	4	40	0	0
15)	" " " " $= \frac{1}{10}$	72	4	45	7	16
16)	as in (14)	72	2	53	0	0
17)	as in (15)	72	2	52	1	2
18)	w/pH7 and 1.25 mM ATP (Sigma Chemicals)	48	2	60	49	82
19)	" "	72	2	54	0	0
20)	" "	48	4	48	4	8
21)	" "	72	4	42	0	0
22)	w/pH 7.5	72	2	45	25	56
23)	w/pH 8.0	72	2	44	0	0
24)	w/pH 8.5	72	2	44	0	0
25)	w/pH 7.5	72	4	45	12	27
26)	w/pH 8.0	72	4	59	0	0
27)	w/pH 8.5	72	4	42	0	0
28)	w/pH 7.5	24	4	44	44	100
29)	w/pH 8.0	24	4	51	0	0
30)	w/pH 7.5	48	4	40	21	53
31)	w/pH 7.5	48	2	23	22	96
32)	w/pH 7.5	72	2	34	11	32
33)	pH 7.5 + 1.25 mM ATP + O_2	72	2	143	130	91
34)	" " " "	72	2	124	120	97

C. Effect of Buffer, ATP, Temperature, Oxygen and Salinity of Post-Preservation Mixing Medium on Sperm Fertility.

Methods.

1. Sperm were incubated for 72 hr at 2C in a variety of media:

a) sperm : pH 7.5 buffer = 1:1

b) sperm : pH 7.5 buffer w/ 1.25 mMATP = 1:1

c) " : " " " + O_2 = 1:1

d) " " " + O_2 = 1:1

2. All preservation was carried out in sealed plastic capsules capable of holding 0.5 ml total solution.

3. Following incubation sperm was expressed onto fresh ova bathed in the following solutions.

a) 10 $^0/_{00}$ sea water

b) 20 $^0/_{00}$ sea water

c) 35 $^0/_{00}$ sea water

d) pH 7 buffer

e) pH 7.5 buffer

Results

(see Table 21)

Table 21: Effect of Incubation Media and Mixing Media on Sperm Preserved 72 hr at 2C.

Incubation Medium		MIXING MEDIA					TOTALS
		$10^{0}/_{00}$	$20^{0}/_{00}$	$35^{0}/_{00}$	pH 7	pH 7.5	
pH 7.5	#ova	40	41	36	38	36	$\frac{191}{4}$ = 2%
	#fert	0	1	0	3	0	
pH 7.5 1.25 mMATP	#ova	24	26	27	25	35	$\frac{137}{3}$ = 2%
	#fert	1	0	0	2	0	
pH 7.5 O_2	#ova	26	17	22	25	26	$\frac{116}{18}$ = 16%
	#fert	7	8	2	0	1	
pH 7.5 O_2	#ova	18	23	18	18	13	$\frac{90}{62}$ = 69%
	#fert	18	22	14	5	3	
TOTALS		$\frac{26}{108}$ =24%	$\frac{32}{107}$ =30%	$\frac{16}{103}$ =16%	$\frac{10}{106}$ = 9%	$\frac{4}{110}$ = 4%	

D. Effect of ATP Concentration.

Methods.

1. Sperm were incubated in the following solutions of ATP in pH 7.5 buffer, at 10C.

   a) 1.25 mM ATP

   b) 1.0 "

   c) 2.0 "

   d) 5.0 "

e) 10.0 mM ATP

f) 0.0 " , i.e. pH 7.5 buffer only

2. Sperm were tested after 72 and 94 hrs with fresh ova.

Results.

Table 22 : Effect of ATP concentration on sperm fertility.

ATP CONC.	72 hr		94 hr	
	# ova	# fertilized	# ova	# fertilized
1.0 mM	48	5	65	6
1.25	57	22	63	0
2.0	39	38	78	0
5.0	39	5	75	0
10.0	46	23	55	0
pH 7.5 only	49	0	58	0

E. Temperature.

Methods.

1. Sperm were stripped and placed in small plastic capsules in a dry state.

2. Capsules were capped and placed in a Lauda-Brinkman K-2/R circulating water bath.

3. The temperature range tested was 0-20C at 2 degree increments.

4. At intervals of 2, 4, 6, 12, 15 and 24 hr, the sperm were removed from the water bath and mixed with fresh ova.

5. After 5-10 min. the ova were rinsed and later checked for evidence of fertilization.

Results.

The relationship between temperature, time of preservation and fertility are given in Table 23 and summarized in Fig. 8. The mortality - abnormality rates resulting from these tests are given in Table 24.

F. Salinity.

Methods.

1. The salinity range tested was the same as that used for the ova (Sect. II E).
2. Sperm : Sea water = 1:10.
3. Sperm were tested at 25C at intervals of 5, 10, 20, 30, 40 min.
4. Sperm were tested at 2C at intervals of 1, 3, 6, 9 hr.

Results.

The relationship between salinity and time of preservation at 25C is given in Table 25 and summarized in Fig. 9 , and at 2C is given in Table 26 and summarized in Fig. 10.

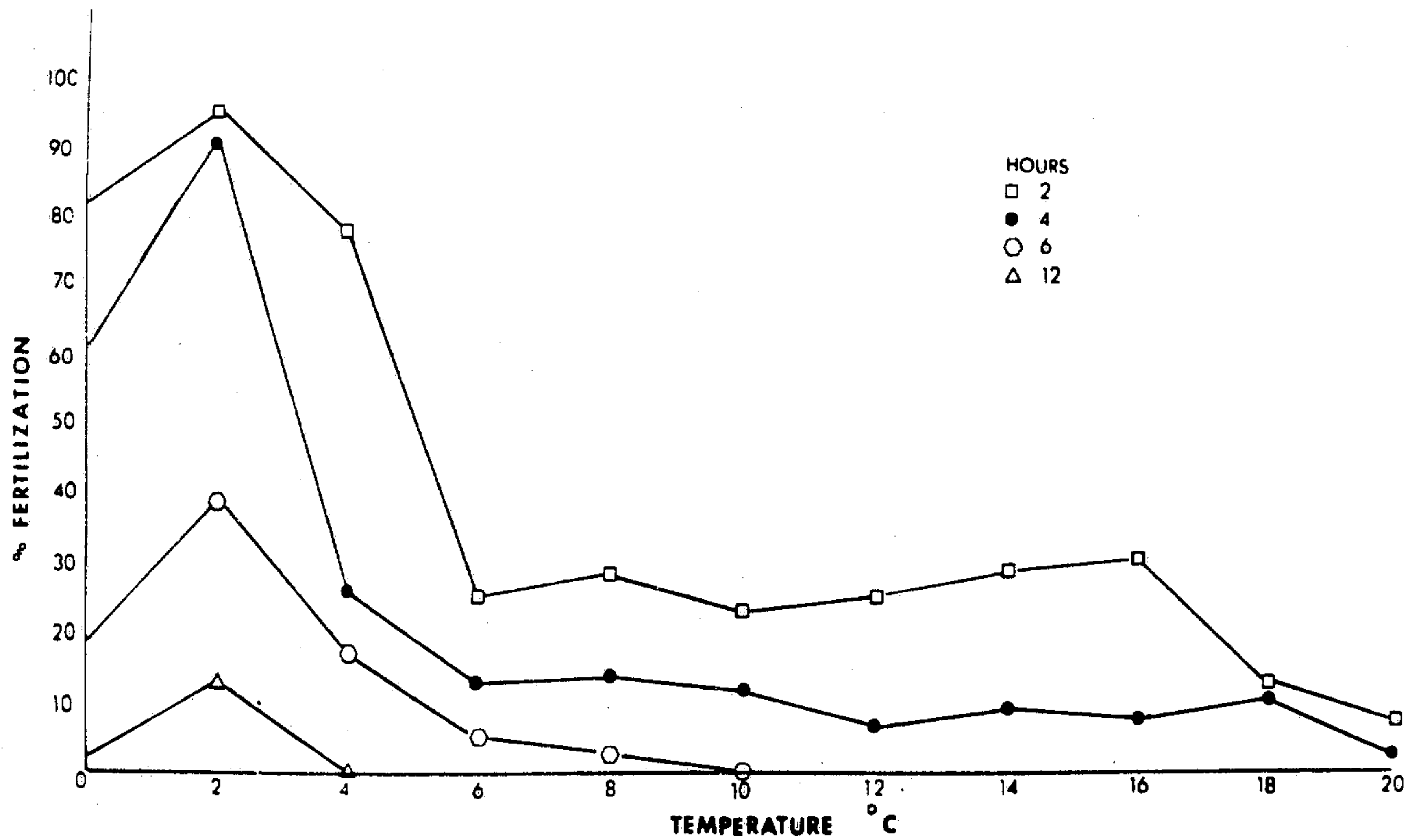
Mortality and abnormality rates from these tests are given in Tables 27 and 28.

Table 23 Percent of fresh ova fertilized with sperm exposed to the range of 0-20°C over various time intervals.

Temp. °C	Exposure Time (Hours) 2	4	6	12	15	24	48	72
0	81.5± 10(a)	60.2± 13	19.2± 9	2.0± 1	0	0		
2	95.6± 4	91.0± 6	38.5± 18	13.1± 5	0	25.6± 33	63.9± 49	36.5± 40
4	77.3± 10	26.1± 7	17.6± 13	0	0	0		
6	25.2± 10	13.1± 4	5.3± 2	0	0	0		
8	28.0± 3	14.2± 9	2.4± 1	0	0	0		
10	22.7± 7	12.1± 4	0	0	0			
12	24.7± 9	6.8± 3	0	0	0			
14	28.8± 13	9.7± 5	0	0	0			
16	30.4± 16	8.3± 4	0	0	0			
18	13.3± 3	10.8± 5	0	0	0			
20	8.4± 4	2.4± 2	0	0	0			

(a) Standard deviations have been rounded to nearest whole number.

FIGURE 8. The effect of temperature on the fertilizing capacity of sperm subjected to the range of 0-20C. Each fertilization percent represents the mean of four replicate tests.



% FERTILIZATION
10C
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20
TEMPERATURE °C
HOURS
2
4
6
12

Table 24 Percentage abortion of embryos from temperature-treated sperm.

Temp. ^oC	2	4	6	12
0	4.0(a)	0	6.3	0
2	0	8.0	0	12.0
4	9.3	5.3	0	-(b)
6	0	5.3	0	-
8	10.3	0	3.7	-
10	2.3	0	-	-
12	4.1	9.7	-	-
14	0	10.0	-	-
16	0	0	-	-
18	0	7.7	-	-
20	6.3	3.2	-	-

(a) Each percent represents mortalities and those embryos which exhibited structural deformaties.

(b) Fertilization did not occur.

Table 25 Percent of fresh ova fertilized with sperm exposed to the range of 0-35°/oo at various time intervals at ambient temperature (22-25°C).

Exposure Time (Minutes)

SALINITY °/oo	5	10	20	30	40
0	0	0	0	0	0
5	26.3 ± 11[a]	11.4 ± 6	3.3 ± 4	1.6 ± 1	0
10	97.2 ± 4	96.0 ± 5	50.0 ± 12	30.0 ±	7.0 ± 3
15	95.9 ± 5	96.0 ± 4	17.9 ± 10	0	0
20	85.6 ± 7	86.3 ± 14	27.0 ± 20	3.2 ± 2	0
25	97.8 ± 2	89.0 ± 3	15.1 ± 12	1.9 ± 2	0
30	98.8 ± 3	95.3 ± 7	34.0 ± 12	3.4 ± 2	0
35	99.2 ± 2	99.2 ± 2	29.8 ± 7	3.4 ± 3	0

(a) Values represent the means. Standard deviations have been rounded to the nearest whole number.

FIGURE 9. Percent fertilization of fresh ova fertilized with sperm exposed over the salinity range of 0-35$^{0}/_{00}$ at ambient temperature (four replicate tests).

