AN ABSTRACT OF THE THESIS OF

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Howard F. Horton

Crassostrea gigas sperm were cyropreserved in liquid nitrogen vapor and subsequently used to fertilize fresh C. gigas eggs. Sperm at a concentration of 2.7×10^9/ml were diluted 1:1 with a 20% dimethyl sulfoxide (DMSO)-sea water (SW) solution (v/v), frozen at ca. -170 C for 24 hr, and thawed in a 22 C water bath. A mean percent fertilization ($\%Z_{\bar{x}}$) of 36.3 (compared to 85.4 in controls) resulted when 7.3×10^5 cryopreserved sperm in 0.07 ml SW were added to 100 fresh C. gigas eggs in 0.2 ml SW. Varying the initial sperm concentration, dilution ratio, DMSO concentration, freezing rate and thawing temperature, as well as adding 0-20% glycerol or 1.0 M glycine (v/v) did not increase $\%Z_{\bar{x}}$, although several different combinations of these factors provided comparable protection. A factorial experiment was used to determine the influence of the seven factors on both $\%Z_{\bar{x}}$ and mean percentage of larvae developing to the D-shape stage ($\%D_{\bar{x}}$). A five-factor analysis of variance

(glycerol and glycine concentrations, freezing rate, thawing temperature, and dilution ratio) was computed for all solutions containing 10% and 20% DMSO and initial sperm concentrations of 2.7×10^9/ml and 3.0×10^8/ml; $\%Z_{\bar{x}}$ and $\%D_{\bar{x}}$ were the dependent variables.

Similar attempts to cryopreserve C. gigas eggs were unsuccessful.

Also reported is a method of fertilization using precise gamete concentrations in small volumes of SW; evidence of polyspermy was minimal. Sperm concentrations between 5.0×10^6/ml and 4.3×10^7/ml were shown to be linearly related to light diffusion readings on a Klett-Summerson Colorimeter.

Cryobiological Investigations of the Gametes of the Pacific Oyster, Crassostrea gigas

by

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CRYOBIOLOGICAL INVESTIGATIONS OF THE GAMETES OF THE PACIFIC OYSTER, CRASSOSTREA GIGAS

INTRODUCTION

Cryobiological investigations have been predominantly concerned with gametes of economically important mammals and birds; Sherman (1964) reviewed both cryopreservative investigations and hypothesized mechanisms of cryoprotection. More recently, Litvan (1972) reviewed proposed mechanisms of cryoprotection and offered a new and more acceptable hypothesis. The structure and function of cryophylactic agents was reviewed by Doebbler (1966).

Cryobiological studies using the gametes of marine and fresh water organisms have been minimal. Low temperature research on fish sperm was reviewed by Graybill and Horton (1969) who reported cryopreservation of the sperm of steelhead trout (Salmo gairdneri). Their study, and that of Ott and Horton (1971) in which sperm of both chinook salmon (Oncorhynchus tshawytscha) and coho salmon (O. kisutch) were cryopreserved, cleary show that cryopreservative techniques and cryoprotective solutions used for gametes of anadromous species are different from those used for the germ cells of terrestrial organisms.

To cross-fertilize individual Pacific oysters (Crassostrea gigas) after sex reversal, Lannan (1971) cryopreserved sperm from this

species using a 20% dimethyl sulfoxide (DMSO)-sea water (SW) solution (v/v); he reported 0-10.3% fertilization of fresh eggs and 0-3.1% development to straight-hinged larvae. Ott (unpublished data) also reported that sperm of C. gigas cryopreserved with a 20% DMSO-SW solution fertilized fresh eggs; however, development was not carried past cleavage and the possibility of latent injury (Sherman 1967) must be considered. A similar examination of echinoderm sperm was reported by Dunn and McLachlan (1973). Neither study quantitatively examined the effects of the freeze-thaw procedure.

The objectives of this study were to determine (1) the cryoprotective properties of several combinations of compounds on C. gigas sperm, (2) the effects of different freezing and thawing rates and sperm concentrations, and (3) the best ratio of cryoprotective solution to sperm suspension. A less extensive investigation was made using the ova of C. gigas. Further, although several parameters in experimental oyster fertilization have been examined by many authors (reviewed by Davis and Calabrese 1964, and Galtsoff 1964), none reported gamete concentrations used, and their methods of fertilization were more suitable for hatchery studies than for small-scale lab investigations. Thus, I developed more useful and precise methods of experimental fertilization.

EXPERIMENTAL

General

Oysters (Crassostrea gigas) were purchased from the Fowler Oyster Co. on Yaquina Bay, Newport, Ore. Although "local" C. gigas occasionally had mature gametes, oysters delivered to the Fowler Co. from Hood Canal in Washington produced consistently higher concentrations of mature gametes.

All SW used was sand-filtered and exposed to ultraviolet light at a flow rate of 1 gal/min. SW of 25-32 ppt salinity and pH range 7.0-8.1 was collected at the Oregon State University Marine Science Center (MSC); because Lannan (1973) reported the MSC experimental oyster hatchery occasionally evidences unusually high larval mortality from late Apr. until early Nov., SW was collected from Nov. 15 to Apr. 1. Except for the sand-filtered flow-through SW system (fluctuating salinity, 24-32 ppt) supplying SW to oyster trays at the MSC, all SW was diluted to 25 ppt with distilled water and stored in Nalgene carboys; all SW used in fertilization experiments was also Millipore-filtered (0.47 μm).

Because Breese (unpublished data) reported that numerous materials were toxic to oyster larvae, the following precautions were taken: glassware was initially acid-washed; used glassware was

carefully cleaned and rinsed several times in both tap and distilled water; all tubing for the carboys was Tygon R3603 (nontoxic by bioassay, unpublished data by Breese); gametes and larvae were confined in glass containers only (except for momentary exposure to stainless-steel syringe needles and nylon screen); all Millipore-filtered SW was stored in glass screw-cap bottles with Parafilm-lined caps (nontoxic by bioassay, unpublished data by Breese).

Procurement of Gametes

To enhance gonad development, oysters were kept in a 16.0 ± 1.0 C "conditioning" tray at the MSC for 3-6 weeks (Loosanoff and Davis 1963). At least one week prior to gamete extraction, 18-36 oysters were transferred to a conditioning tray (recirculating SW) in the Corvallis lab. Mortality rates in the conditioning trays were below 10% for all oysters kept 4-8 weeks.

Oysters were tagged for identification by drilling a 1/32-in. hole in the umbo and attaching a 1/4-x-5/8-in. numbered plastic tag (Howitt Plastics Co., Mollala, Ore. 97038) with monofilament. After several weeks of conditioning, access to the gonads was made by drilling a 3/64-in. hole in the posterior-dorsal region of the right test (flat side, directly over the gonad); gametes were extracted with a glass syringe (2.5 cc fitted with a 20 gauge 1.5-in. needle) containing 0.5 ml SW (Lannan 1971). To protect gametes from large

pressure changes during gonad extractions, a gentle and constant negative pressure was maintained in the syringe; gametes abruptly drawn into the syringe were often damaged and were thus discarded. Only extractions which entered the syringe as dense white cords were used; when gonad extracts entered the syringe in clouds, the material was discarded and a fresh syringe employed.

Extracted gametes were examined by light microscopy; eggs larger than 36 μm and intensely active sperm were used. To prevent spawning after extractions, oysters were isolated for 12-24 hr in 3 l beakers containing SW at 12 C. Oysters without mature gametes were returned to the conditioning tray at the MSC and those with mature gametes were maintained in Corvallis.

Since secretions from oyster gametes are apparently important in fertilization (Galtsoff 1964)--and presumably secretions from the gonads are equally important--the temperature of all donor oysters was brought to 27 $\pm$ 0.5 C; this temperature is in the range recommended for fertilization and larval development by Davis and Calabrese (1964). Oysters were transferred from the conditioning tray to a small holding tank (5 gal) containing SW at 16 C; a 100-watt aquarium heater connected to a thermostat was then used to slowly raise the temperature to 27 $\pm$ 0.5 C. All SW and glassware used in fertilization experiments were stored in an incubator at 27 $\pm$ 0.5 C, and the pH of all SW used ranged from 7.0-7.8. Finally, except for

sperm used in ageing experiments at 4 C, samples were stored at 27 ± 0.5 C.

