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INFLUENCE OF GAMETE DENSITY, SALINITY AND TEMPERATURE ON THE NORMAL EMBRYONIC DEVELOPMENT OF THE MANGROVE OYSTER *CRASSOSTREA RHIZOPHORAE* GUILDING, 1828

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ABSTRACT

Dos Santos, A.E. and Nascimento, I.A., 1985. Influence of gamete density, salinity and temperature on the normal embryonic development of the mangrove oyster *Crassostrea rhizophorae* Guilding, 1828. *Aquaculture*, 47: 335—352.

Laboratory experiments were undertaken to determine the optimal environmental conditions and some of the other factors concerned in the development of *Crassostrea rhizophorae* embryos.

Critical variables such as the number of spermatozoa per ovocyte during fertilization, the time of fertilization after gamete liberation, egg density, temperature and salinity were related to the proportion of normal D-larvae of *C. rhizophorae* in the resulting broods

The highest proportion of normal D-larvae was obtained at concentrations of 500—5000 spermatozoa/ovocyte, under conditions of 25‰ salinity at 25 ± 1°C. The optimal density of eggs, for the production of normal D-larvae, was 10^4—4×10^4 ovocytes/l. If fertilization was delayed for more than 45 min after liberation of spermatozoa the proportion of normal D-larvae was greatly reduced. The experiments demonstrated that the temperature for developing embryos should be below 30°C. At 20 and 25°C there was a high proportion of normal D-larvae 24 h after fertilization. The ideal salinity for embryonic development in *C. rhizophorae* was 25—37‰. Below a salinity of 16‰, less than 2.5% of the D-larvae were normal.

INTRODUCTION

The feasibility of commercial cultivation of the mangrove oyster *C. rhizophorae* has been demonstrated. However, it seems unlikely that sufficient natural spat is available to support commercially viable enterprises in the north east of Brazil (Nascimento, 1983) and this has prompted investigations into the artificial propagation of the species.

Hatchery methods have been well established for other species of the genus, for example *C. virginica* (Loosanoff and Davis, 1963; Dupuy and Rivkin, 1972) and *C. gigas* (Fujita, 1934; Breese and Malouf, 1975), but

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these methods have not yet been adapted for the production of seed of *C. rhizophorae*.

Little is known about the biological processes involved in hatchery practices necessary for the production of *C. rhizophorae*. Apart from work on maturation and spawning (Nascimento, 1978; Nascimento and Lunetta, 1978; Nascimento and Pereira, 1980; Nascimento et al., 1980a,b), research on larval rearing is lacking. It has been demonstrated (Calabrese and Davis, 1970; MacInnes and Calabrese, 1979), that the embryonic-development phase is a critical period in the life cycle of the oyster; this study therefore, focused on this phase. For maximization of the output from a hatchery it is first necessary to ensure that conditions during the fertilization and egg incubation stages are optimal for the production of normal larvae. The objective of this research was to determine the optimum concentration of eggs and spermatozoa during fertilization and the best salinities and temperatures for the production of normal D-larvae of *C. rhizophorae*.

MATERIALS AND METHODS

The experiments were performed on *C. rhizophorae* specimens collected from the mangrove trees in the native environment of the oyster (Salinas da Margarida, 12°52'S, 38°44'W). Gametes were obtained by stripping mature oysters. Because the incidence of hermaphroditism was low (Nascimento, 1980) eggs and sperm could be collected separately, after checking a sample from each gonad with a microscope to determine the sex and the stage of gamete maturation. Eggs and sperm were placed separately in 2-l glass beakers containing glass fibre (GFC)-filtered sea water, having a salinity of 25‰, at 25 ± 1°C. In these containers, gamete densities were determined by counting three samples taken under agitation with a perforated plunger. After fertilization, the embryos were kept in the test containers under each experimental condition for 24 h. All the trials were made in duplicate and repeated 6—10 times.

The pH level in all the experiments was 7.8—8.1, within the range determined as optimal for the development of oyster embryos (Calabrese and Davis, 1966). Dissolved oxygen levels in the beakers ranged from approximately 95% saturation at the beginning of each test to 90.95% saturation after 24 h.

At the end of each 24-h test the water in each beaker was mixed vigorously and a 10-ml sample was removed, preserved in 5% buffered formalin and later examined under a compound microscope. The numbers of embryos that developed normally and abnormally were counted. Responses to the different treatments were recorded as the percentage of embryos failing to develop, or developing in an abnormal manner, in relation to the maximum number of D-larvae expected.

Bivalve embryos are defined as the stages between the fertilized egg and the ciliated trochophore. Normal larvae are defined here as being perfectly D-shaped at the prodissoconk I stage. Abnormal larvae had irregular or mis-

shapen shells, completely or incompletely formed. For statistical treatment those embryos remaining at the end of the 24-h test were also counted as abnormal, since they had not developed to D-larvae, as would normally be expected in 24 h. The percentages of normal D-larvae were submitted to the angular transformation (arcsin$\sqrt{\%}$, according to the method of Sokal and Rohlf, 1969) and then, where necessary, to a one-way or two-way analysis of variance. The comparisons between means were accomplished with the Student-Newman-Keuls test.