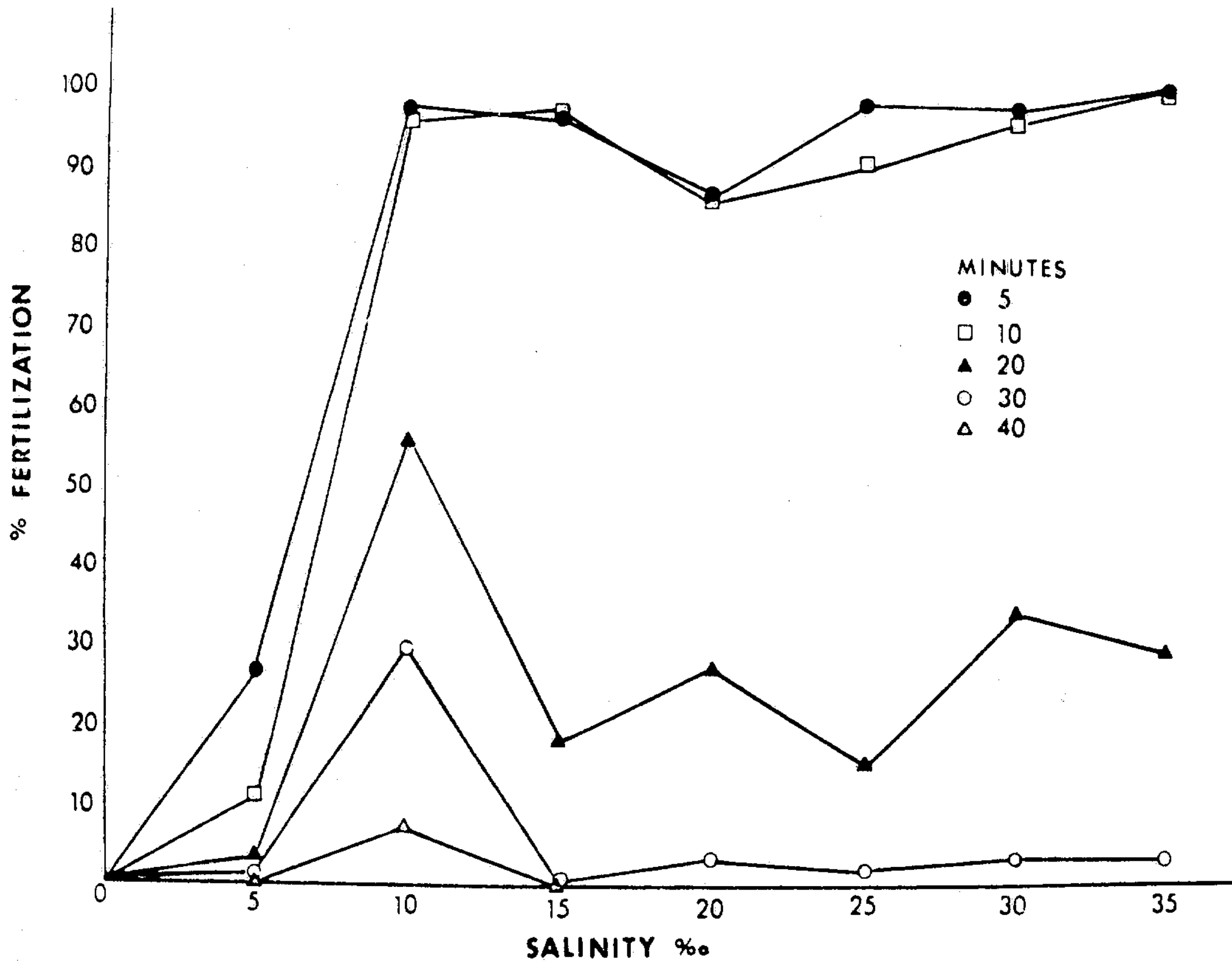

% FERTILIZATION
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
SALINITY ‰
MINUTES
5
10
20
30
40

Table 26 Percent fertilization of fresh ova fertilized with sperm exposed to 0-35 ^{o}/oo sea water at 2°C. The pH of sea water was adjusted with pH 10.00 buffer to 8.0-8.4.

SALINITY (^{o}/oo)	Exposure Time (Hours) 1	3	6
0	0	0	0
5	95.2 $\pm$ 8 (a)	12.3 $\pm$ 7	2.3 $\pm$ 2
10	92.6 $\pm$ 6	15.3 $\pm$ 7	3.7 $\pm$ 2
15	45.5 $\pm$ 13	2.8 $\pm$ 2	0
20	41.4 $\pm$ 8	0	0
25	27 $\pm$ 3	0	0
30	0	0	0
35	0	0	0

(a) Values represent the mean values. Standard deviations have been rounded to nearest whole number.

FIGURE 10. Percent fertilization of fresh ova fertilized with sperm exposed at 2C over the salinity range of 0-30$^0/_{00}$. pH of sea water adjusted to 8.0-8.4.

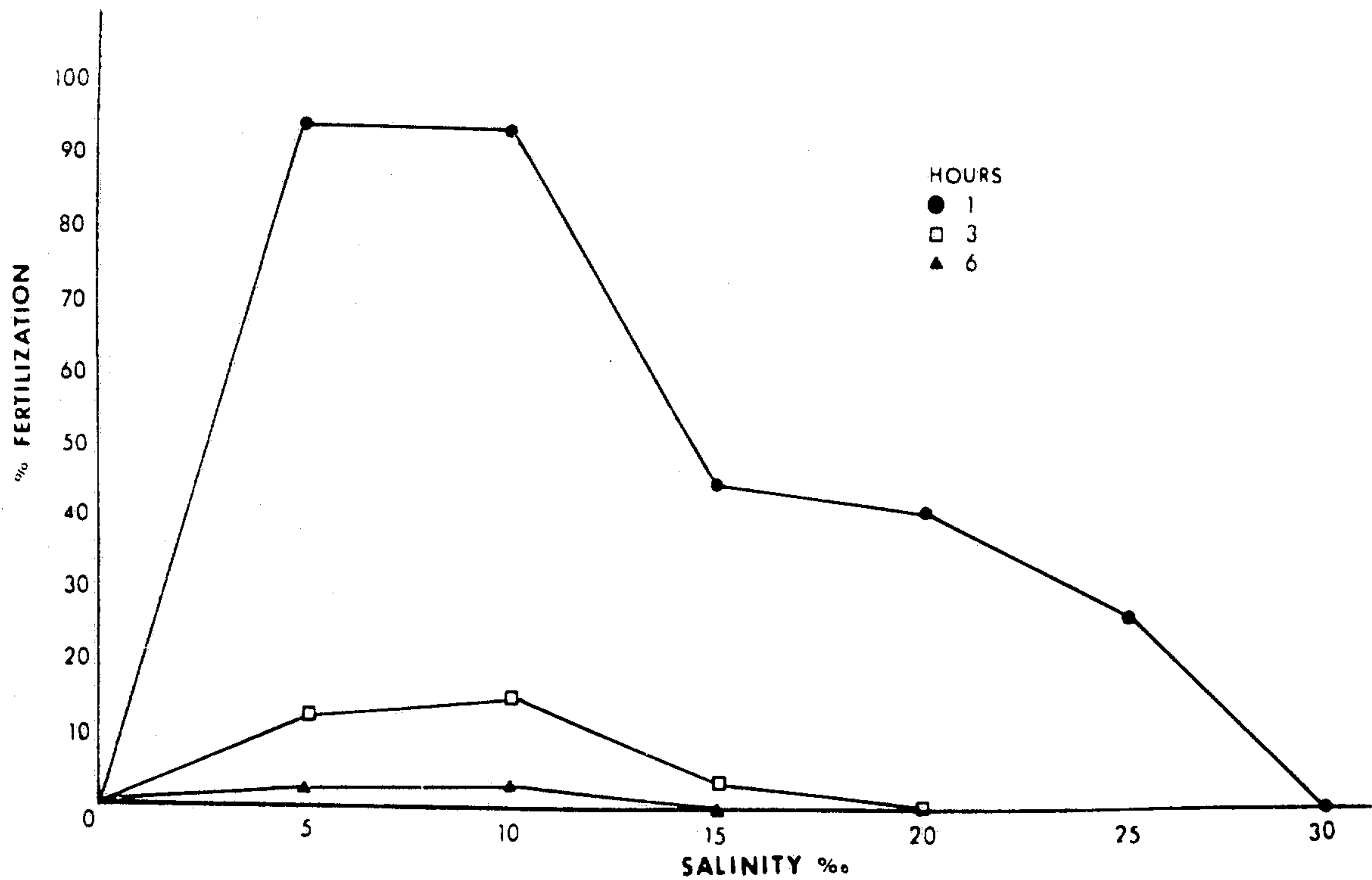

% FERTILIZATION
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
SALINITY ‰
HOURS
1
3
6

Table 27 Percentage abortion of embryos resulting from salinity exposed sperm maintained at 2^oC.

SALINITY o/oo	Exposure Time (Hours) 1	3	6
5	0	5(a)	0
10	1	0	0
15	0	6.7	-(b)
20	0	-	-
25	9.4	-	-

Table 28 Percentage abortion of embryos resulting from salinity exposed sperm maintained at ambient temperature, $22\text{-}25^o$.

SALINITY o/oo	Exposure Time (Minutes) 5	10	20	30	40
0	0	0	0	0	-(b)
10	0	2(a)	0	2.3	1.8
15	2	2	5	0	-
20	0	3	0	12.0	-
25	4	4.2	6.5	0	-
30	4.7	0	6.0	3.4	-
35	5.3	1.7	3.3	0	-

(a) Percentage represent both mortalities and embryos which exhibited structural deformities.

(b) Fertilization did not occur.

G. pH

Methods.

1. pH levels between 6.0 and 9.4 in $10^0/_{00}$ and $22^0/_{00}$ sea water were prepared as in Sect. II F.

2. Sperm:pH-adjusted sea water = 1:10.

3. Preservation tests were run at 25C and checked at 5, 10, 20, 30, 40 min.

4. Sperm were also incubated in pH-adjusted $10^0/_{00}$ sea water at 2C and checked at 1, 3, 6, 9 hr.

Results.

The role of pH and salinity at 25C on sperm preservation are shown in Tables 29 and 30 and summarized in Figs. 11 and 12, and at 2C in Tables 31 and 32 and Figs. 13 and 14. Mortality-abnormality rates are given in Tables 33 and 34.

H. Whole Testis Preservation.

Methods.

1. Males were sacrificed and the testes excised, placed dry in small plastic capsules and the capsules capped.

2. The capsules were placed in a water bath at temperatures from -4C to 10C at two degree increments, and stored for 24, 48, 72, 96 hr intervals before testing with fresh ova in $20^0/_{00}$ sea water.

3. Note: Large robust males with good mature reproductive coloration should be used.

Table 29 Percentage fertilization of fresh ova fertilized with sperm exposed over the pH range 6.0-9.5 at 10‰. Tests were conducted at ambient temperature (22-25°C).

pH	Exposure Time (Minutes) 10	20	30	40
6.0	50.8± 7.0(a)	40.5± 5	3.4± 4	0
6.5	91.2± 3	85.0± 14	6.4± 5	0
6.8	90.7± 11	83.0± 9	12.3± 7	0
7.0	96.7± 4	84.8± 6	42.5± 10	5.9± 3
7.4	89.4± 5	36.3± 5	5.4± 3	0
7.8	83.6± 6	15.4± 7	2.0± 2	0
8.2	86.5± 5	13.1± 5	0	0
8.6	47.3± 12	5.4± 2	0	0
9.0	33.7± 7	0	0	0
9.2	15.8± 3	0	0	0
9.5	0	0	0	0

(a) Standard deviations have been rounded to the nearest whole number.

C-2

Table 30 Percentage fertilization of fresh ova fertilized with sperm exposed to a pH range of 6.0-9.4 at 22 °/oo. Tests were conducted at ambient temperature (22-25 °C).

pH	Exposure Time (Minutes) 10	20	30	40
6.0	36.2 ± 8	27.8 ± 4	0	0
6.5	84.2 ± 4	60.8 ± 7	10.2 ± 5	0
6.8	89.1 ± 6	65.0 ± 14	9.0 ± 5	0
7.0	86.3 ± 9	68.7 ± 10	21.8 ± 8	0
7.4	72.0 ± 11	51.2 ± 9	12.1 ± 4	0
7.8	77.4 ± 9	29.4 ± 6	2.3 ± 2	0
8.2	62.1 ± 7	19.0 ± 9	5.1 ± 1	0
8.6	38.6 ± 7	17.1 ± 5	0	0
9.0	22.0 ± 4	0	0	
9.2	4.3 ± 2	0	0	
9.4	0	0	0	

(a) Standard deviations have been rounded to nearest whole number.

FIGURE 11. Percent fertilization of fresh ova fertilized with sperm held at $10^{0}/_{00}$ exposed to a pH range of 6.0-9.5. Tests were carried out at 22-25C.