Gamete Concentrations

Extractions from the gonads of 3-5 males were transferred to and agitated in a Klett-Summerson sample tube containing 5-8 ml SW; light diffusion through the tube was measured on a Klett-Summerson Colorimeter with a green (#54) filter, and sperm samples were counted in a hemacytometer. Using serial dilutions from several different starting concentrations, I plotted a graph relating sperm concentration to Klett reading (Fig. 1). Because Klett readings below K = 10 were less precise than higher readings, sperm concentrations less than 5.0×10^6/ml were obtained by diluting samples reading K > 10; further, the sperm concentration-Klett reading relationship was not linear above K = 80.

Gonad extractions from 3-5 females were similarly pooled, transferred to a Nytex screen (36 μm mesh), and rinsed with SW to remove small debris and reduce the possibility that some component of the eggs (released when broken during extraction) would cause sperm to agglutinate (Galtsoff 1964). Cleaned eggs were then rinsed into a 250 ml beaker containing 20-50 ml SW. Egg samples were counted under a dissection microscope, diluted to the desired concentration, and maintained at 27 ± 0.5 C. Eggs remaining in SW for more than

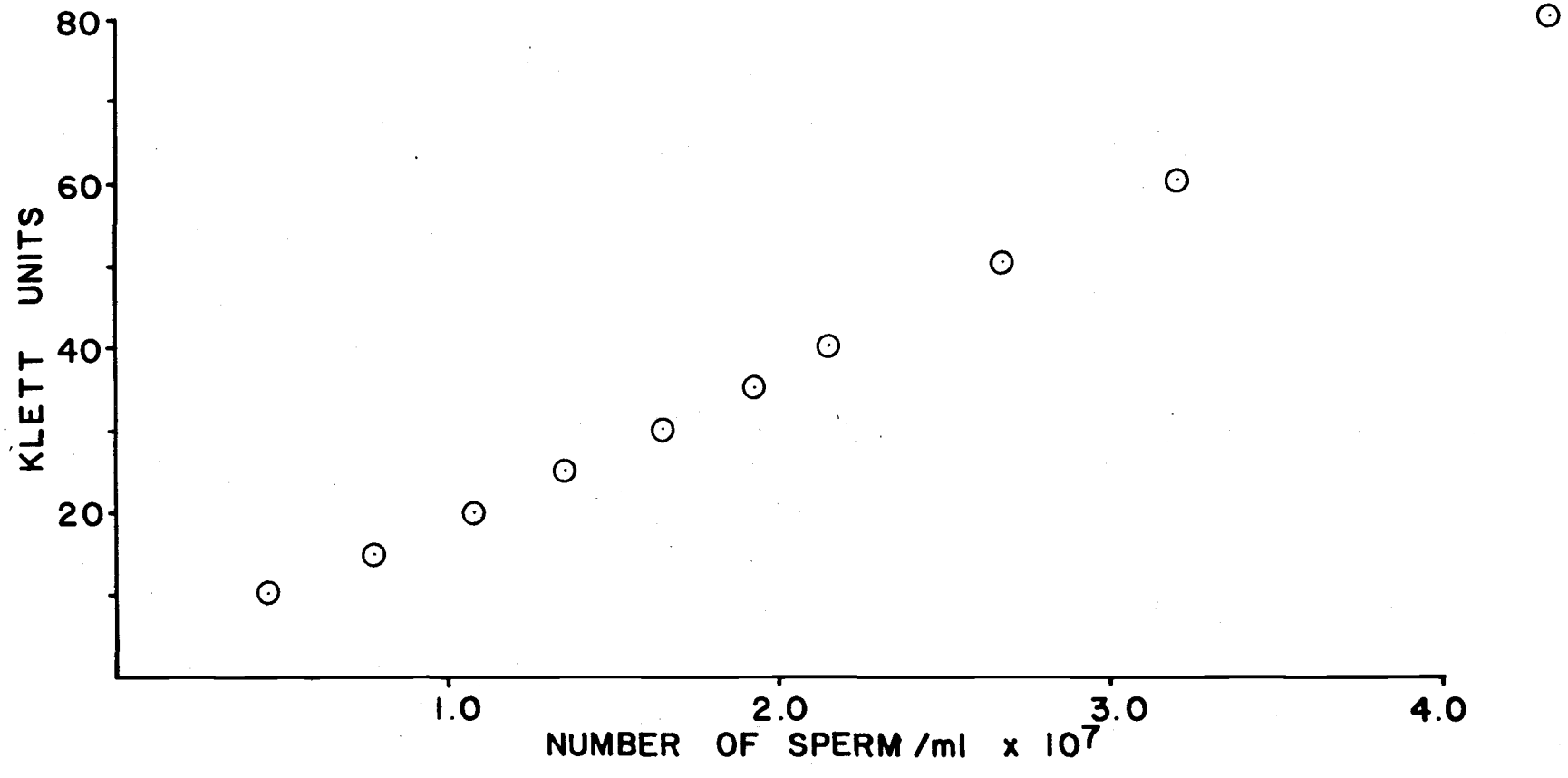

Figure 1. Relationship between concentration of Crassostrea gigas sperm (by hemacytometer count) and light diffusion reading on Klett-Summerson Colorimeter.

1 hr were discarded to reduce the possibility of sperm agglutination resulting from secretions.

Fertilization

Using a Pasteur pipette (45 drops/ml), I transferred predetermined sperm concentrations to numbered Syracuse watch-glasses, then added 0.2 ml of an egg-SW suspension with an automatic pipette. Egg-sperm solutions were flooded with 7 ml SW to dilute the sperm concentration and reduce the possibility of polyspermy. The watch-glasses were then stacked to reduce evaporation and incubated at 27 $\pm$ 0.5 C. Since the number of swimming larvae did not increase after 6 hr post-fertilization time, the number of fertilized eggs was obtained by counting unfertilized eggs remaining on the bottom at 6 hr and subtracting this figure from the number of eggs originally present. After 24 hr the watch-glasses were transferred to a 4 C refrigerator; within 30 min the D-shape (straight-hinged) larvae settled to the bottom and were easily counted.

Freeze-thaw Procedures: Sperm

Cryoprotective solutions (CPS), sperm concentrations (SC), ratios of CPS:SC, and freezing and thawing rates were examined in a factorial experiment which is partially diagrammed in Fig. 2. All solutions were stored at 27 $\pm$ 0.5 C, and all were within the desired