There were five kinds of experiment as described below.

1. Determination of the optimum ratio of sperms per egg

Ten different numbers of sperm/egg (5, 10, 50, 10^2, 5×10^2, 10^3, 5×10^3, 10^4, 5×10^4 and 10^5) were tested. The same egg density ($10^4\ l^{-1}$) was maintained in each of the 20 beakers used in the experiment, which was repeated ten times. Fertilization took place in the test containers after the appropriate volumes of egg and sperm suspensions had been added to the beakers containing glass fibre-filtered (GFC) sea water at $25^0/_{00}$ salinity. The sperm were placed in the test containers within 15 min of being collected from the gonads.

2. Effect of a delay, between spermatozoan liberation and the fertilization process, on the proportion of the resultant normal D-larvae

The best number of sperm/egg (10^2) as determined in Experiment 1 was maintained in this experiment. The same physical conditions were maintained, but the time interval between gamete collection from the gonads and fertilization, was varied to 15, 30, 45, 60, 90 or 120 min. Ten such trials were performed.

3. Determination of the optimum density of eggs for the maximum production of normal D-larvae

Ten different densities (10^4, 2×10^4, 3×10^4, 4×10^4, 5×10^4, 10^5, 15×10^5, 20×10^5, 25×10^5 and 30×10^5) of eggs per litre were tested. All the other factors remained constant as in previous experiments, but 10^2 sperm/egg were used. The time interval between sperm collection and fertilization was again 15 min. Ten such trials were performed.

4. Determination of the appropriate salinity and temperature range for the maximum production of normal C. rhizophorae *D-larvae*

Eleven different salinities (10—40 ppt ($^0/_{00}$) at 3 ppt intervals) and three different temperatures (20, 25 and 30°C) were tested in duplicate with 10^3 sperm/egg (egg density 10^4/l). All other conditions were the same as in

TABLE I

Mean percent values (n = 10 replicates) of normal (N) and abnormal (A) larvae obtained 24 h after fertilization at different concentrations of sperm per ovocyte. Ovocyte density $10^4 l^{-1}$ in all treatments; salinity and temperature 25 ppt and 25 ± 1°C, respectively

Experiments	Concentrations of sperm/ovocyte																			
	5		10		5 × 10		10^2		5 × 10^2		10^3		5 × 10^3		10^4		5 × 10^4		10^5	
	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A
1	14	86	23	77	18	82	50	50	59	41	52	48	51	49	47	53	35	65	24	76
2	12	88	22	78	43	57	73	27	79	21	56	44	53	47	52	48	48	52	17	83
3	23	77	17	83	42	58	54	46	62	38	57	43	58	42	47	53	51	49	32	68
4	11	89	19	81	49	51	45	55	79	21	51	49	72	28	69	31	55	45	10	90
5	12	88	20	80	48	52	53	47	64	36	81	19	55	45	51	49	38	62	21	79
6	21	79	30	70	38	62	55	45	51	49	64	36	53	47	40	60	36	64	14	86
7	23	77	24	76	27	73	47	53	58	42	57	43	47	53	34	66	21	79	5	95
8	53	47	53	47	61	39	38	62	41	59	56	44	54	46	43	57	38	62	6	94
9	44	56	27	73	47	53	48	52	62	38	55	45	58	42	30	70	34	66	5	95
10	22	78	20	80	48	52	69	31	75	25	58	42	69	31	49	51	25	75	4	96
$\bar{X}$	23.5	76.5	25.5	74.5	42.1	57.8	53.2	46.8	63.0	37.0	58.7	41.3	57.0	43.0	46.2	53.8	38.1	61.9	13.8	86.2
S.D.	14.18	14.18	10.40	10.40	12.13	12.13	10.64	10.64	12.15	12.15	8.52	8.52	7.83	7.83	10.78	10.78	10.77	10.77	9.57	9.57

previous experiments. After 24 h the containers were sampled and the results recorded. Ten such trials were performed.

5. Combined effects of salinity and temperature on the embryonic development of C. rhizophorae.

In Experiment 4 the best range of salinities for the production of normal D-larvae was found to be 25–37 ppt. Normally the temperature in the natural environment of *C. rhizophorae* does not go below 20° C or above 30° C. Consequently, in the present experiment the effects were tested of different combinations of a salinity range of 20 to 40 ppt, with 5 ppt intervals in a temperature range of 20—30° C, with 5° C intervals. There were 10^3 sperms/egg and the ovocyte density was 10^4/l. Six such experiments were performed. After 24 h two samples were taken from each beaker, they were fixed and the larvae counted, and the results recorded.

RESULTS

Tables I–III show the results of Experiment 1. The production of normal D-larvae occurs within a large range ($5–10^5$) of sperm/egg. However, the maximum production of normal D-larvae (46.9%—52.8%) was obtained within the range 10^2—5×10^3. Using 5×10, 10^4 and 5×10^4 sperm/egg the results were significantly ($P < 0.05$) lower, varying from 37.9 to 42.8% normal D-larvae. The extreme values of sperm/egg, of 10^5, 5 and 10, led to the production of only 20.7%, 28.3% and 30.0% of normal D-larvae, respectively.