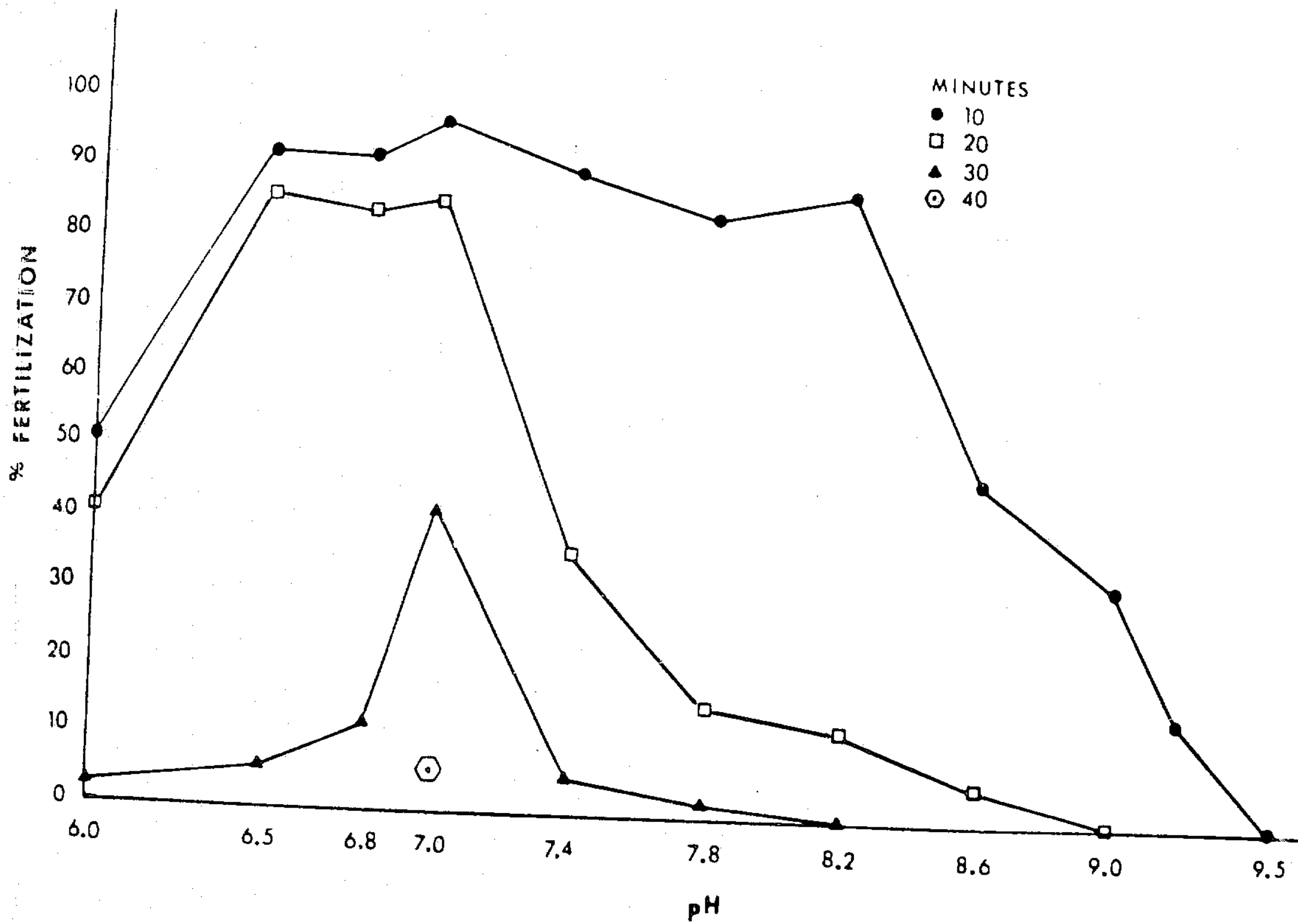

MINUTES
10
20
30
40
% FERTILIZATION
100
90
80
70
60
50
40
30
20
10
0
6.0
6.5
6.8
7.0
7.4
7.8
8.2
8.6
9.0
9.5
pH

FIGURE 12. Percent fertilization of fresh ova fertilized with sperm treated at 22 ‰ sea water over the pH range of 6.0-9.4, at 22-25C.

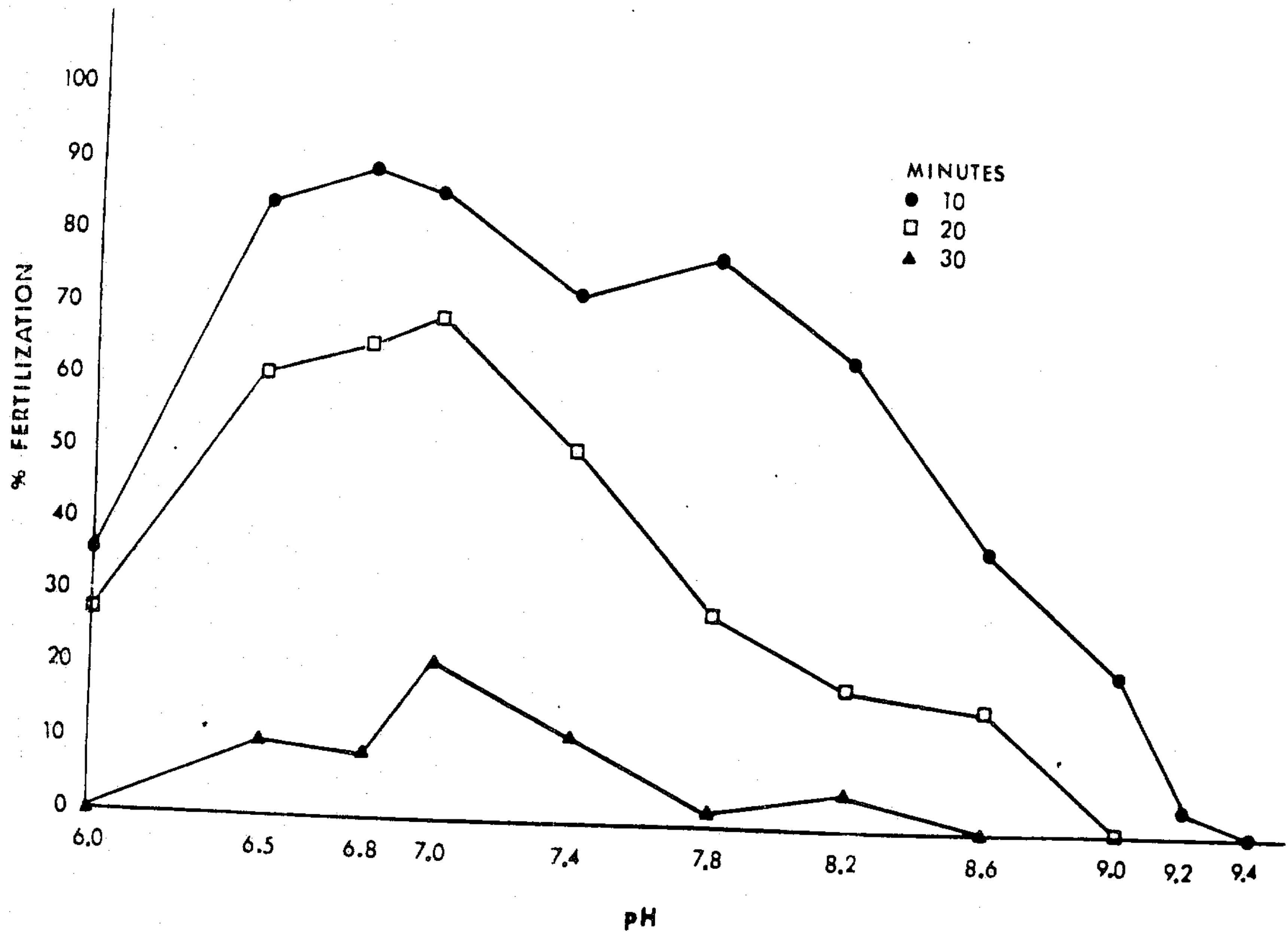

% FERTILIZATION
100
90
80
70
60
50
40
30
20
10
0
6.0
6.5
6.8
7.0
7.4
7.8
8.2
8.6
9.0
9.2
9.4
pH
MINUTES
10
20
30

Table 31 Percent fertilization of fresh ova fertilized with sperm exposed to a pH range of 6.0-9.5 at 2°C and 10°/oo sea water. Values represent the means.

Exposure Time (Hour)

pH	1	3	6	9	12
6.0	82.6± 7.6(a)	42.0± 7.2	6.7± 3.4	0	0
6.5	82.0± 9.4	69.7± 7.5	42.2± 11.4	4.3± 2.1	0
6.8	79.9± 7.8	40.5± 13.8	17.3± 7.3	4.0± 2.1	0
7.0	82.2± 9.6	65.7± 11.7	20.1± 6.3	0	0
7.4	50.2± 11.1	14.1± 5.0	0	0	0
7.8	19.4± 11.8	0	0	0	
8.2	23.8± 13.7	3.5± 1.1	0	0	
8.6	16.5± 5.1	0	0	0	
9.0	5.4± 2.3	0	0	0	
9.2	0	0	0	0	
9.5	0	0	0	0	

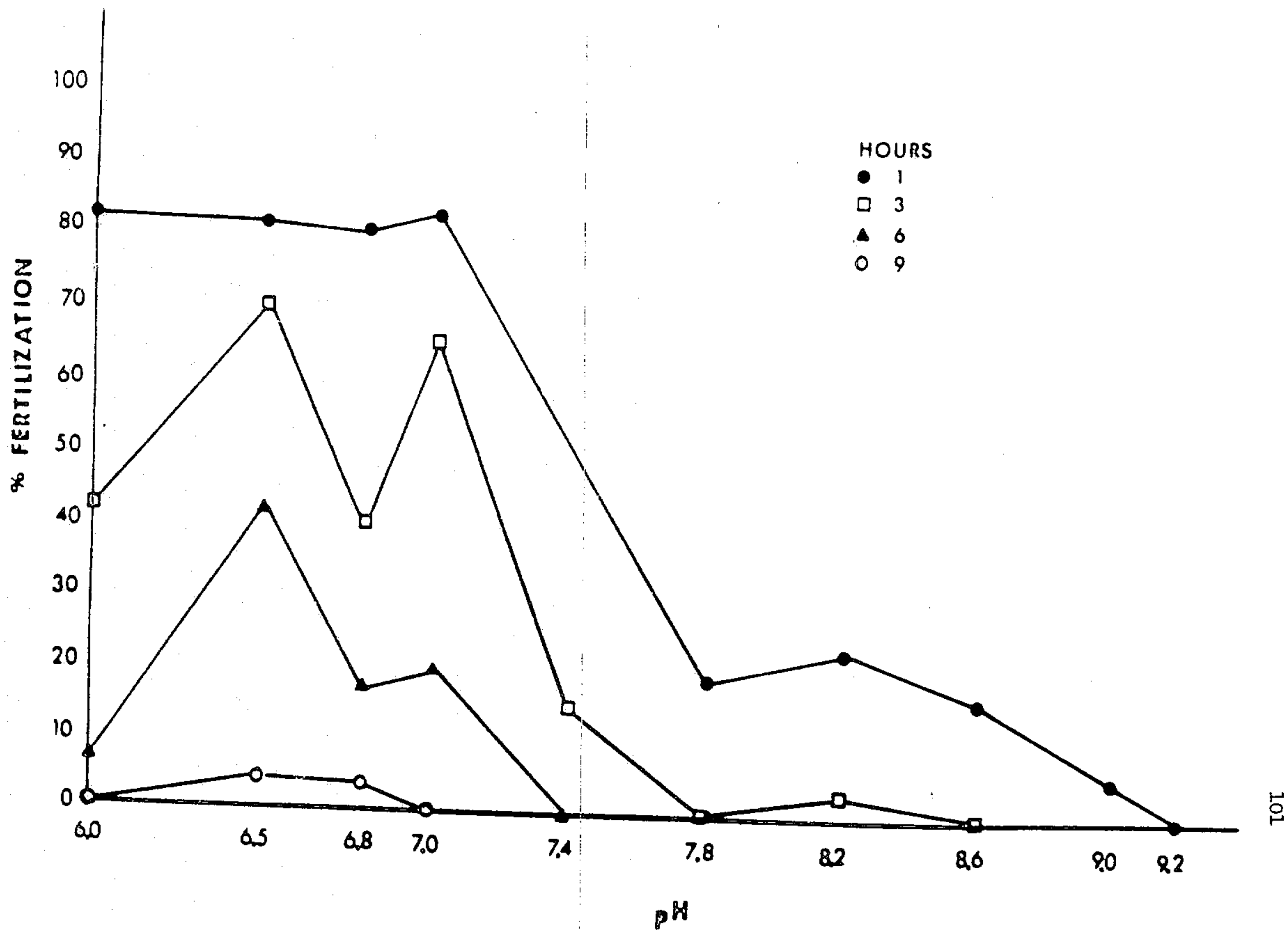
(a) Standard deviations have been rounded to nearest whole number.