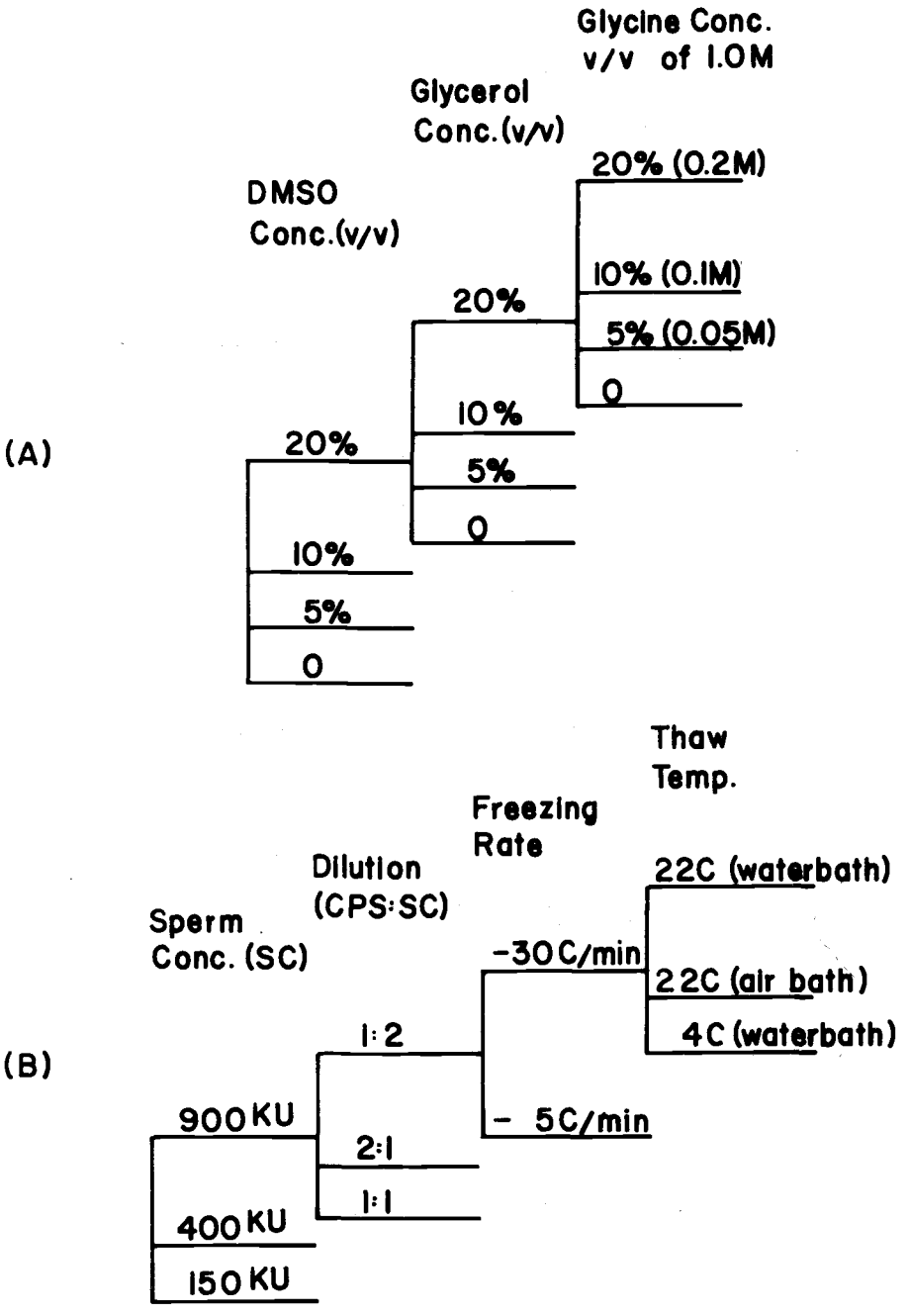

Figure 2. Partial factorial diagram of (A) combinations and concentrations of cryoprotective agents used, and (B) parameters examined.
(KU = Klett-Summerson Colorimeter units: 900 = 2.7×10^9 sperm/ml; 400 = 3.0×10^8 sperm/ml; 150 = 5.7×10^7 sperm/ml; CPS = cryoprotective solution)

7.0-8.0 pH range (Humphrey 1950) without the addition of buffering agents. Sperm in CPS were examined for motility before being subjected to a freeze-thaw regimen; in all cases I observed at least some motile sperm. Sperm-CPS samples (0.5 ml) were pipetted into ampules using a Pasteur pipette fitted in the bulb of an automatic pipette. Temperature reduction methods used were those of Graybill and Horton (1969) except that ampules were not sealed. In preliminary studies Ott (unpublished data) reported that storage of oyster sperm at -170 C provided cryoprotection, whereas storage at -196 C proved detrimental; therefore, all ampules were stored in liquid nitrogen vapor (ca. -170 C) for 24 hr.

Solutions were thawed in one of the following ways: (1) a 22 C water bath for 2 min; (2) an air bath at room temperature (22 C) for 5 min; and (3) a 4 C water bath for 3 min followed by 2 min in a 22 C water bath. All solutions were then warmed to 27 $\pm$ 0.5 C. Thawed sperm-CPS solutions were then diluted and examined to determine whether or not motile sperm were present. To determine how much SW was necessary to dilute each sperm-CPS sample, the following procedure was employed: using SW in place of CPS, I diluted sperm concentrations of K = 900 (2.7×10^9/ml), K = 400 (3.0×10^8/ml), and K = 150 (5.7×10^7/ml) such that SW:SC = 1:1, 1:2, and 2:1; measured amounts of SW were then added until the desired concentration (K = 20) was reached. Thus, after thawing, the solution in each ampule was

transferred to a test tube and this predetermined amount of SW at 27 ± 0.5 C was added. Solutions containing motile sperm were then tested for fertilization capacity (with fresh eggs) using the optimal sperm concentration and dilution time (Tables 1 and 2); three repetitions were performed for each diluted sample.

Controls: Sperm

Although none of the 460 oysters examined were hermaphroditic, sperm-free controls were used in all experiments. To eliminate the possibility of chemical parthenogenesis, I treated fresh eggs (0.2 ml egg-SW suspension/watch-glass) with 0.5 ml of each CPS. To assure egg viability, samples of those used with freeze-thaw sperm were fertilized with fresh sperm; to assess the viability of sperm used in freeze-thaw procedures, samples of untreated sperm were used to fertilize fresh eggs.

Freeze-thaw Procedure and Controls: Eggs

Samples of egg-SW suspensions (10^4 eggs/ml) were diluted 1:1 with each CPS, frozen at -30 C/min, stored at -170 C for 24 hr, and thawed in the 4 C water bath before warming to 27 ± 0.5 C. Prior to freezing, 0.2 ml samples were fertilized with fresh sperm (1 ml containing 3.3×10^7 sperm) to determine if the CPS inhibited fertilization. After warming to 27 ± 0.5 C, thawed samples were diluted with

Table 1. Range and mean percent fertilization (%Z) and percentage of larvae developing to the D-shape stage (%D) resulting from different sperm concentrations combined with fresh eggs (100 ± 4/0.2 ml) of Crassostrea gigas. [1]

Sperm		%Z		%D	
Concentration	Volume (ml)	Range	Mean	Range	Mean
1.1×10^4	0.02	0 - 58	27.6	0-56	25.0
3.3×10^4	0.07	10 - 55	34.4	10-50	29.2
5.5×10^4	0.11	41 - 86	57.4	36-73	48.0
1.1×10^5	0.22	55-100	77.2	50-93	58.6
2.4×10^5	0.02	60-100	72.6	47-88	58.2
7.3×10^5	0.07	75-100	87.0	51-94	68.8
1.2×10^6	0.11	78-100	89.0	35-70	45.2
2.4×10^6	0.22	80-100	89.2	13-72	29.0
5.0×10^6	0.50	80-100	92.4	8-43	21.9
1.1×10^7	1.00	87-100	96.0	0-23	15.0

[1] Sea water of salinity 25 ppt and pH 7.8 ± 0.1; temperature 27 ± 0.5 C; gametes diluted with 7 ml SW at 10 min post-fertilization; 5 repetitions per sperm concentration.

Table 2. Range and mean percent fertilization (%Z) and percentage of larvae developing to the D-shape stage (%D) resulting from reducing the concentration of gametes of Crassostrea gigas at various post-fertilization time intervals. [1]

Flooding time[2] (min post-fertilization)	%Z		%D	
	Range	Mean	Range	Mean
1	41 - 88	67.2	41-72	62.0
5	73-100	85.4	65-94	73.0
10	75-100	87.8	51-94	68.2
15	78-100	89.8	0-68	47.0
30	82-100	96.4	0-20	8.8

[1] Sperm concentration = 7.3×10^5 /0.07 ml; mean egg concentration = 100 $\pm$ 4/0.2 ml sea water (SW); SW of salinity 25 ppt and pH 7.8 $\pm$ 0.1; 5 repetitions per flooding time.

[2] Flooding time = 7 ml SW added to the gamete mixture at indicated time interval.

SW to a concentration of 500 eggs/ml, and 0.2 ml (100 eggs) were combined with 3.3×10^7 sperm (1 ml). To eliminate the possibility of temperature induced parthenogenesis, I used frozen-thawed sperm-free controls.

Analysis of Data

Data on independent variables (freezing rates, thawing rates, initial sperm concentrations, glycine concentrations and glycerol concentrations) and dependent variables (mean percent fertilization [$\%Z_{\bar{x}}$] and mean percentage of larvae developing to the D-shape stage [$\%D_{\bar{x}}$]) were entered into a CDC-3300 computer; to reduce the variance in each repetition, the following variable transformations were made: arcsin $\sqrt{\%Z}$ = degrees of fertilization, and arcsin $\sqrt{\%D}$ = degrees of D-shape larvae. A 5-factor analysis of variance for both $\%Z_{\bar{x}}$ and $\%D_{\bar{x}}$ (transformed) was obtained for K = 900 and K = 400 in solutions containing 10% DMSO, and K = 900 and K = 400 in solutions containing 20% DMSO. This program (OSU computer program NANOVAX) provided sums of squares, mean squares, degrees of freedom, grand means, individual means, and means of two-way interactions. The grand means, individual means, and means of two-way interactions are presented in the Appendix; Table 3 presents F values of individual factors and combinations of factors derived from the mean squares. Back-transformations of the data in

Table 3. F-values of independent variables and combinations of variables used in a factorial experiment testing the effects of dilution ratio (U), freezing rate (E), thawing rate (T), glycerol concentration (L), and glycine concentration (N) on mean percent fertilization ($\%Z_{\bar{x}}$) and mean percentage of larvae developing to the D-shape ($\%D_{\bar{x}}$) using fresh eggs and cryopreserved sperm of Crassostrea gigas.