Experiment 2 (Tables IV—VI) showed that if fertilization was delayed for more than 45 min after gamete collection from the gonads, the proportion of normal D-larvae was reduced from about 50% to 34.7%, 25.2% and 16.4%, respectively if the sperm were used 60, 90 and 120 min after liberation (Table VI). ANOVA results indicated a significant difference ($P < 0.05$) between the treatments. The Student-Newman-Keuls test results did not

TABLE II

Results of the analysis of variance between the mean percent values of normal D-larvae obtained 24 h after fertilization at different ratios of sperm/ovocyte according to the data in Table I. Data were transformed using the regular transformation (arcsin $\sqrt{\%}$) before analysis of variance

Total of experiments	Sources of variation	d.f.	SS	MS	F value	Tabulated value
10	Between groups	9	10156.55	1128.51	24.02***	1.96
	Within groups	90	4228.37	46.98		
	Total	99	14384.92			

***$P < 0.001$.

TABLE III

Results of Student-Newman-Keuls test between the mean percent values of normal D-larvae obtained 24 h after fertilization at different ratios of sperm/ovocyte. The values united by lines do not differ significantly ($P > 0.05$). Mean values are presented with ± 1 standard deviation

Concentrations of sperm/ovocyte									
10^5	5	10	5×10^4	5×10	10^4	10^2	5×10^3	10^3	5×10^2
Percentage means									
13.8 ± 9.57	23.5 ± 14.18	25.5 ± 10.40	38.1 ± 0.77	42.1 ± 12.13	46.2 ± 10.78	53.2 ± 10.64	57.0 ± 7.83	58.7 ± 8.52	63.0 ± 12.15
Transformed means arcsin $\sqrt{\%}$									
20.74 ± 7.99	28.27 ± 9.22	30.05 ± 6.42	37.95 ± 6.48	40.26 ± 7.38	42.80 ± 6.32	46.91 ± 6.26	49.09 ± 4.63	50.14 ± 5.33	52.76 ± 7.37

TABLE IV

Mean percent values (n = 10 replicates) of normal (N) and abnormal (A) larvae obtained 24 h after fertilization with different time intervals between collection of sperm and fertilization. Ratio of sperm per ovocyte, 10^3 in all treatments: salinity and temperature, 25 ppt and 25 ± 1°C respectively

Experiments	Time intervals (min) from sperm collection to fertilization											
	15		30		45		60		90		120	
	N	A	N	A	N	A	N	A	N	A	N	A
1	34.5	65.5	54.0	46.0	54.0	46.0	19.5	80.5	13.0	87.0	5.0	96.0
2	60.5	39.5	74.0	26.0	69.0	31.0	26.5	73.5	4.5	95.5	5.0	95.0
3	37.5	62.5	37.5	62.5	26.0	64.0	31.0	69.0	8.0	92.0	3.5	96.5
4	56.5	43.5	64.0	36.0	67.5	32.5	21.5	78.5	8.0	92.0	3.5	96.5
5	92.0	8.0	72.0	28.0	65.5	34.5	37.5	62.5	44.5	55.5	16.5	83.5
6	74.0	26.0	72.0	28.0	69.0	31.0	56.0	44.0	23.0	77.0	11.5	88.5
7	47.5	52.5	64.0	36.0	61.0	39.0	46.0	54.0	23.0	77.0	8.0	92.0
8	92.0	8.0	69.0	31.0	51.0	49.0	23.0	77.0	33.0	67.0	10.0	90.0
9	77.0	23.0	69.0	31.0	52.5	47.5	29.5	70.5	15.0	85.0	6.5	93.5
10	59.0	41.0	57.5	42.5	64.0	36.0	37.5	62.5	23.0	77.0	15.0	85.0
$\bar{X}$	63.1	36.9	63.3	36.7	58.9	41.1	32.8	67.2	19.5	80.5	8.4	91.6
S.D.	20.46	20.46	11.14	11.14	10.56	10.56	11.60	11.60	12.45	12.45	4.67	4.67

TABLE V

Results of analysis of variance between mean percent values of normal D-larvae 24 h after fertilization with different time intervals from the collection of sperm to fertilization. Data transformed using the regular transformation (arcsin $\sqrt{\%}$) before analysis of variance

Total of experiments	Sources of variation	d.f.	SS	MS	F value	Tabulated value
10	Between groups	5	12487.77	2497.55	36.11***	2.39
	Within groups	54	3735.06	69.17		
	Total	59	16222.83			

***$P < 0.001$.

TABLE VI

Results of Student-Newman-Keuls test between mean percent values of normal D-larvae obtained at different time intervals from the collection of sperm to fertilization. Values united by lines do not differ significantly ($P > 0.05$). Mean values are presented with ± 1 standard deviation

Time intervals (min)	120	90	60	45	30	15
Percentage means	8.4 ± 4.67	19.5 ± 12.45	32.8 ± 11.60	58.9 ± 10.56	63.3 ± 11.14	63.1 ± 20.46
Transformed means arcsin $\sqrt{\%}$	16.36 ± 4.76	25.19 ± 9.15	34.69 ± 7.06	50.23 ± 6.17	52.84 ± 6.58	53.48 ± 13.52