Table 32 Percent fertilization of fresh ova fertilized with sperm exposed to a pH range of 6.0-9.4 at 22 °/oo sea water and 28°C. Values represent the means.

pH	Exposure Time (Hours) 1	3	6	9
6.0	69.3± 4 (a)	0	0	0
6.5	91.3± 8	20.4± 10	4.2± 2	0
6.8	93.1± 7	23.5± 11	2.3± 2	0
7.0	95.4± 5	16.1± 8	1.5± 1	0
7.4	95.2± 3	27.0± 9	3.7± 2	0
7.8	28.1± 7	2.1± 1	0	0
8.2	18.3± 5	4.1± 2	0	0
8.6	10.4± 5	1.8± 1	0	0
9.0	13.2± 4	0	0	0
9.2	0	0	0	0
9.4	0	0	0	0

(a) Standard deviations have been rounded to nearest whole number.

FIGURE 13. Percent fertilization of fresh ova fertilized by sperm exposed to $10^{0}/_{00}$ at 2C over the pH range 6.0-9.2.



% FERTILIZATION
100
90
80
70
60
50
40
30
20
10
0
HOURS
1
3
6
9
6.0
6.5
6.8
7.0
7.4
7.8
8.2
8.6
9.0
9.2
pH

FIGURE 14. Percent fertilization of fresh ova fertilized by sperm exposed to $22^{0}/_{00}$ at 2C over the pH range 6.0-9.2.

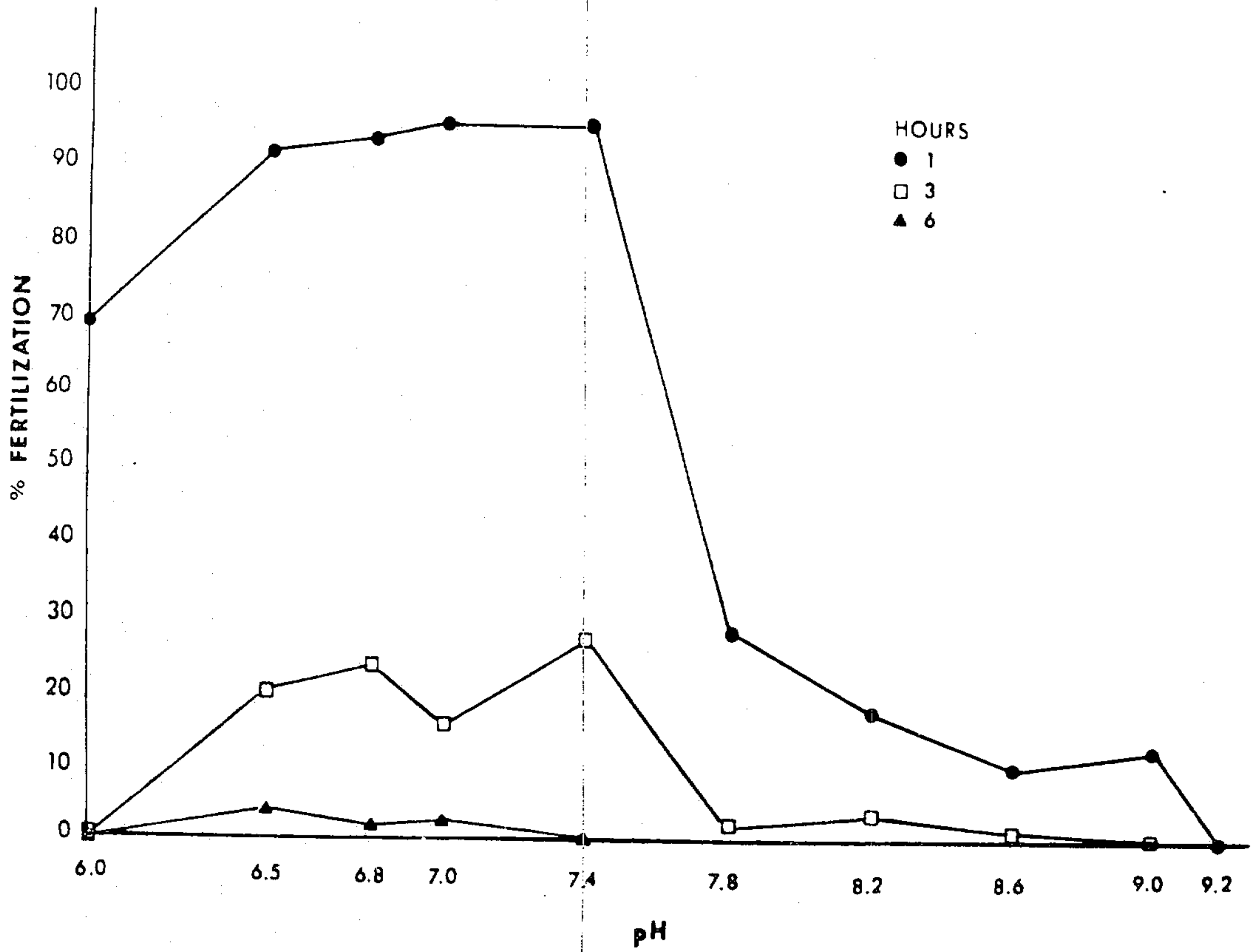

100
90
80
70
60
50
40
30
20
10
0
% FERTILIZATION
HOURS
1
3
6
6.0
6.5
6.8
7.0
7.4
7.8
8.2
8.6
9.0
9.2
pH

Table 33 Percent abortion of embryos from pH-treated sperm.
A. Sperm exposed to 10o/oo sea water at 22-25°C.
B. Sperm exposed to 22o/oo sea water at 22-25°C.

Exposure Time (Minutes)

	pH	10	20	30	40
A.	6.0	0	0	0	-(b)
	6.5	0.6(a)	2.4	4.7	-
	6.8	0	3.5	9.0	-
	7.0	0	0	5.2	0
	7.4	0	0	0	-
	7.8	3.5	15.0	0	-
	8.2	4.7	8.9	0	-
	8.6	5.7	13.0	-	-
	9.0	20.3	-	-	-
	9.2	40.7	-	-	-
B.	6.0	8.3	5.3	0	-(b)
	6.5	0	5.7	0	-
	6.8	7.4	4.5	10.0	-
	7.0	3.7	3.0	0	-
	7.4	4.0	0	0	-
	7.8	0	5.3	0	-
	8.2	4.3	2.1	0	-
	8.6	0	9.7	-	-
	9.0	20.0	-	-	-
	9.2	24.3			

(a) Values represent total mortalities and embryos exhibiting structural deformaties.

(b) Fertilization did not occur.

Table 34 Percentage abortion of embryos resulting from pH-treated sperm.

A. Sperm exposed to 10‰ sea water at 2°C.
B. Sperm exposed to 22‰ sea water at 2°C.

Exposure Time (Hours)

A.

pH	1	3	6	9
6.0	5.1 (a)	0	8.5	- (b)
6.5	4.0	0	3.7	10
6.8	5.0	0	7.6	0
7.0	0	0	0	-
7.4	8.3	0	-	-
7.8	0	-	-	-
8.2	0	0	-	-
8.6	0	-	-	-
9.0	0	-	-	-

B.

pH	1	3	6	9
6.0	0	- (b)	-	-
6.5	3 (a)	7	0	-
6.8	0	5.3	0	-
7.0	0	3.8	0	-
7.4	6	9	0	-
7.8	5.5	0	-	-
8.2	0	0	-	-
8.6	0	0	-	-
9.0	0	-	-	-
9.5	-	-	-	-

(a) Values include total mortalities and embryos with structural deformities.
(b) No fertilization recorded.

Results.

Table 35 : Percent fertility of sperm from whole testis preservation.

Time	10C	8C	6C	4C	2C	OC	-2C	-4C
24	86	85	96	90	90	98	92	95
48	96	30	91	89	*	99	99	99
72	4	7	95	60	93**	96	99	46
96	0	19	88	70	72	88	83	26
Total # ova	364	247	661	389	373	467	490	553
# fertilized	241	95	610	297	327	445	466	380
% abnormal	3	3	15	6	2	3	7	33

* not attempted

** average of 3 replicates

IV. DISCUSSION OF OVA AND SPERM PRESERVATION TESTS.

A. Ova Preservation

Best results for ova preservation can be obtained by "dry" techniques, using only the small amounts of ovarian fluid that accompanies the ova upon stripping to keep the ova moist. Containers containing the ova should be sealable and watertight to prevent both dessication or entry of extraneous fluids. Ideally, the container should be completely filled with ova, with minimum air-ova interface, again to prevent dessication. A gas permeable material would probably be of advantage, since the exchange of O_2 and CO_2 would be allowed.

The temperature of storage is also fairly critical. In the experiments performed, the range 10-14 C proved to be optimum. In this range ova retained fertilizability of up to 67% after 72 hrs., with recorded fertility up to 168 hrs at 12C. Refinement of technique could probably enhance these values. Abnormality and abortion rates were also minimal within this temperature range.

Techniques using other than dry preservation such as salines, pH-adjusted media, sugar solutions, etc. generally gave unsatisfactory results in terms of long time storage and preservation of fertilizability. Ova tended to water harden, become activated, or perhaps die after exposure to any kind of aqueous medium. Non-aqueous media were not tested, however.

B. Sperm Preservation

Sperm preservation proved to be more difficult since the process of obtaining the sperm, i.e. stripping, also initiated activity and motility in the sperm. Under normal circumstances the motility is short lived, of the order of minutes. Once the sperm lost their motility they would be unable to effect fertilization. Again, all aqueous media tested proved unsatisfactory for preservation, even those which included some ATP as an energy source.

The best results for stripped sperm preservation were obtained using dry storage, i.e. only the sperm and accompanying spermatic fluids, and low temperatures, the latter acting to slow or inhibit the motility. Temperatures below 6C gave good results, with the best preservation at approx. 2C. The preservation time, however, was only of the order of a few hours, e.g. 13% fertility after 12 hrs, rather than days as with the ova. Hence, preservation of stripped sperm was judged to be unfeasable with respect to the objectives of this study.

Hence it was decided to leave the sperm intact, within the testes, and preserve the testes themselves. This would prevent the activation of the sperm caused by stripping. Excellent results were obtained by preserving whole, excised testes, from robust, mature males. Sperm retained fertilizability of over 90% when stored in the temperature range of 0-4C for 72 hrs, and fertility was still in excess of 70% after 96 hrs.

Thus, whole testis preservation at low temperature is regarded

as the best procedure for maintaining sperm fertility. Abnormality and abortion rates of the resulting embryos were also very minimal using this procedure.

V. EMBRYO PRESERVATION TESTS.

All tests thus far have dealt with the preservation of individual gametes separately. The following tests study the possibility of using temperature to arrest development of already fertilized zygotes. This would provide an alternative method of studying embryogenesis by delaying the onset of embryogenesis until the desired time.

Methods.

1. Large batches of at least 500 fresh ova were fertilized with fresh sperm.
2. The resulting zygotes were immediately divided into 11 equal lots in cappable test tubes, one lot to act as a control group and the other 10 to act as test groups.
3. The experimental lots were designated as follows:

   a) freshly fertilized

   b) germinal disc stage

   c) 2-cell stage

   d) 4-cell stage

   4) 64-cell stage

   f) 10 hour post fertilization

   g) 12 " " "

   h) 18 " " "

   i) 24 " " "

   j) 48 " " "

4. Each lot was allowed to develop at 25C until it reached its designated stage.

5. Upon reaching the designated stage a given lot was placed in a water bath at the test temperature, ranging from 2-12C at 2 degree intervals.

6. At 24, 48, 72 hr intervals 1/3 of the embryos in each tube were removed along with 5 ml of test water, placed in a Petri dish allowed to reach 25C, and monitored for subsequent embryonic development, abortions, abnormalities, and hatching.

Results.

(see Tables 36-41)

Table 36 : Effect of storage at 2C on the embryogenesis of *F. heteroclitus* zygotes.