Source of Variation	df[1]	$F_{0.95}$	10% DMSO[2] 400 KU[3]		10% DMSO 900 KU[4]		20% DMSO[5] 400 KU		20% DMSO 900 KU	
			$\%Z_{\bar{x}}$	$\%D_{\bar{x}}$	$\%Z_{\bar{x}}$	$\%D_{\bar{x}}$	$\%Z_{\bar{x}}$	$\%D_{\bar{x}}$	$\%Z_{\bar{x}}$	$\%D_{\bar{x}}$
U	2	3.00	175.80	66.98	210.30	56.95	397.90	341.70	585.10	405.50
E	1	3.84	3.88	5.90	0.39	1.04	6.91	6.99	0.24	0.02
UE	2	3.00	1.17	1.63	0.78	0.26	2.09	1.85	2.30	5.56
T	2	3.00	0.24	1.68	11.30	3.08	223.30	185.40	475.90	349.32
UT	4	2.37	0.13	0.59	3.71	1.10	27.31	24.21	39.78	24.48
ET	2	3.00	2.93	2.06	1.39	1.27	3.74	2.67	19.14	9.90
UET	4	2.37	1.35	1.46	1.25	0.71	4.24	3.40	0.78	0.90
L	3	2.60	9.26	9.75	34.19	12.32	560.20	426.40	838.50	506.10
UL	6	2.10	3.02	3.25	9.76	4.48	30.96	35.79	54.78	41.29
EL	3	2.60	0.70	0.50	0.20	0.23	4.82	5.38	14.99	8.59
UEL	6	2.10	0.33	0.18	0.91	0.65	4.33	3.76	10.47	6.80
TL	6	2.10	0.70	0.78	2.18	1.51	23.56	30.18	20.30	21.94
UTL	12	1.75	0.31	0.51	2.44	1.30	6.40	5.36	16.09	10.92
ETL	6	2.10	0.51	1.13	0.97	1.28	1.32	1.46	2.80	2.92
UETL	12	1.75	0.95	1.21	2.88	1.30	3.36	3.46	5.56	4.02
N	3	2.60	3.61	4.41	0.66	1.15	30.92	23.50	36.90	45.22
UN	6	2.10	2.10	2.12	0.62	1.76	0.70	0.72	7.23	4.24
EN	3	2.60	5.33	4.97	0.11	0.33	2.65	2.44	2.44	1.00
UEN	6	2.10	1.44	1.75	1.88	0.55	0.94	1.21	8.56	6.71
TN	6	2.10	1.37	0.95	1.85	1.91	3.69	4.09	8.53	8.36
UTN	12	1.75	0.71	0.72	1.48	1.34	1.52	1.22	2.50	2.55
ETN	6	2.10	1.03	2.73	0.71	0.81	1.03	1.19	1.44	1.19
UETN	12	1.75	1.12	1.04	1.71	1.45	1.54	1.73	0.97	1.18
LN	9	1.88	0.77	0.67	8.32	4.11	9.18	5.50	8.50	5.99
ULN	18	1.59	0.90	0.48	2.52	2.03	2.12	1.09	5.54	2.53
ELN	9	1.88	0.94	1.28	1.24	0.76	1.95	1.76	5.60	3.88
UELN	18	1.59	1.30	0.99	1.14	0.68	0.59	0.96	1.73	1.65
TLN	18	1.59	0.62	0.59	1.77	0.86	2.63	2.27	5.69	3.75
UTLN	36	1.40	0.78	0.94	1.44	0.74	0.84	0.95	2.31	1.93
ETLN	18	1.59	1.34	1.26	1.31	0.93	1.76	1.02	1.60	1.37
UETLN	36	1.40	1.05	1.04	2.28	1.56	1.71	1.27	1.87	1.84

[1] df = degrees of freedom

[2] 10% DMSO = cryoprotective solutions containing 10% dimethyl sulfoxide (v/v)

[3] 400 KU = 400 Klett-Summerson Colorimeter units (= initial sperm concentration of 3.0×10^8/ml)

[4] 900 KU = 900 Klett-Summerson Colorimeter units (= initial sperm concentration of 2.7×10^9/ml)

[5] 20% DMSO = cryoprotective solutions containing 20% dimethyl sulfoxide (v/v)

the Appendix can be made using Table A 16 in Snedecor and Cochran (1967).

RESULTS-DISCUSSION

Experimental Fertilization Methods

The salinity (25 ppt) and temperature (27 C) chosen for fertilization and early larval development were within the ideal range reported by Davis and Calabrese (1964); however, these authors did not report gamete concentrations. Further, since Galtsoff (1964) reported high sperm concentrations may result in polyspermy, I first examined the problem of sperm concentration and the need for dilution in small containers. Flooding the watch-glasses with 7 ml SW 5 min after the addition of sperm resulted in minimal polyspermy, probably by reducing the number of sperm-egg collisions; the %Z and %D resulting from dilutions at other time intervals are presented in Table 2. In Table 1 I have recorded %Z and %D obtained using different sperm concentrations. In experiments producing a large decrease from %Z to %D (Tables 1 and 2), aberrant forms were observed (e.g., swimming chains of cells, trochophores persisting past 48 hr), suggesting polyspermy had occurred. Three drops of sperm suspension at K = 20 (each drop from the Pasteur pipette = 0.022 ml) provided the desired number of sperm (7.3×10^5).

Sperm Concentration

Gonad extractions often contained large pieces of gonadal tissue

as well as gametes. The presence of a variable amount of tissue undoubtedly resulted in less than precise colorimetric measurements; however, attempts to obtain samples with less debris by centrifugation resulted in agglutination, and gravity filtration resulted in an increased number of sperm with bent or broken tails. Counting sperm from different extractions revealed that the number of sperm/ml at K = 20 ranged from 9.9×10^6 to 1.1×10^7; thus I considered this method of extraction and estimation of sperm concentration sufficiently precise.

Life Extenders

Attempts to cryopreserve sperm from other organisms has in nearly all cases required a life extender, i.e., a chemical or group of chemicals which increases the effective life span of spermatozoa by reducing their activity or increasing their ability fo fully utilize endogenous reserves (Sherman 1964; Ott and Horton 1971). In experiments preliminary to cryopreservative investigations, I aged sperm in SW (K = 20) for 7 days at 4 C, and a separate sample for 2 days at 27 C; in both cases sperm retained fertilization capacity--although %Z was quite low--thus obviating the need for more complex life extenders. Unstressed sperm, i.e., sperm fresh from the gonad, would presumably be most suitable for cryopreservative studies; thus, regardless of longevity, all sperm were subjected to the freeze-thaw

regimen less than 1.5 hr after extraction. Dunn and McLachlan (1973) recently reported cryopreservation of echinoderm sperm in DMSO-SW solutions, i.e., without additional chemicals to extend sperm life.

Viability of Thawed Sperm

After thawing and dilution, no motile sperm were observed in samples containing the initial sperm concentration of 5.6×10^7 (K = 150); similarly, no motile sperm were found in samples containing 0 or 5% DMSO. Since I made no attempt to fertilize fresh eggs with these frozen-thawed sperm, they were omitted from the computer analysis. I observed at least some motile sperm in all other samples, although the % motility was often quite low and the activity of individual sperm was appreciably less than that of SW controls. Since evaluation of both percent motility and sperm mobility is subjective, fertilization capacity was the main criterion for successful cryopreservation. Further, as noted by Graham and Pace (1967), survival of motile cells does not indicate that fertilization will ensue.

The means of %Z and %D (both transformed to arcsin $\sqrt{\%}$) resulting from fertilization experiments (using sperm treated with the combinations of cryophylactic agents shown in Fig. 2A [0 and 5% DMSO omitted] and subjected to the freeze-thaw regimen shown in Fig. 2B [K = 150 omitted]) are listed in the Appendix. Statistical

F values of individual and combined factors are presented in Table 3, as are the $F_{0.95}$ values. The influence of significant factors (dilution ratio, thawing temperature, glycine concentration, and glycerol concentration) on $\%Z_{\bar{x}}$ and $\%D_{\bar{x}}$ are illustrated in Fig. 3 and 4. Several significant two-way interactions are similarly graphed in Fig. 5, 6, 7, and 8. In all cases the slope of the $\%D_{\bar{x}}$ line approximates that of the $\%Z_{\bar{x}}$ line for a given sperm and DMSO concentration. This indicates that cryopreserved sperm capable of fertilizing fresh eggs do not exhibit latent injury, i. e., (using cryopreserved sperm) the decrease from $\%Z_{\bar{x}}$ to $\%D_{\bar{x}}$ is similar to the predictable decrease from $\%Z_{\bar{x}}$ to $\%D_{\bar{x}}$ observed using fresh sperm. As noted previously, the loss of some larvae is also dependent on sperm concentration; however, the possibility of polyspermy has been avoided by using concentrations of sperm which would result in negligible polyspermy even if all sperm present were cryopreserved.