TABLE VII

Mean percent values (n = 10 replicates) of normal (N) and abnormal (A) larvae obtained 24 h after fertilization, using different ovocyte densities. Ratio of sperm per ovocyte, 10^3 in all treatments; salinity and temperature, 25 ppt and 25 ± 1°, respectively

Experiments	Ovocyte densities/l																			
	10^4		2×10^4		3×10^4		4×10^4		5×10^4		10^5		15×10^5		20×10^5		25×10^5		30×10^5	
	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A
1	59.5	40.5	73.5	26.5	66.2	33.8	72.0	28.0	62.2	37.8	56.4	43.6	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
2	56.0	44.0	60.0	40.0	42.2	57.8	32.4	67.6	27.2	72.8	26.0	74.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
3	48.5	51.5	55.0	45.0	42.5	57.5	56.4	43.6	37.9	62.1	22.8	77.2	16.8	83.2	3.9	96.1	48.7	51.3	1.9	98.1
4	77.5	22.5	69.5	30.5	78.5	21.5	68.9	31.1	59.4	40.6	46.1	53.9	39.0	61.0	39.0	61.0	11.2	88.8	1.7	98.3
5	67.5	32.5	63.0	37.0	74.2	25.8	54.3	45.7	51.1	48.9	13.3	86.7	0.7	99.3	0.0	100.0	0.0	100.0	0.0	100.0
6	50.5	49.5	75.0	25.0	64.0	36.0	70.9	29.1	61.7	38.3	28.6	71.4	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
7	67.0	33.0	61.7	38.3	69.3	30.7	77.6	22.4	68.7	31.3	31.9	68.1	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
8	66.0	34.0	46.5	53.5	42.5	57.5	46.5	53.5	32.1	67.9	24.6	75.4	36.6	63.4	0.0	100.0	0.0	100.0	0.0	100.0
9	82.0	18.0	77.0	23.0	71.2	28.8	69.0	31.0	57.7	42.3	34.5	65.5	0.0	100.0	14.6	85.4	0.0	100.0	0.0	100.0
10	64.5	35.5	50.8	49.2	37.2	62.8	36.0	64.0	29.4	70.6	36.8	63.2	20.7	79.3	0.0	100.0	0.0	100.0	0.0	100.0
$\bar{X}$	63.9	36.1	63.2	36.8	58.8	41.2	58.4	41.6	48.7	51.3	32.1	67.9	11.8	88.2	5.8	94.2	6.0	94.0	0.4	99.6
S.D.	10.74	10.74	10.48	10.48	15.79	15.79	15.89	15.89	15.56	15.56	12.29	12.29	15.73	15.73	12.56	12.56	15.41	15.41	0.76	0.76

show any significant difference ($P > 0.05$) when the sperm were used for fertilization 15—45 min after liberation from the gonads. However, there was a significant difference between these and all of the other treatments (60—120 min after collection).

TABLE VIII

Results of analysis of variance between mean percent values of normal D-larvae obtained at different ovocyte densities. Data transformed using the regular transformation (arcsin $\sqrt{\%}$) before analysis of variance

Total of experiments	Sources of variation	d.f.	SS	MS	F value	Tabulated value
10	Between groups	9	42434.71	4714.97	43.65***	1.96
	Within groups	90	9720.55	108.01		
	Total	99	52155.26			

***$P < 0.001$.

Experiment 3 revealed that egg densities higher than 10^5 eggs/l markedly depressed the yield of normal D-larvae as compared with densities between 10^4 and 5×10^4 (Tables VII—IX). These groups of treatments were significantly different ($P < 0.05$) (Table IX). The highest proportion of normal D-larvae produced was 53.3% at 10^4 eggs/l but this value was not significantly different ($P > 0.05$) from those obtained with 2×10^4, 3×10^4, 4×10^4 and 5×10^4/l, giving, respectively, 52.8%, 50.3%, 50.0% and 44.2% normal D-larvae. These high values dropped to 13.2% and 1.5% respectively when the egg densities were increased to 15×10^5 and 30×10^5/l.

The isolated effects of different salinity and temperature on the embryonic development of *C. rhizophorae* were tested in Experiment 4 (Tables X—XII). Salinities between 25 and 37 ppt yielded the highest transformed percentages of normal D-larvae (48.7%—54.8%) (Table XII) which did not differ significantly ($P < 0.05$). Salinities of 10—19 ppt yielded the lowest percentages of normal D-larvae (0—6.7%, not significantly different). The percentages of normal D-larvae (Table XIII) obtained under different temperatures (20, 25 and 30°C) were statistically different (Table XIV). The Student-Newman-Keuls test showed no significant difference between the results obtained at 20 and 25°C, but these were both significantly higher than the results obtained at 30°C (Tables XIV and XV). The values of 49.4 and 50.1% of normal D-larvae obtained respectively at 20 and 25°C dropped to 6.05 at 30°C.