	25C	2 C - 24 HR									
	CONTROL	FF[1]	GD[2]	2C	4C	64C	10 hr	12 hr	18 hr	24 hr	48 hr
total # zygotes	20	46	30	30	31	30	50	50	52	30	26
% initial mortality	5	100	97	97	100	63	6	8	6	20	42
% total mortality[3]	15	100	97	97	100	70	22	22	23	30	54
% abnormality	0	-	3	0	-	10	12	4	2	7	0
% hatching	85	-	0	3	-	20	66	74	75	63	46

	25C	2 C - 48 HR									
total # zygotes		50	30	30	30	30	49	50	50	29	25
% initial mortality		100	100	100	100	90	53	76	50	48	52
% total mortality		100	100	100	100	94	76	86	72	70	72
% abnormality		-	-	-	-	3	2	0	2	3	0
% hatching		-	-	-	-	3	22	14	26	27	28

	25C	2 C - 72 HR									
total # zygotes		50	30	31	30	32	56	50	47	31	23
% initial mortality		100	100	100	100	100	93	94	98	74	61
% total mortality		100	100	100	100	100	93	94	98	78	65
% abnormality		-	-	-	-	-	0	4	0	3	5
% hatching		-	-	-	-	-	7	2	2	19	30

[1] Fresh Fertilized

[2] Germinal Disc

[3] First 48 hrs after removal from water bath.

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Table 37 : Effect of storage at 4C on the embryogenesis of *F. heteroclitus* zygotes.

	25C	4 C – 24 HR									
	CONTROL	FF[1]	GD[2]	2C	4C	64C	10 hr	12 hr	18 hr	24 hr	48 hr
total # zygotes	104	30	23	24	24	25	49	50	40	28	24
% initial mortality[3]	3	33	61	71	71	36	12	4	40	4	13
% total mortality	48	53	74	96	75	56	39	32	60	32	33
% abnormality	7	17	9	0	21	16	2	6	35	0	17
% hatching	45	30	17	4	4	28	59	62	5	68	50

	25C	4 C – 48 HR									
total # zygotes		29	25	25	25	25	29	49	51	24	25
% initial mortality		86	92	100	100	88	72	59	27	42	16
% total mortality		90	92	100	100	88	79	73	55	63	48
% abnormality		3	4	–	–	12	7	0	0	0	0
% hatching		7	4	–	–	0	14	27	45	37	52

	25C	4 C – 72 HR									
total # zygotes		28	28	27	25	25	30	50	73	21	24
% initial mortality		82	100	100	100	100	97	88	85	81	50
% total mortality		89	100	100	100	100	97	90	92	90	67
% abnormality		0	–	–	–	–	0	4	0	0	0
% hatching		11	–	–	–	–	3	6	8	10	33

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Table 38: Effect of storage at 6C on the embryogenesis of *F. heteroclitus* zygotes.

	25C	6 C - 24 HR									
	CONTROL	FF[1]	GD[2]	2C	4C	64C	10 hr	12 hr	18 hr	24 hr	48 hr
total # zygotes	168	52	31	31	30	29	38	39	41	30	37
% initial mortality	0	29	94	97	83	55	3	18	2	7	14
% total mortality	21	37	94	97	83	55	11	28	12	13	16
% abnormality	10	7	0	0	0	14	2	3	8	7	14
% hatching	69	56	6	3	17	31	87	69	80	80	70

	25C	6 C - 48 HR									
total # zygotes		51	31	30	30	31	31	39	38	29	29
% initial mortality		82	100	100	97	54	35	8	8	45	17
% total mortality		92	100	100	100	58	48	21	16	48	28
% abnormality		2	-	-	-	19	4	0	0	0	0
% hatching		6	-	-	-	23	48	79	84	52	72

	25C	6 C - 72 HR									
total # zygotes		46	29	28	31	31	34	42	41	29	26
% initial mortality		85	100	100	100	84	71	36	5	55	19
% total mortality		91	100	100	100	94	88	62	61	66	31
% abnormality		9	-	-	-	6	12	0	2	3	4
% hatching		0	-	-	-	0	0	38	37	31	65

Table 39 : Effect of storage at 8C on the embryogenesis of *F. heteroclitus* zygotes.

	25C	8 C - 24 HR									
	CONTROL	FF[1]	GD[2]	2C	4C	64C	10 hr	12 hr	18 hr	24 hr	48 hr
total # zygotes	56	26	49	51	50	49	34	52	21	20	19
% initial mortality	0	19	43	35	38	4	0	2	10	5	0
% total mortality	36	73	90	82	76	92	79	31	100	80	100
% abnormality	0	19	0	14	8	8	0	0	-	0	-
% hatching	64	8	10	4	16	0	21	69	-	20	-

	25C	8 C - 48 HR									
total # zygotes		48	49	36	51	48	36	51	25	20	21
% initial mortality		17	84	33	100	13	3	4	8	5	19
% total mortality		85	92	92	100	88	28	27	84	85	67
% abnormality		7	4	2	-	8	0	0	0	5	0
% hatching		8	4	6	-	4	72	73	16	10	33

	25C	8 C -72 HR									
total # zygotes		54	50	43	47	56	40	48	28	20	18
% initial mortality		100	100	100	100	48	5	8	21	15	50
% total mortality		100	100	100	100	77	60	52	78	35	67
% abnormality		-	-	-	-	11	2	0	2	0	0
% hatching		-	-	-	-	12	38	48	20	65	33

Table 40 : Effect of storage at 10C on the embryogenesis of *F. heteroclitus* zygotes.

	25C	10 C - 24 HR									
	CONTROL	FF[1]	GD[2]	2C	4C	64C	10 hr	12 hr	18 hr	24 hr	48 hr
total # zygotes	63	16	15	16	15	10	49	51	54	10	10
% initial mortality	25	25	53	50	60	10	6	2	2	0	0
% total mortality	51	38	60	63	67	60	55	34	50	20	10
% abnormality	9	6	0	0	0	0	0	4	2	0	0
% hatching	40	56	40	37	33	40	45	62	48	80	90

	25C	10 C - 48 HR									
total # zygotes		16	15	15	15	10	45	53	52	10	10
% initial mortality		31	87	73	80	40	9	4	10	0	10
% total mortality		38	87	80	80	60	67	57	71	20	50
% abnormality		6	0	0	0	0	2	0	0	0	0
% hatching		56	13	20	20	40	31	43	29	80	50

	25C	10 C -72 HR									
total # zygotes		16	18	18	15	10	55	51	50	10	10
% initial mortality		56	77	83	93	40	4	8	10	0	0
% total mortality		80	77	83	93	50	24	33	34	30	0
% abnormality		2	5	0	0	20	16	0	2	0	0
% hatching		18	18	17	7	30	60	67	64	70	100

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Table 41: Effect of storage at 12C on the embryogenesis of *F. heteroclitus* zygotes.

	25C	12 C - 24 HR									
	CONTROL	FF[1]	GD[2]	2C	4C	64C	10 hr	12 hr	18 hr	24 hr	48 hr
total # zygotes	76	30	21	20	20	25	40	40	37	24	20
% initial mortality	3	13	24	60	30	0	3	5	19	0	10
% total mortality	8	17	24	60	100*	8	8	8	22	4	10
% abnormality	0	3	0	0	0	0	7	5	5	0	5
% hatching	92	80	76	40	0	92	85	88	73	96	85

	25C	12 C - 48 HR									
	CONTROL	FF[1]	GD[2]	2C	4C	64C	10 hr	12 hr	18 hr	24 hr	48 hr
total # zygotes		26	20	20	17	20	39	41	38	20	28
% initial mortality		19	45	50	47	5	21	7	11	0	32
% total mortality		23	45	50	71	5	23	10	13	0	32
% abnormality		0	0	0	0	15	5	7	3	5	0
% hatching		77	55	50	29	80	72	83	84	95	68

	25C	12 C - 72 HR									
	CONTROL	FF[1]	GD[2]	2C	4C	64C	10 hr	12 hr	18 hr	24 hr	48 hr
total # zygotes		32	19	19	20	30	30	34	36	31	27
% initial mortality		34	63	47	65	10	20	24	39	3	4
% total mortality		34	63	47	70	10	20	24	39	6	4
% abnormality		3	5	0	0	20	3	3	8	0	4
% hatching		63	32	53	30	70	77	73	53	94	92

* Dish killed off by fungus infection.

VI. ASTP VISUAL ORIENTATION TESTS

A. BIBLIOGRAPHY OF PERTINENT FISH VISION LITERATURE

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B. Protocol of Visual Acclimation Procedures

Introduction

This experiment was designed to investigate the relative roles of the vestibular and visual systems in orientation and swimming behavior under zero gravity. Hence, it was decided to rear freshly hatched fry under a predetermined visual parameter, i.e. acclimate the fry to a given visual background. Prior to the ASTP flight, the fry would be packaged in plastic bags with one side containing the background to which they were acclimated. The purpose of the experiment was: a) determine if the fry could use a known visual cue to enable them to orient in the absence of gravitational cues, b) to determine if fry reared with a definite visual background would adapt to zero gravity more rapidly than control or blinded fry.

Methods

1. Large batches of ova were freshly fertilized, incubated at 22C for 21 days and then mechanically disturbed to obtain maximum simultaneous hatching on day 21.

2. The hatchees were divided into 5 equal lots and each lot placed in a separate 10 gal tank, for acclimation to a different visual background.

3. The fry were acclimated to the following visual parameters.

a) control group - no special visual background

b) horizontal bar background on sides of tank - 1/4" black and white bars

c) vertical bar background on sides of tank - 1/4" black and white bars

d) black overhead background - roof and top half of sides painted black with light from below

e) blinded fish.

4. A new group of fry were hatched every 2 weeks, until ultimately a series of 5 tanks for each condition in (3) above were established.

5. By ASTP liftoff time - fry ranging in age from 3-11 weeks, in 2 week intervals, were available.

6. It was decided that the 3 week old fry were the ideal size for packaging and use on the ASTP mission.

7. Fry were placed in large plastic bags with 500 ml sea water with one side of the bag painted with the acclimatization background for that group. The bags were placed in a light tight container and transported to NASA, Houston, for packaging.

8. ASTP E.I.P. stated that the final package had 5 compartments, each with 6 fry, and that the compartments were designated as follows:

Compartment 1 - Normal controls

Compartment 2 - Vertical bar acclimated

Compartment 3 - Horizontal bar acclimated

Compartment 4 - Black overhead acclimated

Compartment 5 - Blinded

C. Protocol for Film Analysis

1. A Vanguard Analyser was used to facilitate a detailed analysis of the position and locomotor activity of each fish.

2. The duration of time in seconds and the number of frames a given fish was visible for each day's filming sequence were determined, according to the following information provided by NASA:

Flight Film - Sequences in Order Seen

Roll CI 28

a) Day 6 - fish filmed at 24 frames per second

b) Day 7 - fish filmed at 24 frames per second

Roll CI 29

c) Day 2 - fish lost because of improper setting of film magazine

d) Day 8 - fish filmed at 12 frames per second

e) Day 9 - fish filmed at 12 frames per second

3. The position of each fish was drawn, using tracing paper, at least every 6 frames (i.e. every 1/4-1/2 sec). Thus, a frame-by-frame diagram for each fish was established, showing the orientation of that fish relative to its original starting point and its position 1/4-1/2 sec previously.

a) The numbers within the fish body outline represent a sequential 6 frame series, starting with 0, each number thereafter representing a 6 frame step.

b) Occasionally intermediate frame positions are also drawn, for particularly active fish and these are designated by a subscript

e.g. 5_0, 5_1, 5_2, 5_3, 5_4, 5_5, etc., so that each interval could be divided into 6 subintervals if necessary.

c) Only position changes are noted. If a fish does not move in a given 6 frame interval, the number is not recorded in the body position outline, but is recorded in the margin next to (rather than below) the previous number.

4. The frame-by-frame diagrams were used to construct summary diagrams for each fish on each day. Each summary diagram shows:

a) the number of frames the fish was visible

b) the time the fish was visible

c) the starting or 0 point, i.e. the position of the fish as it first became visible

d) the pathway and direction of swimming activity of the fish, as indicated by arrows.

D. Results of ASTP Film Analysis

1. Film from Day 2 was missing. Unfortunately this was probably the most critical day since this was the earliest possible time that fish could be monitored in what was probably their most active and aggravated state. All subsequent days were to be compared to Day 2 from the standpoint of

   a) relative quality (looping vs. orientated) and quantity of activity

   b) comparison of the effectiveness of the various visual parameters in orientation and adaptation to zero gravity.