General

The main objective of this investigation was to develop a successful methodology for the cryopreservation of oyster gametes, thus I have made no attempt to substantiate existing hypotheses of cryoprotective mechanisms or propose new ones. The parameters chosen in this study have been examined in cryobiological investigations of germ cells of other organisms; their importance in providing some degree of cryoprotection is widely recognized.

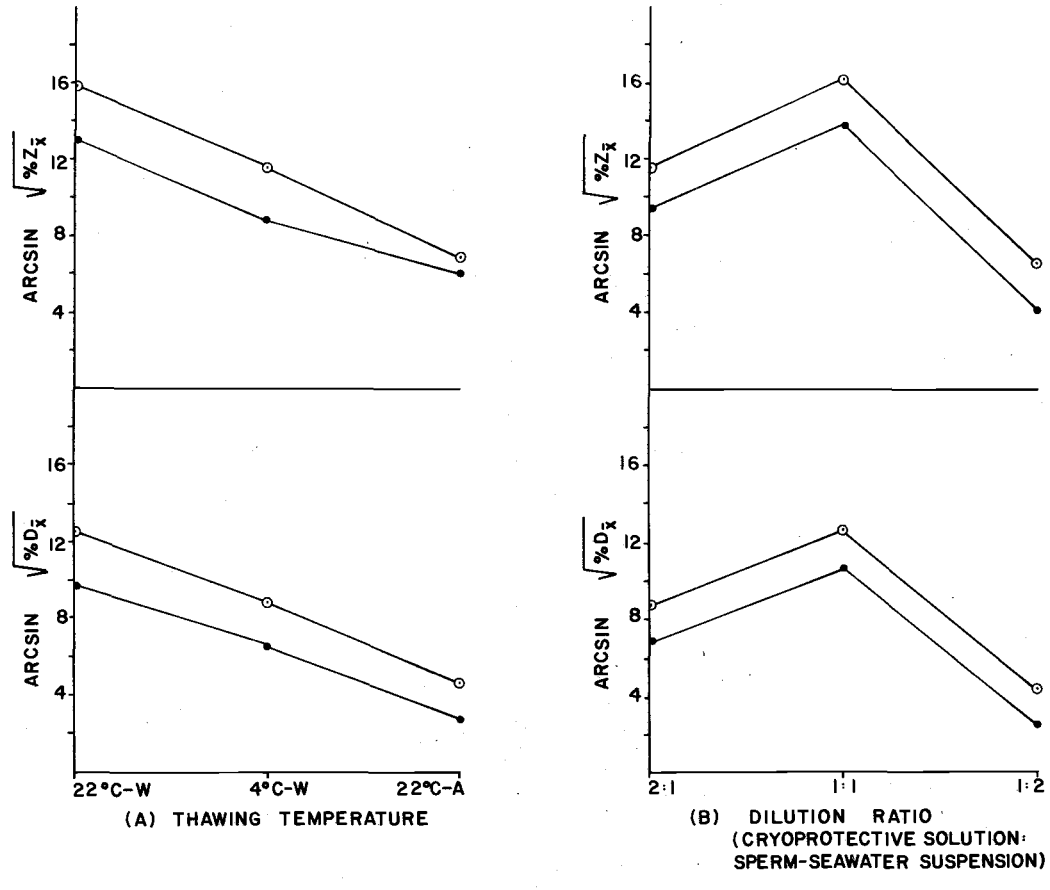

Figure 3. Influence of (A) thawing temperature, and (B) dilution ratio on both arcsin $\sqrt{\%Z_{\bar{x}}}$ ($\%Z_{\bar{x}}$ = mean percent fertilization) and arcsin $\sqrt{\%D_{\bar{x}}}$ ($\%D_{\bar{x}}$ = mean percentage of larvae developing to D-shape) using fresh eggs and cryopreserved sperm of Crassostrea gigas (means plotted are those of all cryoprotective solutions containing 20% dimethyl sulfoxide subjected [in factorial experimentation] to all other freeze-thaw procedures, using 3 repetitions per fertilization). W = water bath; A = air bath; o = initial sperm concentration of 2.7×10^9/ml; ● = initial sperm concentration of 3.0×10^8/ml.

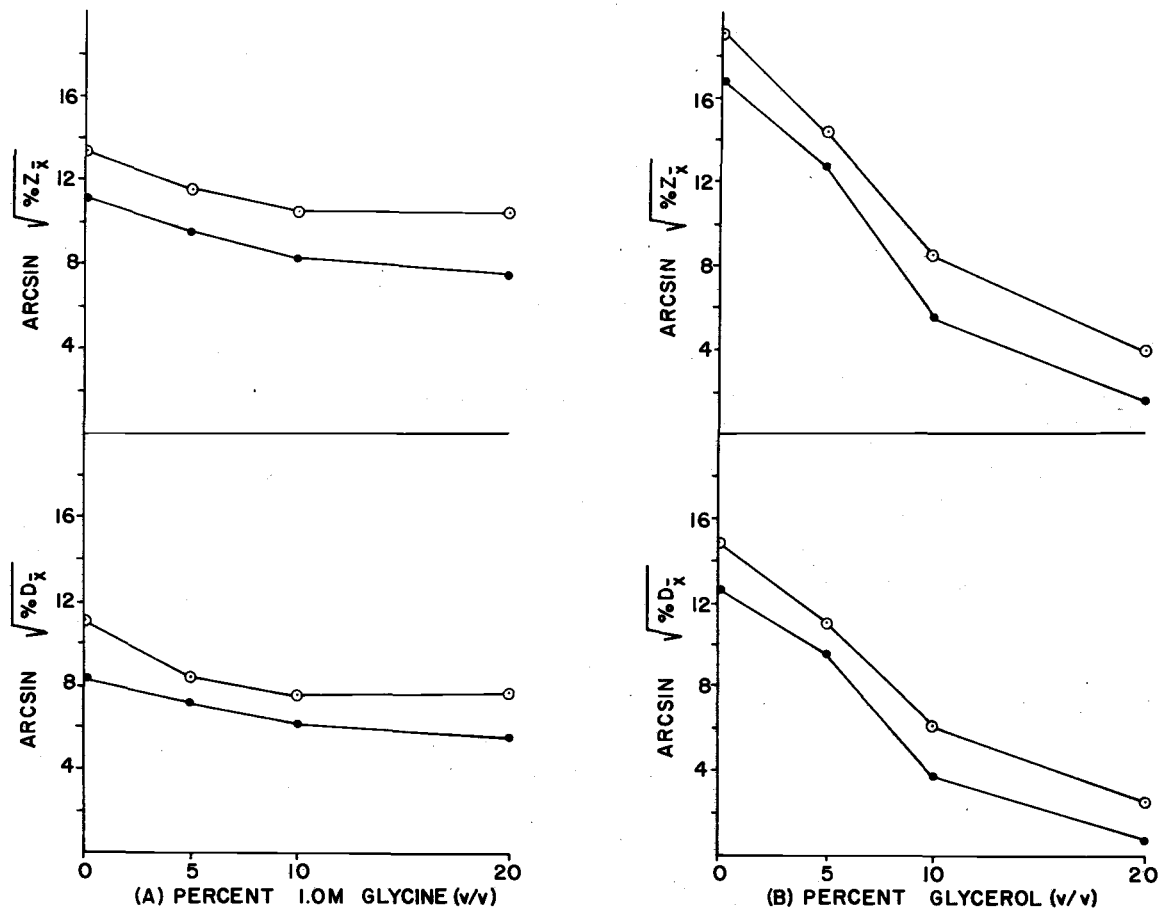

Figure 4. Influence of (A) glycine, and (B) glycerol concentrations on both arcsin $\sqrt{\%Z_{\bar{x}}}$ ($\%Z_{\bar{x}}$ = mean percent fertilization) and arcsin $\sqrt{\%D_{\bar{x}}}$ ($\%D_{\bar{x}}$ = mean percentage of larvae developing to D-shape) using fresh eggs and cryopreserved sperm of Crassostrea gigas (means plotted are those of cryoprotective solutions containing 20% dimethyl sulfoxide subjected [in factorial experimentation] to all freeze-thaw procedures using 3 repetitions per fertilization). o = initial sperm concentration of 2.7×10^9/ml; ● = initial sperm concentration of 3.0×10^8/ml.