Because the salinity-tolerance of oyster embryos can vary with temperature, both factors were tested in combination in Experiment 5 (Table XVI). No significant differences ($P < 0.05$) were observed among the salinities tested (20—40 ppt, with 5 ppt intervals) at each temperature (20, 25 and 30°C). However there were significant differences ($P < 0.05$) between the temperatures at all the salinities tested (Table XVII). The lowest values were at 30°C

TABLE IX

Results of Student-Newman-Keuls test between mean percent values of normal D-larvae obtained at different ovocyte densities. Values united by lines do not differ significantly ($P > 0.05$). Mean values presented with ± 1 standard deviation

Ovocyte densities/l									
30×10^5	25×10^5	20×10^5	15×10^5	10^5	5×10^4	4×10^4	3×10^4	2×10^4	10^4
Percentage means									
0.4 ± 0.76	6.0 ± 15.41	5.8 ± 12.56	11.8 ± 15.73	32.1 ± 12.29	48.7 ± 15.56	58.4 ± 15.89	58.8 ± 15.79	63.2 ± 10.98	63.9 ± 10.74
Transformed means arcsin $\sqrt{\%}$									
154 ± 3.25	6.38 ± 14.66	7.25 ± 13.34	13.19 ± 16.60	34.18 ± 7.66	44.21 ± 9.13	50.01 ± 9.4	50.28 ± 9.35	52.82 ± 6.30	53.26 ± 6.59

TABLE X

Mean percent (n = 10 replicates) of normal (N) and abnormal (A) larvae exposed to different salinities for 24 h after fertilization. In all treatments concentration of sperm/egg (10^3), egg density (10^4/l) and temperature (25 ± 1°C) were the same

Experiments	Salinity (‰)																					
	10		13		16		19		22		25		28		31		34		37		40	
	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A
1	0	100	0	100	0.0	100.0	6.0	94.0	45.0	55.0	58.5	41.5	64.0	36.0	64.0	36.0	70.0	30.0	66.5	33.5	68.5	31.5
2	0	100	0	100	0.0	100.0	6.0	94.0	42.5	57.5	78.5	21.5	75.0	25.0	53.5	46.5	48.5	51.5	77.5	22.5	43.5	56.5
3	0	100	0	100	1.5	98.5	1.5	98.5	57.5	42.5	57.5	42.5	58.5	41.5	78.5	21.5	76.5	23.5	73.5	26.5	44.5	55.5
4	0	100	0	100	0.5	99.5	2.0	98.0	26.5	73.5	70.5	29.5	65.0	35.0	63.5	36.5	54.5	45.5	66.0	34.0	42.0	58.8
5	0	100	0	100	0.0	100.0	0.0	100.0	38.5	61.5	53.5	36.5	72.5	27.5	84.5	15.5	72.5	27.5	61.0	39.0	37.0	63.0
6	0	100	0	100	0.0	100.0	0.0	100.0	28.5	71.5	53.5	36.5	69.0	31.0	61.0	39.0	60.5	39.5	63.5	36.5	29.5	70.5
7	0	100	0	100	0.0	100.0	0.0	100.0	12.5	87.5	62.0	38.0	67.5	32.5	58.5	41.5	74.0	26.0	39.5	60.5	44.5	55.5
8	0	100	0	100	0.5	99.5	0.0	100.0	17.5	82.5	37.0	63.0	62.0	38.0	50.0	50.0	44.0	56.0	16.5	83.5	24.0	76.0
9	0	100	0	100	0.5	99.5	3.0	97.0	43.0	57.0	51.0	49.0	62.0	38.0	62.5	37.5	56.0	44.0	49.5	50.5	43.0	57.0
10	0	100	0	100	1.0	99.0	5.5	94.5	45.5	54.5	64.0	36.0	71.5	28.5	74.0	26.0	73.0	27.0	51.0	49.0	49.5	50.5
$\bar{X}$	0	100	0	100	0.4	99.6	2.4	97.6	35.7	64.3	59.6	40.4	66.7	33.3	65.0	35.0	63.0	37.0	56.5	43.5	42.5	57.4
S.D.	0	0	0	0	0.52	0.52	2.58	2.58	14.03	14.03	11.27	11.27	5.30	5.30	10.89	10.89	11.74	11.74	18.13	18.13	11.91	11.91

(Table XVIII). The values at 20°C and 25°C which were significantly higher than at 30°C, did not differ significantly ($P > 0.05$) from one another. The percentages of normal D-larvae obtained at 20° and 25°C were 33.6%—45.5%, while at 30°C they dropped to 7.9—11.7% (Table XIX).

TABLE XI

Results of the analysis of variance between the mean percent values of normal D-larvae obtained 24 h after fertilization at different salinities. Data were transformed using the regular transformation (arcsin $\sqrt{\%}$) before analysis of variance

Total of experiments	Sources of variation	d.f.	SS	MS	F value	Tabulated value
10	Between groups	10	56741.06	5674.11	138.77***	1.92
	Within groups	99	4047.89	40.89		
	Total	109	60788.95			

***$P < 0.001$.

DISCUSSION

It is of primary importance if oyster seed is to be produced in the laboratory that the fertilized eggs should give a high proportion of normal and healthy larvae. Loosanoff and Davis (1963) showed, for bivalves, that the percentage of normal larvae decreased with increased egg densities. In addition Gruffydd and Beaumont (1970), working on *Pecten maximus*, found that not only the ovocyte density but also the ratio of sperm to egg were important factors for the production of normal D-larvae. The best conditions for the fertilization and egg incubation stages for *C. rhizophorae* were therefore sought in the present work.