2. Analysis of Days 6, 7, 8, 9 gave the following results.

   a) Tables 42-45 represent an analysis of each day representing the conditions in each compartment on the given day.

   b) Table 46 analyses each compartment and compares certain behavioral parameters over the 4 day test period.

   c) Tables 47-66 analyzes the activity of each fish in each compartment for each day's filming. It should be noted, however, that the number designations given to each fish, i.e. fish #1, fish #2, etc., are completely arbitrary from day to day, since it is virtually impossible to actually identify individuals.

   d) Table 67 compares the total amount of looping behavior for each day of the test in all compartments combined. If anything, there was an increase in looping with increasing time, since the smallest amount of looping occurred on Day 6.

Table 42: Summary of Conditions on Day 6.

	COMPARTMENT				
	1	2	3	4	5
Number of fish visible	5	5	5	5	6
Number of fish alive	5	5	5	5	5
Total mortality	1	1	1	1	1
Number of fish looping spontaneously	0	0	1	1	1
Number of fish looping after disturbance	2	0	0	0	2
Total # of fish looping	2	0	1	1	3
Back always to camera	4	4	2	2	5[3]
Belly to Camera	1[1]	1	3[1]	1[2]+2[1]	0
Time Filmed (sec.)	13	10-25	33	19	29

[1]intermittently

[2]entirely

[3]difficult to tell because of black ventral coloration of blinded animals.

Table 43: Summary of Conditions on Day 7.

	COMPARTMENT				
	1	2	3	4	5
Number of fish visible	5	5	5	5	6
Number of fish alive	5	5	5	5	4
Total mortality	1	1	1	1	2
Number of fish looping spontaneously	2	1	2	1	4
Number of fish looping after distrubance	0	0	2	1	4
Total # of fish looping	2	1	2	1	4
Back always to camera	5	3	5	1	4
Belly to camera	0	1+1[1]	0	4[1]	0
Time filmed (sec.)	9	22	23	24	30

Table 44: Summary of Conditions on Day 8.

	COMPARTMENT				
	1	2	3	4	5
Number of fish visible	5	5	5	3	6
Number of fish alive	5	5	5	2	4
Total mortality	1	1	1	4	2
Number of fish looping spontaneously	0	1	0	0	0
Number of fish looping after distrubance	3	2	0	0	3
Total # of fish looping	3	3	0	0	3
Back always to camera	5	5	3	2	4
Belly to camera	0	0	1+1[1]	0	0
Time filmed (sec.)	9	17	17	18	19

Table 45: Summary of Conditions on Day 9.

	COMPARTMENT				
	1	2	3	4	5
Number of fish visible	5	5	5	4	6
Number of fish alive	5	5	5	2	4
Total mortality	1	1	1	4	2
Number of fish looping spontaneously	0	0	0	0	0
Number of fish looping after distrubance	3	4	2	0	1
Total # of fish looping	3	4	2	0	1
Back always to camera	5	4	3	2	4
Belly to camera	0	1[1]	1+1[1]	0	0
Time filmed (sec.)	10	22	18	21	24

Time Looping

Table 46: Compartment by Compartment Comparison of Activity of ASTP Fish.

A. Compartment 1	DAY 6	DAY 7	DAY 8	DAY 9
Number of fish living	5	5	5	5
Number of fish looping	2	2	3	3
# of fish with belly to camera	1	0	0	0
# of fish with back to camera	4	5	5	5

B. Compartment 2	6	7	8	9
Number of fish living	5	5	5	5
Number of fish looping	0	1	3	4
# of fish with belly to camera	1	2	0	1
# of fish with back to camera	4	3	5	4

C. Compartment 3	6	7	8	9
Number of fish living	5	5	5	5
Number of fish looping	1	2	0	2
# of fish with belly to camera	3	0	2	1
# of fish with back to camera	2	5	3	4

D. Compartment 4	6	7	8	9
Number of fish living	5	5	2	2
Number of fish looping	1	1	0	0
# of fish with belly to camera	3	4	0	0
# of fish with back to camera	2	1	2	2

E. Compartment 5	6	7	8	9
Number of fish living	5	4	4	4
Number of fish looping	3	4	3	1
# of fish with belly to camera	0	0	0	0
# of fish with back to camera	5	4	4	4

Table 47: Fish-by-Fish Analysis of Behavior of Fish in Compartment 1, Day 6.

DAY 6 - COMPARTMENT 1						
FISH	1	2	3	4	5	6
Time visible (sec.)	13	13	13	13	9	
Total time looping (sec.)	0	0	3.5	3	0	
Percent time looping	-	-	27	23	-	
Number of loops	-	-	8	6	-	
Number of loops/sec.	-	-	2.3	2	-	
Looping to left	-	-	yes	no	-	
Looping to right	-	-	yes	yes	-	

Table 48: Fish-by-Fish Analysis of Behavior of Fish in Compartment 2, Day 6.

DAY 6 - COMPARTMENT 2						
FISH	1	2	3	4	5	6
Time visible (sec.)	25	25	25	25	10	
Total time looping (sec.)	0	0	0	0	0	
Percent time looping	-	-	-	-	-	
Number of loops	-	-	-	-	-	
Number of loops/sec.	-	-	-	-	-	
Looping to left	-	-	-	-	-	
Looping to right	-	-	-	-	-	

Table 49: Fish-by-Fish Analysis of Behavior of Fish in Compartment 3, Day 6.

DAY 6 - COMPARTMENT 3						
FISH	1	2	3	4	5	6
Time visible (sec.)	41	32	31	33	33	
Total time looping (sec.)	0	0	23.6	0	0	
Percent time looping	-	-	76	-	-	
Number of loops	-	-	8	-	-	
Number of loops/sec.	-	-	0.3	-	-	
Looping to left	-	-	yes	-	-	
Looping to right	-	-	no	-	-	

Table 50 : Fish-by-Fish Analysis of Behavior of Fish in Compartment 4, Day 6.

DAY 6 - COMPARTMENT 4						
FISH	1	2	3	4	5	6
Time visible (sec.)	19	19	19	17	19	
Total time looping (sec.)	0	0	4.75	0	0	
Percent time looping	-	-	25	-	-	
Number of loops			2			
Number of loops/sec.	-	-	0.4	-	-	
Looping to left	-	-	no	-	-	
Looping to right	-	-	yes	-	-	

Table 51: Fish-by-Fish Analysis of Behavior of Fish in Compartment 5, Day 6.

DAY 6 - COMPARTMENT 5						
FISH	1	2	3	4	5	6
Time visible (sec.)	29	19[1]	29	29	29	29
Total time looping (sec.)	0	0	2.5	0	9.75	7
Percent time looping	-	-	9	-	34	24
Number of loops	-	-	6	-	11	10
Number of loops/sec.	-	-	2.4	-	1.1	1.4
Looping to left	-	-	yes	-	yes	yes
Looping to right	-	-	yes	-	yes	yes

[1]dead

Table 52: Fish-by-Fish Analysis of Behavior of Fish in Compartment 1, Day 7.

DAY 7 - COMPARTMENT 1						
FISH	1	2	3	4	5	6
Time visible (sec.)	9	9	9	9	3	
Total time looping (sec.)	0	9	0	0	3	
Percent time looping	-	100	-	-	100	
Number of loops	-	8	-	-	5[1]	
Number of loops/sec.	-	0.9	-	-	1.7	
Looping to left	yes		-	-	no	
Looping to right	no		-	-	yes	

[1] estimate only - fish last in glare part of time

Table 53: Fish-by-Fish Analysis of Behavior of Fish in Compartment 2, Day 7.

DAY 7 - COMPARTMENT 2						
FISH	1	2	3	4	5	6
Time visible (sec.)	22	22	4	22	10	
Total time looping (sec.)	0	0	4	0	0	
Percent time looping	-	-	100	-	-	
Number of loops	-	-	6	-	-	
Number of loops/sec.	-	-	1.5	-	-	
Looping to left	-	-	yes	-	-	
Looping to right	-	-	no	-	-	

Table 54 : Fish-by-Fish Analysis of Behavior of Fish in Compartment 3, Day 7.

DAY 7 - COMPARTMENT 3						
FISH	1	2	3	4	5	6
Time visible (sec.)	23	24	23	23	23	
Total time looping (sec.)	0	4.8	23	0	0	
Percent time looping	-	20	100	-	-	
Number of loops	-	5	30	-	-	
Number of loops/sec.	-	1.0	1.3			
Looping to left	-	yes	yes	-	-	
Looping to right	-	yes	yes	-	-	

Table 55: Fish-by-Fish Analysis of Behavior of Fish in Compartment 4, Day 7.

DAY 7 - COMPARTMENT 4						
FISH	1	2	3	4	5	6
Time visible (sec.)	24	24	24	15	24	
Total time looping (sec.)	0	0	0	0	8.25	
Percent time looping	-	-	-	-	34	
Number of loops	-	-	-	-	6	
Number of loops/sec.	-	-	-	-	0.7	
Looping to left	-	-	-	-	no	
Looping to right	-	-	-	-	yes	

Table 56: Fish-by-Fish Analysis of Behavior of Fish in Compartment 5, Day 7.

DAY 7 - COMPARTMENT 5						
FISH	1	2	3	4	5	6
Time visible (sec.)	30	30	30	30	30	13
Total time looping (sec.)	0[1]	19.75	25.5	8.75	22.25	0[1]
Percent time looping	-	66	85	29	74	-
Number of loops	-	27	24	19	14	-
Number of loops/sec.	-	1.4	0.9	2.1	0.6	
Looping to left	-	yes	yes	yes	yes	-
Looping to right	-	yes	yes	yes	yes	-

[1]fish is dead

Table 57: Fish-by-Fish Analysis of Behavior of Fish in Compartment 1, Day 8.

DAY 8 - COMPARTMENT 1						
FISH	1	2	3	4	5	6
Time visible (sec.)	9	9	9	9	9	
Total time looping (sec.)	7.5	0	45	7.5	0	
Percent time looping	83	-	50	83	-	
Number of loops	6	-	3	4	-	
Number of loops/sec.	0.8	-	0.7	0.5	-	
Looping to left	yes	-	yes	no	-	
Looping to right	yes	-	yes	yes	-	

Table 58: Fish-by-Fish Analysis of Behavior of Fish in Compartment 2, Day 8.

DAY 8 - COMPARTMENT 2						
FISH	1	2	3	4	5	6
Time visible (sec.)	17	11	17	17	17	
Total time looping (sec.)	14	11	0	0	17	
Percent time looping	82	100	-	-	100	
Number of loops	15	7	-	-	12[2]	
Number of loops/sec.	1.1	0.6	-	-	0.7	
Looping to left	yes	yes	-	-	yes	
Looping to right	yes	yes	-	-	yes	

[2]approximate - lost in glare part of time

Table 59: Fish-by-Fish Analysis of Behavior of Fish in Compartment 3, Day 8.

DAY 8 - COMPARTMENT 3						
FISH	1	2	3	4	5	6
Time visible (sec.)	17	17	17	17	5	
Total time looping (sec.)	0	0	0	0	0	
Percent time looping	-	-	-	-	-	
Number of loops	-	-	-	-	-	
Number of loops/sec.	-	-	-	-	-	
Looping to left	-	-	-	-	-	
Looping to right						

Table 60: Fish-by-Fish Analysis of Behavior of Fish in Compartment 4, Day 8.

DAY 8 - COMPARTMENT 4						
FISH	1	2	3	4	5	6
Time visible (sec.)	17	7.5	17			
Total time looping (sec.)	0	0	0[1]			
Percent time looping	-	-	-			
Number of loops	-	-	-			
Number of loops/sec.	-	-	-			
Looping to left	-	-	-			
Looping to right	-	-	-			

[1]dead

Table 61: Fish-by-Fish Analysis of Behavior of Fish in Compartment 5, Day 8.

DAY 8 — COMPARTMENT 5						
FISH	1	2	3	4	5	6
Time visible (sec.)	19.5	18.5	19	20	19	19
Total time looping (sec.)	16.5	0[1]	0	15	0[1]	10
Percent time looping	85	-	-	75	-	50
Number of loops	17	-	-	22	-	15
Number of loops/sec.	1.0	-	-	1.5	-	1.5
Looping to left	yes	-	-	yes	-	yes
Looping to right	yes	-	-	yes	-	yes

[1]possibly dead

Table 62: Fish-by-Fish Analysis of Behavior of Fish in Compartment 1, Day 9.

DAY 9 - COMPARTMENT 1						
FISH	1	2	3	4	5	6
Time visible (sec.)	12	10.5	10.5	10.5	10.5	
Total time looping (sec.)	0	5	8.5	0	8	
Percent time looping	-	48	81	-	76	
Number of loops	-	7	10	-	9	
Number of loops/sec.	-	1.4	1.2	-	1.1	
Looping to left	-	no	yes	-	no	
Looping to right	-	yes	yes	-	yes	

Table 63: Fish-by-Fish Analysis of Behavior of Fish in Compartment 2, Day 9.