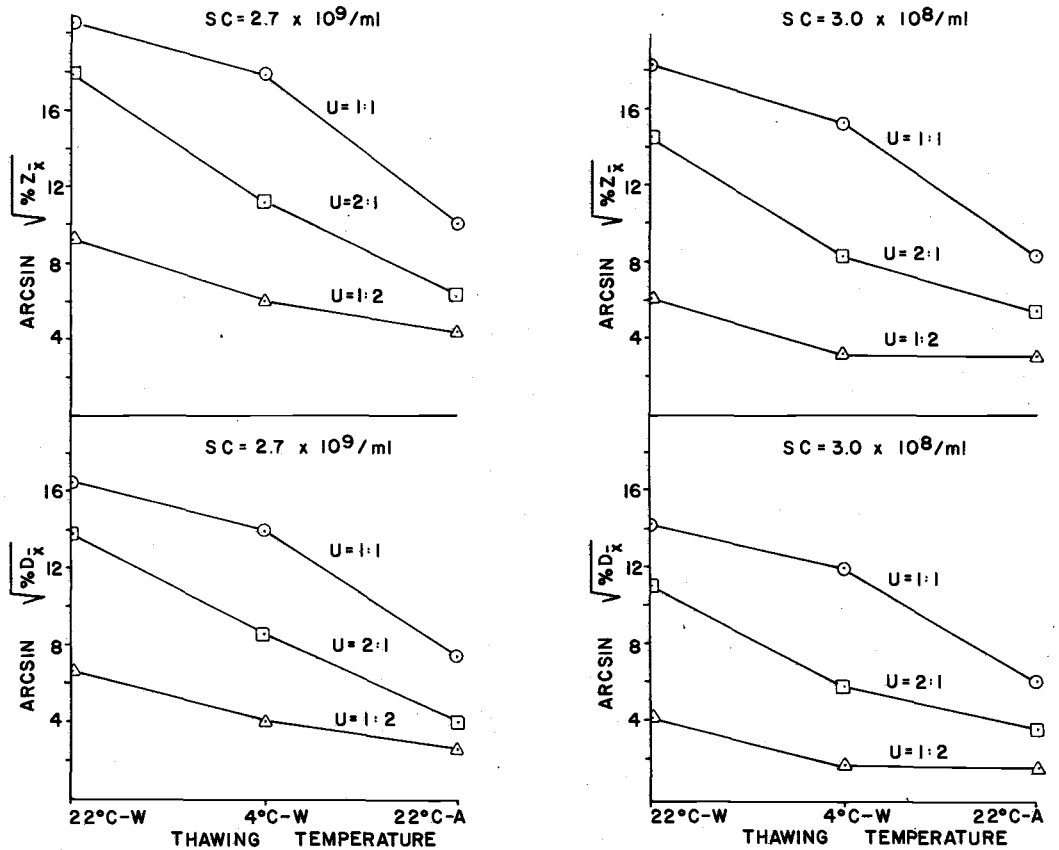

Figure 5. Influence of the interaction between thawing temperature and dilution ratio (U = dilution ratio = cryoprotective solution:sperm-sea water suspension) on both arcsin $\sqrt{\%Z_{\bar{x}}}$ ($\%Z_{\bar{x}}$ = mean percent fertilization) and arsin $\sqrt{\%D_{\bar{x}}}$ ($\%D_{\bar{x}}$ = mean percentage of larvae developing to D-shape) using fresh eggs and cryopreserved sperm of Crassostrea gigas (means plotted are those of cryoprotective solutions containing 20% dimethyl sulfoxide subjected [in factorial experimentation] to all other freeze-thaw procedures using 3 repetitions per fertilization). SC = initial sperm concentration.

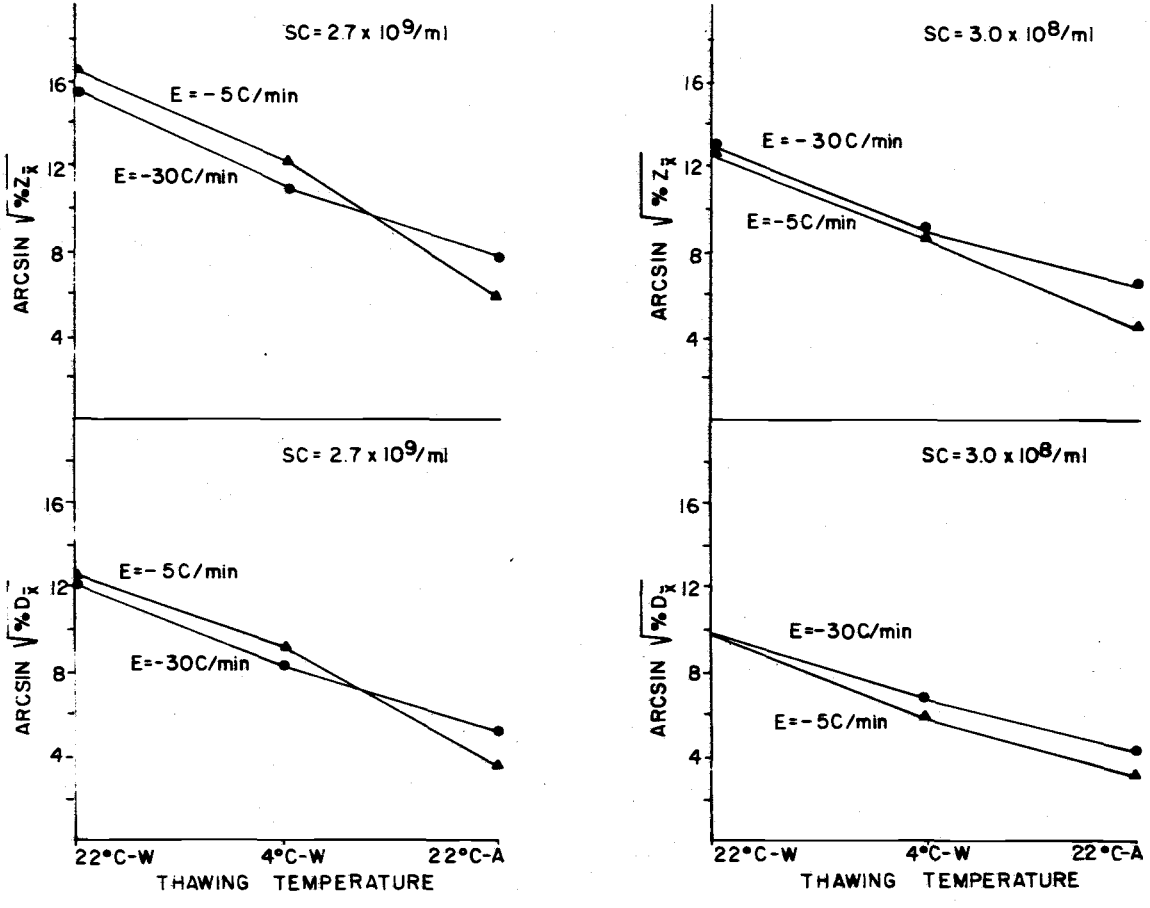

Figure 6. Influence of the interaction between thawing temperature and freezing rate (E) on both arcsin $\sqrt{\%Z_{\bar{x}}}$ ($\%Z_{\bar{x}}$ = mean percent fertilization) and arsin $\sqrt{\%D_{\bar{x}}}$ ($\%D_{\bar{x}}$ = mean percentage of larvae developing to D-shape) using fresh eggs and cryopreserved sperm of Crassostrea gigas (means plotted are those of cryoprotective solutions containing 20% dimethyl sulfoxide subjected [in factorial experimentation] to all other freeze-thaw procedures using 3 repetitions per fertilization). SC = initial sperm concentration.