While Loosanoff and Davis, 1963, indicated that the best concentration for fertilization of *C. virginica* ovocytes was 30 000/l,we found the best densities for *C. rhizophorae* to be 10 000—40 000 ovocytes/l, based on the yield of normal D-larvae. These densities were lower than the 100 000 ovocytes/l used by Helm and Millican (1977) for *C. gigas* which produced 80% of normal D-larvae. The same egg concentration (100 000/l) in the present experiment yielded only 32.1% of normal *C. rhizophorae* D-larvae.

The work reported here indicates that the best densities of sperm per egg for fertilization and for the development of *C. rhizophorae* embryos to normal D-larvae were 10^2—5×10^3. It was demonstrated that higher or lower numbers of sperms per egg adversely affect the yield. According to Gruffydd and Beaumont (1970) the mechanisms causing abnormalities, as a result of higher sperm or egg concentrations, are unknown, but possible explanations can be offered. The presence of waste products and the resultant lack of sufficient oxygen around the developing eggs could cause mortalities and lower

TABLE XII

Results of Student-Newman-Keuls test between the mean percent values of normal D-larvae obtained 24 h after fertilization at different salinities. Values united by lines do not differ significantly ($P > 0.05$). Mean values presented with ± standard deviation

Salinity ($^0/_{00}$)										
10	13	16	19	22	40	37	25	34	31	28
Percentage means										
0 ± 0	0 ± 0	0.4 ± 0.52	2.4 ± 2.58	35.7 ± 14.03	42.6 ± 11.91	56.5 ± 18.13	59.6 ± 11.27	63.0 ± 11.74	65.0 ± 10.89	66.7 ± 30
Transformed means arcsin $\sqrt{\%}$										
0 ± 0	0 ± 0	3.09 ± 2.83	6.71 ± 6.25	36.26 ± 8.87	40.66 ± 7.07	48.68 ± 11.06	50.66 ± 6.73	52.70 ± 7.01	54.00 ± 6.85	54.81 ± 3.25

TABLE XIII

Mean percent (n = 10 replicates) of normal (N) and abnormal (A) larvae exposed to different temperatures for 24 h after fertilization. In all treatments, concentration of sperm/egg, egg density and salinity were 10^3, 10^4/l and 30 ppt, respectively

Experiments	Temperature (° C)					
	20		25		30	
	N	A	N	A	N	A
1	71.5	28.5	61.0	39.0	4.0	96.0
2	50.0	50.0	52.0	48.0	7.5	92.5
3	59.5	40.5	78.0	22.0	1.5	98.5
4	68.0	32.0	56.5	43.5	0.0	100.0
5	67.0	33.0	71.0	29.0	1.0	99.0
6	38.5	61.5	46.5	53.5	0.5	99.5
7	46.5	53.5	45.5	54.5	0.5	99.5
8	50.5	49.5	45.5	54.5	0.5	99.5
9	53.5	46.5	54.5	45.5	0.5	99.5
10	70.5	29.5	75.5	24.5	0.5	99.5
$\bar{X}$	57.5	42.5	58.5	41.5	1.7	98.3
S.D.	11.42	11.42	12.35	12.35	2.35	2.35

TABLE XIV

Results of analysis of variance between the mean percent values of normal D-larvae obtained 24 h after fertilization at different temperatures. Data were transformed using the regular transformation (arcsin $\sqrt{\%}$) before analysis of variance

Total of experiments	Sources of variation	d.f.	SS	MS	F value	Tabulated value
10	Between groups	2	12743.2	6371.6	159.21***	3.35
	Within groups	27	1080.48	40.02		
	Total	29	13823.68			

***$P < 0.001$.

TABLE XV

Results of Student-Newman-Keuls test between the mean percent values of normal D-larvae obtained 24 h after fertilization at different salinities. The values united by lines do not differ significantly ($P > 0.05$). Mean values are presented with ± 1 standard deviation

Temperature (° C)	30	20	25
Percentage means	1.7 ± 2.35	57.5 ± 11.42	58.5 ± 12.35
Transformed means arcsin $\sqrt{\%}$	6.05 ± 4.53	49.44 ± 6.70	50.08 ± 7.40

TABLE XVI

Mean percent (n = 10 replicates) of normal (N) and abnormal (A) larvae exposed to different combinations of salinity (ppt) and temperature (°C). In all treatments the concentration of sperm/egg and the egg density were 10^3 and 10^4/l respectively