DAY 9 - COMPARTMENT 2						
FISH	1	2	3	4	5	6
Time visible (sec.)	22	22	22	22	22	
Total time looping (sec.)	8	17.5	10.5	12	0	
Percent time looping	36	80	48	55	-	
Number of loops	7	13	9	9	-	
Number of loops/sec.	0.9	0.8	0.9	0.8	-	
Looping to left	yes	yes	yes	yes	-	
Looping to right	yes	yes	yes	yes	-	

Table 64 : Fish-by-Fish Analysis of Behavior of Fish in Compartment 3, Day 9.

DAY 9 - COMPARTMENT 3						
FISH	1	2	3	4	5	6
Time visible (sec.)	18	18	18	18	18	
Total time looping (sec.)	7	7	0	0	0	
Percent time looping	39	39	-	-	-	
Number of loops	8	14	-	-	-	
Number of loops/sec.	1.1	2	-	-	-	
Looping to left	yes	yes	-	-	-	
Looping to right	yes	yes	-	-	-	

Table 65: Fish-by-Fish Analysis of Behavior of Fish in Compartment 4, Day 9.

DAY 9 - COMPARTMENT 4						
FISH	1	2	3	4	5	6
Time visible (sec.)	21	21	21	20		
Total time looping (sec.)	0	0[1]	0[1]	0		
Percent time looping	–	–	–	–		
Number of loops	–	–	–	–		
Number of loops/sec.	–	–	–	–		
Looping to left	–	–	–	–		
Looping to right	–	–	–	–		

[1] dead

Table 66: Fish-by-Fish Analysis of Behavior of Fish in Compartment 5, Day 9.

DAY 9 — COMPARTMENT 5						
FISH	1	2	3	4	5	6
Time visible (sec.)	24.5	24.5	24.5	12.5	12.5	24.5
Total time looping (sec.)	0	0[1]	0[1]	3	0	0[1]
Percent time looping	-	-	-	24	-	-
Number of loops	-	-	-	6	-	-
Number of loops/sec.	-	-	-	2	-	-
Looping to left	-	-	-	yes	-	-
Looping to right	-	-	-	no	-	-

[1] dead

Table 67: Comparison of Total Looping Behavior on Day-by-Day Basis.

	D 6	D 7	D 8	D 9
Number of Fish Alive	25	24	21	21
Number of Fish Looping	7	10	9	10
Percent of Fish Looping	28	42	43	48

Table 68: Comparison of Total Looping Behavior on Compartment-by-Compartment Basis.

	1	2	3	4	5
Total # of Fish-Days[1]	20	20	20	14	17
Avg. # Fish/Cmpt./Day	5	5	5	3.5	4.25
Total # of Fish-Day Looping[2]	10	8	5	2	11
Avg. # Fish-Looping/Cmpt.	2.5	2	1.25	0.5	2.75
Percent of Fish Looping/Cmpt.	50	40	25	14	65

[1]Total of live fish present in that compartment over 4 day period.

[2] " " - " looping " " " " " " "

The increase in looping behavior thereafter, however, may be due to an increased tendency on the part of the astronauts to "force" the fish to loop by tapping or shaking the bags. Thus, I feel the increased looping is not indicative, since undistrubed fish may have oriented normally.

e) Table 68 compares the total looping behavior on a compartment-by-compartment basis over the total test period. The greatest amounts of looping occurred in Cmpt. 1 and 5, i.e. the controls and blinded fish. The least amount of looping occurred in Cmpt. 4, i.e. black background fishes. The ability of these fish to orient is also supported by the fact that most of them oriented with their backs to the black background, Table 46 D., Days 6 and 7, unlike most of the fishes in other compartments.

Whether the percent looping behavior of the fishes in Cmpts. 2 and 3 is significantly less than that of the controls could probably be proven statistically. Thus, it would appear that prior visual acclimation did permit those fishes to orient somewhat better than fishes with no visual acclimation.

f) Tables 69 and 70 present both a day-by-day and compartment-by-compartment analysis of the total time duration of looping and the number of loops made. I can see no discernable patterns here, other than the smaller amounts of time and lesser number of loops made by the fishes in

Cmpt. 4 (Table 70).

Table 69: Day-by-Day Comparison of Duration and Quality of Looping.

	D 6	D 7	D 8	D 9
Total # fish looping	7	10	9	10
Avg. percent time looping	31	71	79	53
Avg. number of loops/sec.	1.4	1.2	0.9	1.2

Table 70: Compartment-by-Compartment Comparison of Duration and Quality of looping.

	Compartment				
	1	2	3	4	5
Average # fish looping/ cmpt./day	2.5	2	1.25	0.5	2.75
Average percent time looping/fish	67	63	55	30	51
Average # loops/sec./fish	1.3	0.9	1.1	0.6	1.5

Conclusions.

The success of the two main objectives of the experiment can be summarized as follows:

a) No conclusions can be drawn as to whether prior visual acclimation would allow those fishes to become visually oriented more rapidly than non-acclimated fishes. The quantity of disorientation was observed to increase rather than decrease over the test period. But, as pointed out, this may be due to the fact that the astronauts increased their efforts to "force" the fishes to become disoriented by tapping or shaking the bags.

b) Prior visual acclimation, however, did seem to enable such fishes to orient better than non-acclimated fish, since the least amounts of looping was shown by fishes in Cmpts. 2, 3, and especially 4.

VII. ELECTRON MICROSCOPY

The relationships between the various ovum envelopes and the mechanism of fertilization was studied by scanning electron microscopy (SEM).

Methods.

Only mature ova were used for this investigation and were obtained by stripping gravid females. The ova were fixed in 2% glutaraldehyde (in $25^{0}/_{00}$ Instant Ocean solution) for 2 hours at room temperature and then rinsed with distilled water; a graded ethanoldehydration sequence prior to 3 changes in amyl acetate and subsequent critical point drying in CO_2 was used to bring the specimens to dryness. The ova were mounted on aluminum stubs, coated with gold/palladium in an ion sputtering apparatus and examined on a JOEL JSM U-3 Scanning Electron Microscope.

Results.

1. The outer surface of the ovum is covered with a dense pile of short (0.3-0.6μ dia) fibrils (Fig. 1a). Several thicker (0.8-1.0μ dia.), longer fibrils are present in the area peripheral to the micropyle. These fibrils are part of the outermost, jelly coat layer of the ovum.

2. The chorion lies directly beneath the jelly coat. The outermost portion of the chorion is a thin, homogeneous zone about 0.3-0.4μ thick (Figs. 2a, 3c). Immediately below this homogeneous

zone is a heterogeneous portion of the chorion comprised of several lamellae, numbering from a few as 4 in some ova (Fig. 2c) to up to 9-10 in others. Each lamellus appears to be continuous throughout the chorion. Thinner lamellae are found at the inner and outer extremes of the chorion, especially when there are a larger number (Fig. 2a). The entire lamellar zone is about 9-12μ thick.

3. Internal to the lamellar zone is a homogeneous, chorionic crystalline zone. This zone was not always detected (Figs. 2c, 3a, c). When present, it varies from a maximum of 13μ in thickness (Fig. 2a, d), to a relatively thin 1μ (Fig. 3b).

4. The micropyle apparatus consists of a relatively smooth-sided, funnel-shaped vestibule (Fig. 1c) at the bottom of which is a 4-5μ dia. opening, the micropyle, surrounded by a slightly elevated lip (Fig. 1b). The micropyle canal traverses the chorion, diminishing in diameter as it approaches the ovoplasm. The inner micropyle aperture rests directly against the ovoplasm (Fig. 1d). The canal sides are irregular, showing evidence of the lamellated structure of the chorion (Figs. 1b, d).

Conclusions.

1. The presence of a thick, impenetrable chorion surrounding the plasma membrane precludes any access of sperm to the ovum nucleus other than by means of the micropyle apparatus.

2. The size of the micropyle apertures, outer 4-5μ dia., inner 2-3μ dia., are such that although several sperm may approach the outer

aperture and possibly enter, the canal becomes progressively narrower and it is conceivable that only one sperm makes contact with the plasma membrane.

3. The absence of fibrils in the region of the micropyle is an adaptation facilitating fertilization. These fibrils are sticky and would collect sperm and prevent their access to the micropyle.

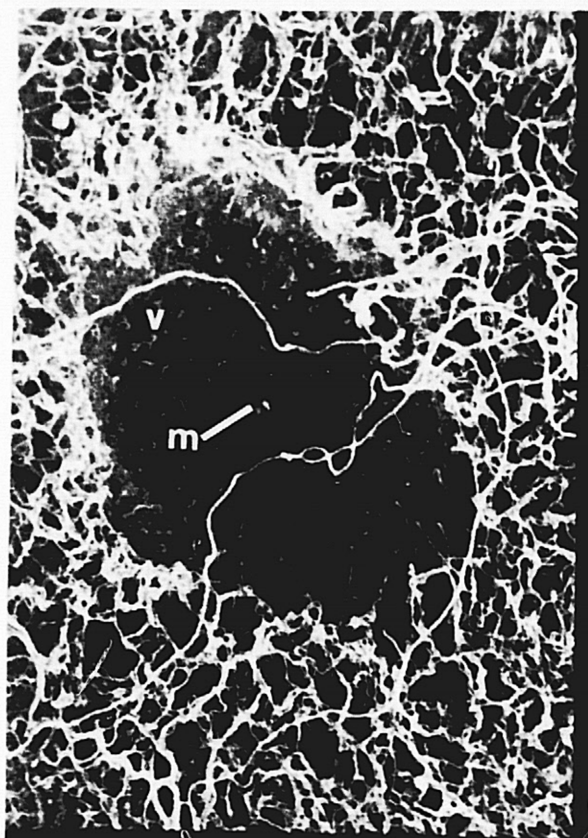
FIGURE 1.

a. Surface view of ovum showing fibrils and fibril free vestibule (v) surrounding micropyle (m) X500.

b. External micropyle aperture with lip (l) and inner view of micropyle canal, X4000.

c. Side view of vestibule with micropyle at bottom, X500.