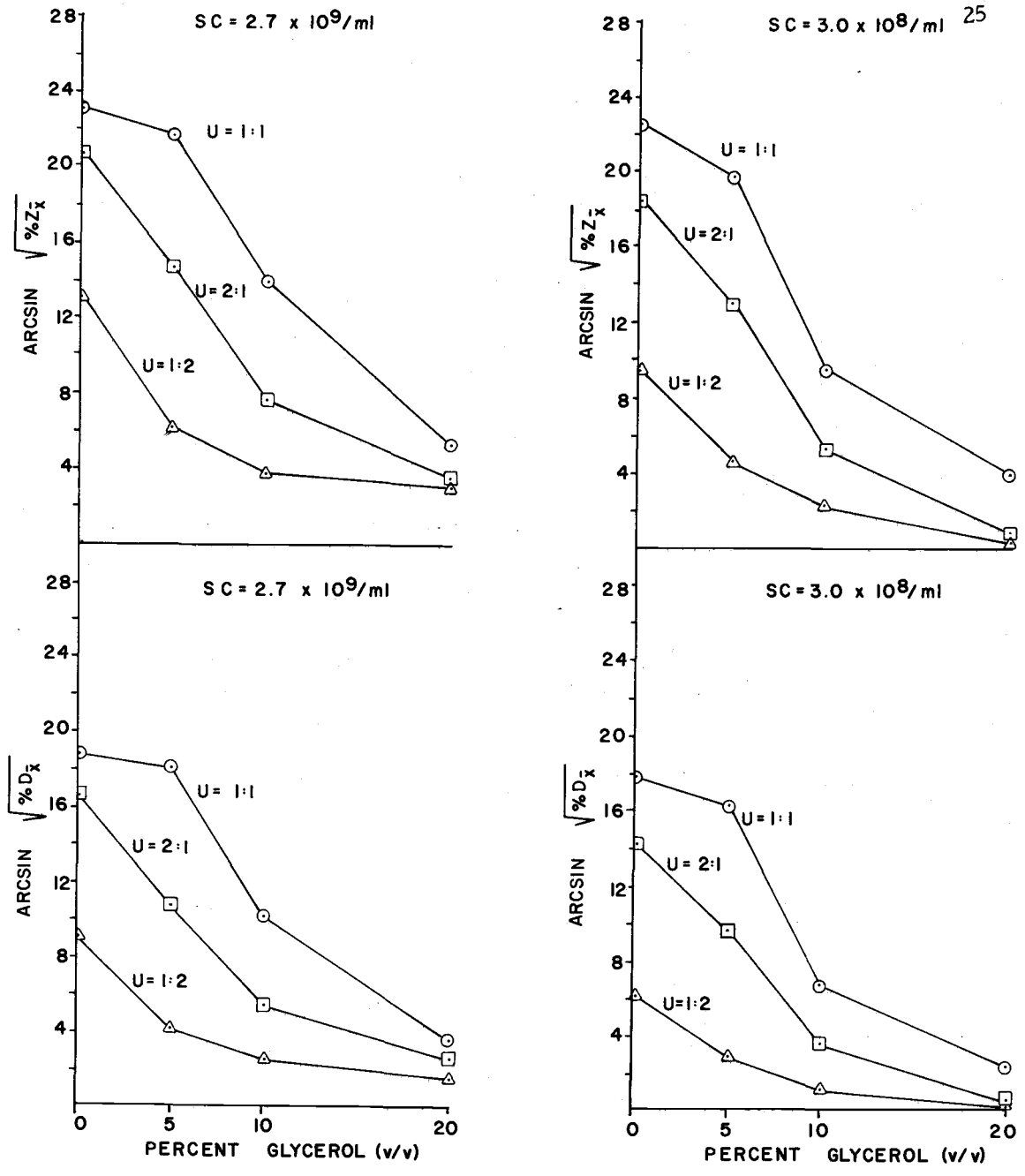

Figure 7. Influence of the interaction between glycerol concentration (v/v) and dilution ratio (dilution ratio = U = cryoprotective solution:sperm-sea water suspension) on both arcsin $\sqrt{\%Z_{\bar{x}}}$ ($\%Z_{\bar{x}}$ = mean percent fertilization) and arcsin $\sqrt{\%D_{\bar{x}}}$ ($\%D_{\bar{x}}$ = mean percentage of larvae developing to D-shape) using fresh eggs and cryopreserved sperm of Crassostrea gigas (means plotted are those of cryoprotective solutions containing 20% dimethyl sulfoxide subjected [in factorial experimentation] to all other freeze-thaw procedures using 3 repetitions per fertilization). SC = initial sperm concentration.

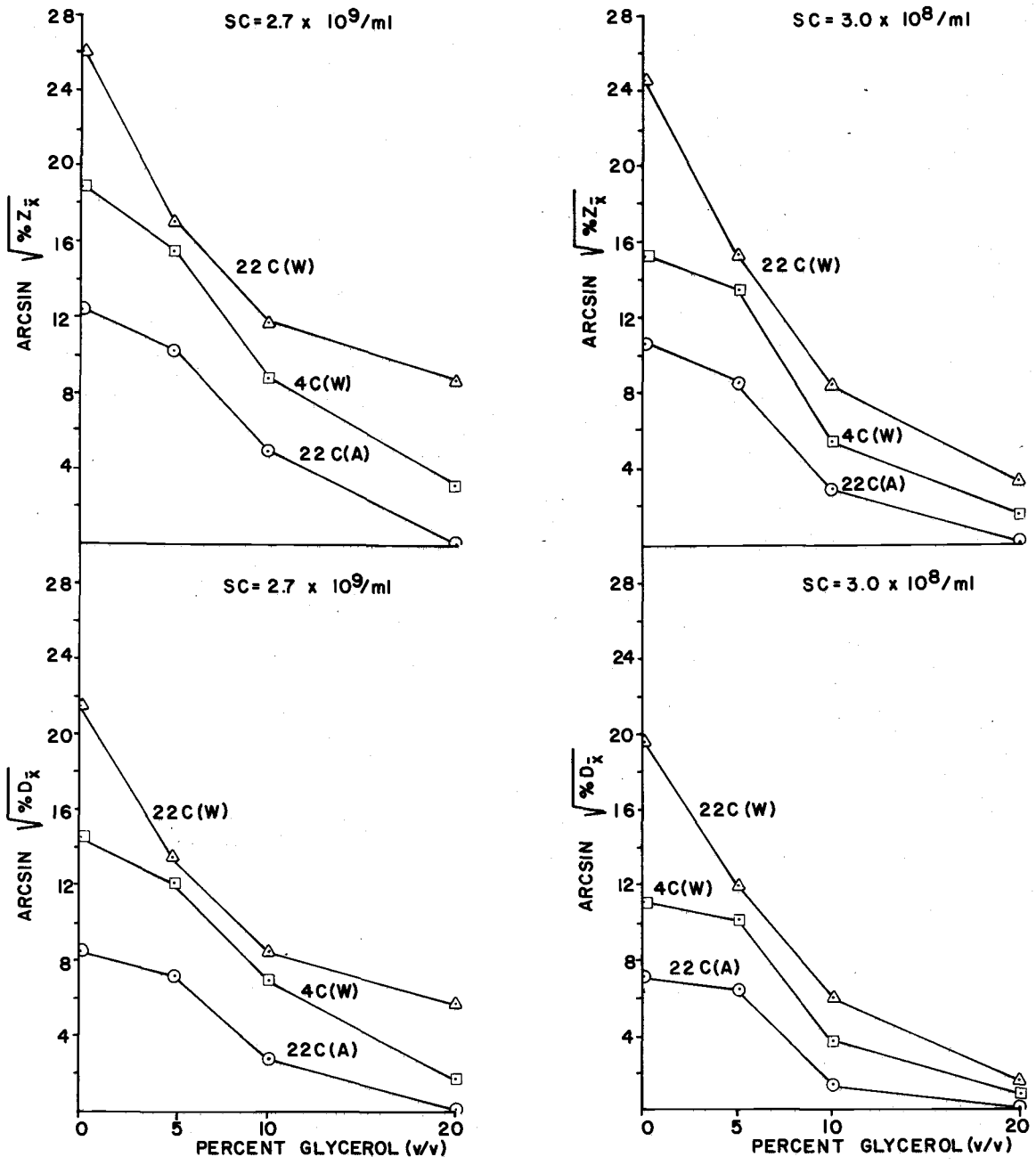

Figure 8. Influence of the interaction between glycerol concentration (v/v) and thawing temperature on both arcsin $\sqrt{\%Z_{\bar{x}}}$ ($\%Z_{\bar{x}}$ = mean percent fertilization) and arcsin $\sqrt{\%D_{\bar{x}}}$ ($\%D_{\bar{x}}$ = mean percentage of larvae developing to D-shape) using fresh eggs and cryopreserved sperm of Crassostrea gigas (means plotted are those of cryoprotective solutions containing 20% dimethyl sulfoxide subjected [in factorial experimentation] to all other freeze-thaw procedures using 3 repetitions per fertilization). SC = initial sperm concentration; W = water bath; A = air bath.