Temperature (°C)					20					
Salinity (‰)	20		25		30		35		40	
	N	A	N	A	N	A	N	A	N	A
1	36.0	64.0	62.0	38.0	52.0	48.0	59.0	41.0	63.0	37.0
2	28.5	71.5	33.0	67.0	34.0	66.0	41.0	59.0	39.0	61.0
3	51.5	48.5	49.5	50.5	50.5	49.5	52.5	47.5	50.5	49.5
4	30.5	69.5	44.5	55.5	54.5	45.5	51.5	48.5	51.5	48.5
5	29.0	71.0	49.0	51.0	44.0	56.0	49.0	51.0	40.0	60.0
6	31.0	69.0	35.5	64.5	39.5	60.5	42.0	58.0	30.5	69.5
$\bar{X}$	34.4	65.6	45.6	54.4	45.8	54.2	49.2	50.8	45.8	54.2
S.D.	8.78	8.78	10.56	10.56	7.98	7.98	6.80	6.80	11.53	11.53
Temperature (°C)					25					
Salinity (‰)	20		25		30		35		40	
	N	A	N	A	N	A	N	A	N	A
1	25.5	74.5	47.5	52.5	42.5	57.5	39.5	60.5	28.5	71.5
2	22.5	77.5	39.0	61.0	37.5	62.5	32.5	67.5	21.0	79.0
3	53.5	46.5	47.5	52.5	66.5	33.5	60.5	39.5	47.5	52.5
4	20.5	79.5	35.5	64.5	41.5	58.5	41.5	58.5	30.5	69.5
5	42.5	57.5	59.5	40.5	66.5	33.5	59.5	40.5	54.5	45.5
6	22.5	77.5	38.0	62.0	50.5	49.5	38.5	61.5	28.5	71.5
$\bar{X}$	31.2	68.8	44.5	55.5	50.8	49.2	45.3	54.7	35.1	64.9
S.D.	13.59	13.59	8.90	8.90	12.83	12.83	11.75	11.75	12.94	12.94
Temperature (°C)					30					
Salinity (‰)	20		25		30		35		40	
	N	A	N	A	N	A	N	A	N	A
1	1.0	99.0	4.0	96.0	2.5	97.5	1.0	99.0	3.0	97.0
2	3.0	97.0	5.0	95.0	4.0	96.0	2.5	97.5	2.0	98.0
3	2.0	98.0	3.0	97.0	1.0	99.0	4.0	96.0	2.5	97.5
4	2.0	98.0	0.0	100.0	3.0	97.0	6.5	93.5	1.0	99.0
5	1.0	99.0	3.5	96.5	5.0	95.0	8.0	92.0	5.0	95.0
6	3.0	97.0	7.0	93.0	7.0	97.0	5.0	95.0	3.0	97.0
$\bar{X}$	2.0	98.0	3.8	96.2	3.7	96.3	4.5	95.5	2.8	97.2
S.D.	0.89	0.89	2.32	2.32	2.09	2.09	2.57	2.57	1.33	1.33

TABLE XVII

Results of the analysis of variance between the mean percent values of normal D-larvae obtained 24 h after fertilization at different combinations of temperature and salinity. Data transformed using the regular transformation (arcsin $\sqrt{\%}$) before analysis of variance

Total of experiments	Sources of variation	d.f.	SS	MS	F value	Tabulated value
6	Between groups total	14	19792.48	1413.75		
	Temperature	2	18911.47	9455.74	181.63***	3.20
	Salinity	4	642.81	160.70	3.09*	2.59
	Interaction	8	238.2	29.78	0.57 n.s.	2.16
	Error	45	2342.92	52.06		
	Total	59	22135.4			

***$P < 0.001$.
* $P < 0.05$.
n.s. = not significant.

TABLE XVIII

Results of Student-Newman-Keuls test between mean percent values of normal D-larvae obtained 24 h after fertilization at different combinations of temperature and salinity considering the temperatures for each salinity tested. Mean values presented with ± 1 standard deviation

Salinity 20 ppt			
Temperature (°C)	30	25	20
Percentage means	34.4 ± 8.78	31.2 ± 13.59	2.0 ± 0.89
Transformed means arcsin $\sqrt{\%}$	7.59 ± 1.90	33.60 ± 8.26	35.82 ± 5.18
Salinity 25 ppt			
Temperature (°C)	30	25	20
Percentage means	3.8 ± 2.32	44.5 ± 8.90	45.6 ± 10.56
Transformed means arcsin $\sqrt{\%}$	10.09 ± 5.29	41.82 ± 5.15	42.43 ± 6.14
Salinity 30 ppt			
Temperature (°C)	30	20	25
Percentage means	3.7 ± 2.09	45.8 ± 7.89	50.8 ± 12.83
Transformed means arcsin $\sqrt{\%}$	10.77 ± 3.31	42.53 ± 4.63	45.52 ± 6.75
Salinity 35 ppt			
Temperature (°C)	30	25	20
Percentage means	4.5 ± 2.57	45.3 ± 11.75	49.2 ± 6.80
Transformed means arcsin $\sqrt{1}$	11.75 ± 3.89	42.28 ± 6.81	44.35 ± 4.17
Salinity 40 ppt			
Temperature (°C)	30	25	20
Percentage means	2.8 ± 1.33	35.1 ± 12.49	45.8 ± 11.53
Transformed means arcsin $\sqrt{\%}$	9.33 ± 2.35	36.08 ± 7.77	42.52 ± 6.72

TABLE XIX

Results of Student-Newman-Keuls test between the mean percent values of normal D-larvae obtained 24 h after fertilization at different combinations of temperatures and salinities, considering the salinities at each temperature tested. Mean values are presented with ± 1 standard deviation