d. Side view of micropyle canal (mc) with portion of cell cytoplasm (c) adjacent to inner micropyle aperture, X2000.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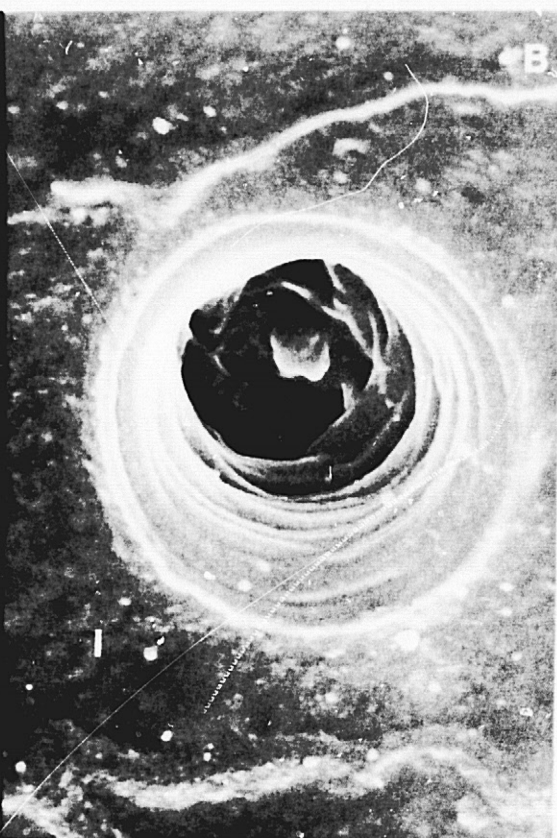
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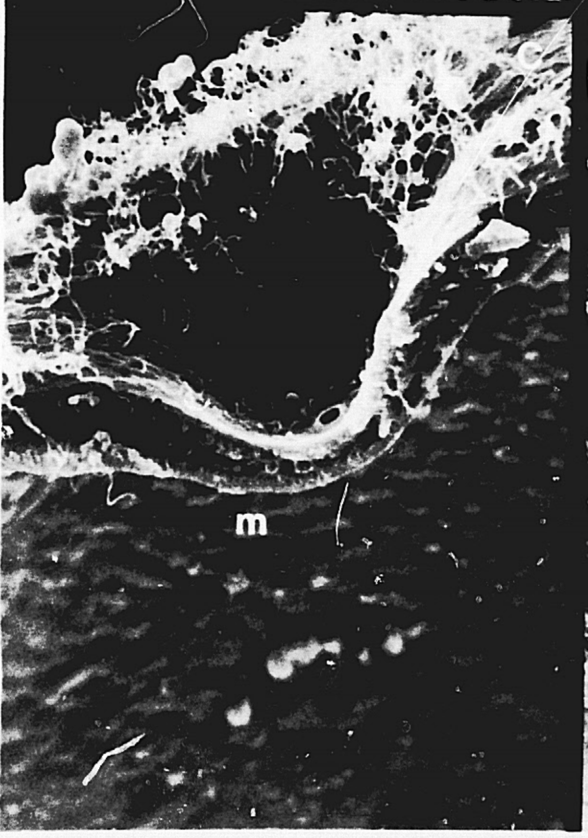
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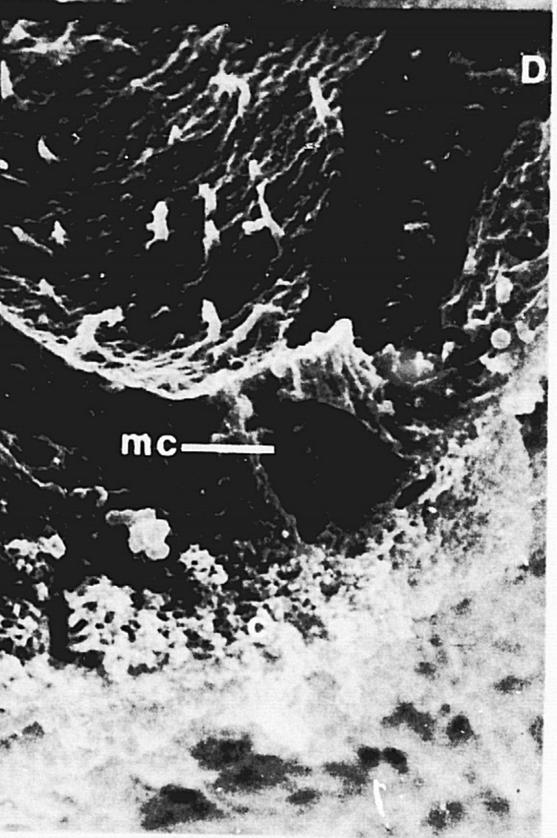
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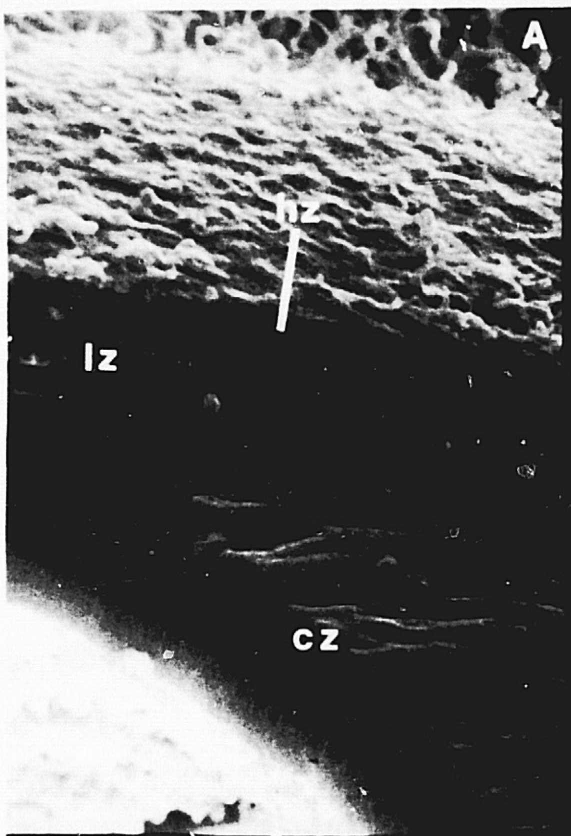
FIGURE 2.

a. Transverse view of chorion homogeneous zone (hz), lamellar zone (lz), and tongue of chorion crystalline zone (cz), X1000.

b. Transverse view of chorion lamellar zone with 7 lamellae, X2000.

c. Transverse view of chorion lamellar zone with 4 lamellae showing herringbone pattern, X1000.

d. Transverse view of lamellar and crystalline zones of chorion, X1000.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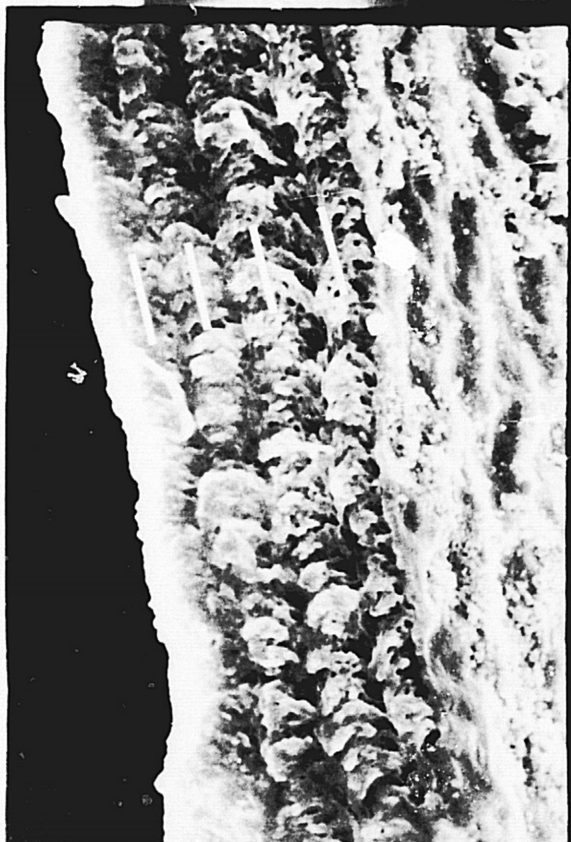

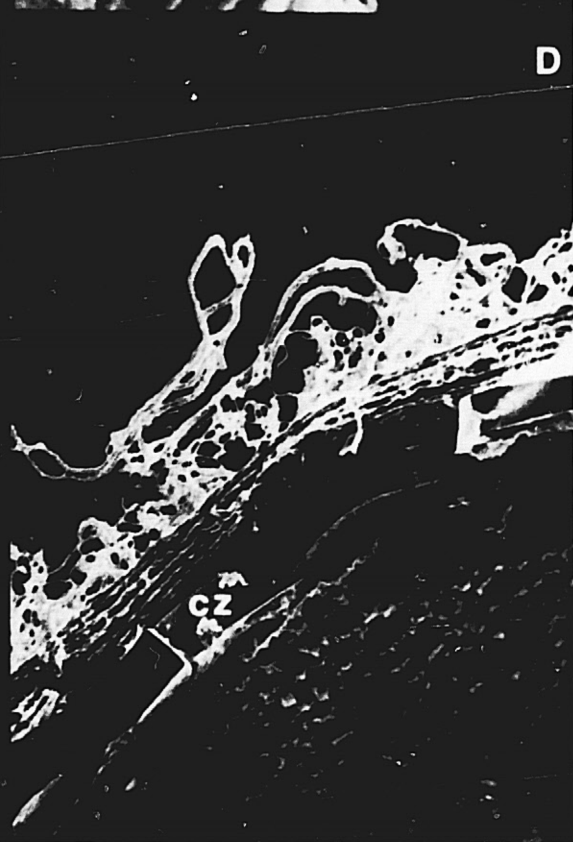
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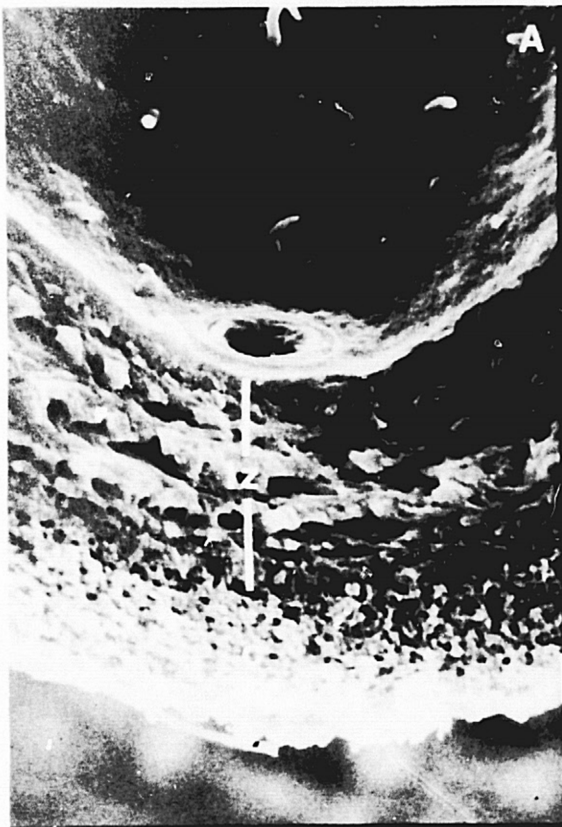
FIGURE 3.

a. Transverse view of lamellar zone of chorion with 10 lamellae and with underlying cytoplasm (c), X2000.

b. Transverse view of chorion showing extremely thin crystalline zone (cz), X2000.

c. Chorion homogeneous zone (hz) and lamellar zone (lz) with fibrillar protein matrix, X500.

d. Chorion lamellar zone (lz) showing fibrillar protein matrix (f = jelly coat fibrils), X1500.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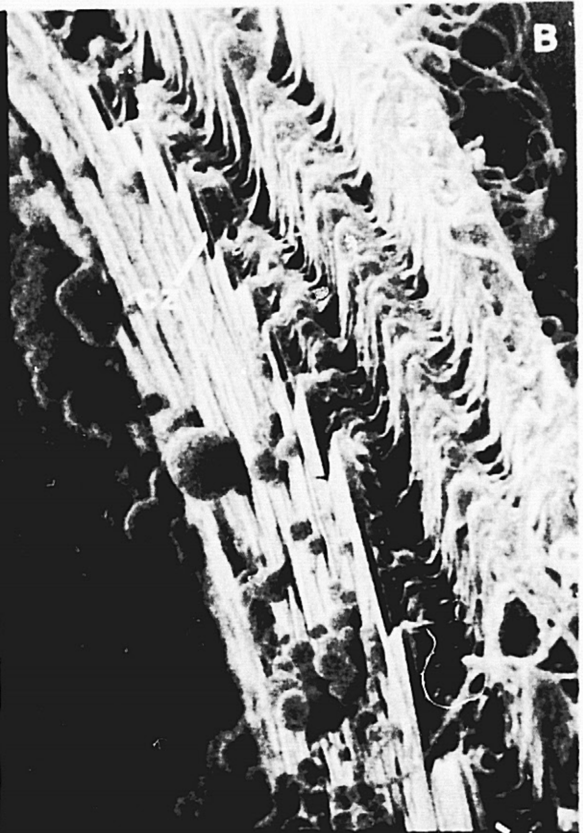
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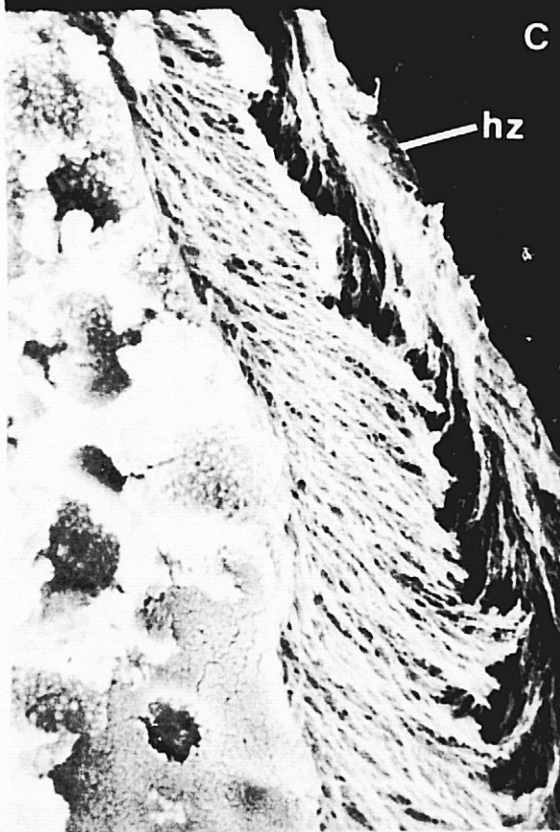
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hz

D
f
lz