Temperature 20° C					
Salinities (‰)	20	25	40	30	35
Percentage means	34.4 ± 8.78	45.6 ± 10.56	45.8 ± 11.53	45.8 ± 7.98	49.2 ± 6.80
Transformed means arcsin $\sqrt{\%}$	35.82 ± 5.18	42.43 ± 6.14	42.52 ± 6.72	42.53 ± 4.63	44.35 ± 4.17
Temperature 25° C					
Salinities (‰)	20	40	25	35	30
Percentage means	31.2 ± 13.59	35.1 ± 12.94	44.5 ± 8.90	45.3 ± 11.75	50.8 ± 12.83
Transformed means arcsin $\sqrt{\%}$	33.60 ± 8.26	36.08 ± 7.77	41.82 ± 5.15	42.28 ± 6.81	45.52 ± 6.75
Temperature 30° C					
Salinities (‰)	20	40	25	30	35
Percentage means	2.0 ± 0.89	2.8 ± 1.33	3.8 ± 2.32	3.7 ± 2.09	4.5 ± 2.57
Transformed means arcsin $\sqrt{\%}$	7.95 ± 1.90	9.33 ± 2.35	10.09 ± 5.29	10.77 ± 3.31	11.75 ± 3.75

the yields when there are high egg concentrations. Polyspermy could be a contributory cause of low larval yield resulting from high sperm concentrations. However, Stiles and Longwell (1973), working on *C. virginica*, found that the incidence of polyspermy did not correlate with development of eggs to straight-hinge larvae, incidence of abnormal larvae, or to setting failure.

In the present research, when utilizing the best concentrations of sperm and eggs, the percentage of embryos reaching the stage of prodissoconk I was 38—81% (percentages of abnormalities between 62.0 and 19.0%). Stiles and Longwell (1973), working on *C. virginica*, found that abnormalities of fertilization, meiosis and cleavage, and heteroploidy occurred in 10—86% of the eggs in different mass-spawned groups in the laboratory. In these groups the average number of sperm/egg did not approach 20, at which the incidence of chromosome and division abnormalities would be expected to increase. Such rates of abnormalities may therefore be considered as natural.

The oysters used as parents in these experiments on *C. rhizophorae* were not selected by size, or because they had spawned previously, since for oysters no significant difference has been noted between the quality of gametes from individuals of different sizes (Loosanoff and Davis, 1963) or from the first and last spawning (Davis and Chanley, 1956). However, ovocyte quality (Nascimento, 1978) and good condition of the parents (Galtsoff, 1964) were taken into account in our experiments.

From the species *C. gigas* Helm and Millican (1977) found that if fertilization was delayed for more than 60—90 min after gamete liberation, the proportion of larvae which developed was greatly reduced. The present research corroborated these results for *C. rhizophorae*, showing however no significant differences in the percentages of normal D-larvae formed when the

sperm fertilized the eggs 15–45 min after collection from the gonads.

The propagation of desirable commercial molluscs depends upon an adequate knowledge of their spawning and of the environmental requirements of their embryos and larvae. Temperature and salinity are two environmental factors of primary importance for the successful development of oyster larvae (Galtsoff, 1964). Because *C. rhizophorae* is a tropical species generally occupying a habitat in which the water temperature is not below 20° nor above 30°C, one experiment was done within this temperature range. The results showed no statistical differences between the mean values of normal D-larvae obtained at 20° or 25°C, but the results obtained at 30°C were significantly lower. Thus, for the successful development of *C. rhizophorae* embryos the temperature should be lower than 30°C. These data are in agreement with those obtained by Helm and Millican (1977) who found that the temperature for *C. gigas* embryonic development should not exceed 25°C. Davis and Calabrese (1964) found that *C. virginica* larvae are well able to tolerate temperatures between 17.5 and 30°C when reared at 25 ppt of salinity. *C. rhizophorae* embryos develop well in a salinity range of 25—37 ppt, at temperatures of 20°—25°C, while *C. gigas* embryos develop well at 19—27 ppt (Amemiya, 1928), the larvae developing best at 25°C (Helm and Millican, 1977). *C. virginica* embryos do not develop normally in salinities lower than 17.5 ppt (Davis and Calabrese, 1964) and our research shows that *C. rhizophorae* embryos do not develop at salinities lower than 19 ppt. As the range of temperature tested was narrow, these results were confirmed when the combined effects of temperature and salinity were examined.

CONCLUSIONS

1. The highest proportion of normal D-larvae of *C. rhizophorae* was obtained at concentrations between 500 and 5000 sperm per egg under conditions of 25 ppt salinity and at a temperature of 25.0 ± 1.0°C.
2. The best densities of eggs for the production of normal D-larvae were 10^4—4×10^4 per litre.
3. Fertilization should occur within 45 min of the sperm liberation from the gonad. Delays of 45—120 min lead to reduction of the yield of normal D-larvae.
4. The optimum temperature for the development of *C. rhizophorae* embryos is below 30°C. At 20 and 25°C there is a high proportion of normal D-larvae 24 h after fertilization.
5. The best salinities for the embryonic development of *C. rhizophorae* were between 25 and 37 ppt. Below a salinity of 16 ppt the percentage of normal D-larvae was less than 2.5%.

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