DMSO and Glycerol

I found both glycerol and DMSO toxic to unfrozen oyster sperm in concentrations $\geq$ 25% (v/v). Glycerol is apparently toxic even at low concentrations as indicated in Fig. 3: increases in glycerol concentration result in a decreasing $\%Z_{\bar{x}}$. Barner (1964) reported mannitol functions as an osmotic antagonist to DMSO in mammalian systems; I found no decrease in toxicity to either fresh or frozen oyster sperm in solutions of 20, 25, 30, and 40% DMSO or similar concentrations of glycerol containing 100-900 mg of mannitol/100 ml.

DMSO and glycerol are cryophylactic agents which function intracellularly (Rowe 1966; Doebbler 1966); they also provide some extracellular protection at faster freezing rates due to the formation of vitreous ice (Litvan 1972). (The lower limits of "fast" freezing rates apparently vary from organism to organism and cell to cell; freezing rates from 120-5,000 C/min are generally accepted as "fast.") Litvan also discusses the probable primary mechanisms of cryoprotection provided by agents such as DMSO and glycerol, and discusses the primary causes of the failure of cells to withstand the rigors of freeze-thaw treatment.

Glycine

The influence of glycine concentration on $\%Z_{\bar{x}}$ and $\%D_{\bar{x}}$ graphed in Fig. 4A indicates that increased glycine concentration results in

decreased $\%Z_{\bar{x}}$ and $\%D_{\bar{x}}$; an increase from 10% to 20% glycine does not significantly alter $\%Z_{\bar{x}}$. At the 10% and 20% concentrations glycine reduces the negative slope indicating that higher concentrations may provide a greater measure of protection in solutions containing 20% DMSO.

Glycine was chosen as an additive since amino acids apparently provide some degree of extracellular cryoprotection (Doebbler 1966). Workers studying the respiration of both oyster and sea-urchin sperm (Tyler and Atkinson 1950; Tyler and Rothschild 1951; Tyler 1953; Jeffrey 1954) reported that low concentrations of glycine caused sperm to remain more intensely active and remain motile longer than controls; glycine prolonged the fertilization capacity as well. These investigators suggested that sperm are more capable of utilizing endogenous reserves in the presence of glycine.

Tyler (1953) reported that amino acids act as metalchelating agents, and in experiments ruling out calcium-glycine binding suggested that the heavy metals Cu and Zn were bound by glycine. Thus, investigations in which the concentration of Cu and Zn is minimal and/or the concentration of glycine is > 20% may result in increased survival of oyster sperm.

Freezing Rate

Fast freezing rates often result in the formation of vitreous

extracellular ice, but may cause severe membrane stress due to a rapid removal of deadsorbed intracellular water (Litvan 1972). Litvan hypothesized that extracellular vapor pressure (vp) is low due to ice formation, while intracellular unfrozen adsorbed water has a higher vp. The resulting Δvp is reduced when intracellular water is desorbed, removed from the cell, and frozen extracellularly. Slower freezing rates reduce the possibility of membrane rupture by allowing a slower reduction of Δvp; however, more water is desorbed at the slower rates and consequently dehydration is increased while vitreous ice formation is decreased. Since even slight ruptures of the cell membrane might result in damage to the Na^+ pump and concomittant loss of K^+ ions, I attempted to find a moderate freezing rate, one which would minimize both membrane stress and dehydration. Freezing rates of -30 C/min and -5 C/min resulted in virtually no difference in sperm survival (Appendix) and relatively high levels of $\%Z_{\bar{x}}$ and $\%D_{\bar{x}}$ were obtained using both rates. Apparently both rates are within the moderate range; an unequivocal statement is impossible without further experimentation.

Thawing Temperature

Similar deleterious effects are evidenced with fast and slow thaw rates (Litvan 1972); the data graphed in Fig. 3A suggest that both

the 4 C water bath and the 22 C water bath are in the moderate range, although the latter resulted in slightly higher survival rates.

Dilution Ratio

Diluting the initial sperm concentrations reduced the salinity below that considered optimal by Davis and Calabrese (1964); however, dilution with SW immediately after thawing returned the salinity to the desirable range. As noted by Choong and Wales (1962), although moderate dilutions may have no effect on the viability of unshocked spermatozoa, such dilutions increase the susceptibility of these cells to cold shock (Sherman [1964] defined cold shock, or temperature shock, as "the manifestation of deleterious effects of rapid cooling above the freezing temperature"). The lower $\%Z_{\bar{x}}$ and $\%D_{\bar{x}}$ using dilution ratios of 2:1 and 1:2 (as compared to 1:1 in Fig. 3B) may result from either temperature shock or increased toxicity from the cryophylactic agents employed at the former dilution, and from an insufficient concentration of cryoprotective agent for the latter. Finally, since inorganic salts provide cryoprotection for some organisms (Rowe 1966), lower salt concentration (2:1 dilution) may provide less extracellular protection, whereas at the 1:2 dilution the adverse effect of a lower concentration of DMSO may be partially abated by the increased salt concentration. Investigations using higher salinities may be difficult to evaluate since

experiments using fresh gametes in artificial SW resulted in low %Z in this lab and at the MSC (Lund, personal communication).

Eggs

No fertilization resulted from experiments using fresh sperm with eggs subjected to the freeze-thaw regimen. The cryopreservation of eggs has proven an elusive endeavor (Sherman 1964); however, the remarkable cryopreservation of mouse embryos reported by Whittingham, Leibo and Mazur (1972) indicates that with further understanding of the mechanisms of cryoprotection future attempts to cryopreserve ova will most likely prove successful.

Recommendations

Since both geneticists and hatchery workers may be interested in cryopreserving oyster sperm, I have entered in Table 4 the most successful results of fertilization experiments using cryopreserved sperm. Only a slight increase in $\%Z_{\bar{x}}$ results from increasing the initial sperm concentration one order of magnitude; due to the frequent difficulty in obtaining sperm concentrations of 2.7×10^9/ml (K = 900), I would recommend using a concentration of K = 400. Similarly, since only small differences in $\%Z_{\bar{x}}$ result from the different freezing rates and since the equipment used to obtain rates of -5°C/min is an added expense, I further recommend the -30 C/min freezing rate.

Table 4. Largest means of % fertilization ($\%Z_{\bar{x}}$) and percentage of larvae developing to the D-shape stage ($\%D_{\bar{x}}$) attained using cryopreserved sperm subjected to various treatments (footnoted below) and fresh eggs of Crassostrea gigas (3 repetitions).

DMSO[1]	L[2]	N[3]	SC[4]	U[5]	E[6]	T[7]	$\%Z_{\bar{x}}$	$\%D_{\bar{x}}$
20	0	0	900	1:1	-30	22W	33.0	27.7
20	0	0	900	2:1	-30	22W	27.7	21.0
20	0	0	900	1:1	- 5	22W	36.3	28.0
20	0	0	900	2:1	- 5	22W	28.0	22.2
20	0	0	900	1:1	- 5	4W	20.3	15.7
20	5	0	900	2:1	-30	22W	20.0	16.0
20	5	0	900	1:1	-30	22W	24.3	19.3
20	0	0	400	1:1	-30	22W	32.7	25.0
20	0	0	400	2:1	-30	22W	30.0	26.7
20	0	0	400	1:1	- 5	22W	32.0	26.3
20	0	0	400	2:1	- 5	22W	23.7	17.7
20	0	0	400	1:1	- 5	4W	20.0	14.7
20	5	0	400	1:1	-30	22W	23.7	19.3
20	5	0	400	1:1	- 5	22W	20.0	13.7

[1] DMSO = % dimethyl sulfoxide (v/v)

[2] L = % glycerol (v/v)

[3] N = % glycine (1.0 M, v/v)

[4] SC = initial sperm concentration in Klett-Summerson colorimeter units: 400 = 3.0×10^8/ml; 900 = 2.7×10^9/ml

[5] U = dilution ratio (cryoprotective solution:sperm solution)

[6] E = freezing rate: -30 = -30 C/min; -5 = -5 C/min

[7] T = thawing temperature: 22W = 22 C water bath; 4W = 4 C water bath

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APPENDIX

APPENDIX

Means of percent fertilization and percentage of larvae developing to the D-shape stage obtained from a 5-factor analysis of variance (OSU computer program NANOVAX).

The following explanations and definitions refer to data in this Appendix.

(a) All means are expressed as arcsin $\sqrt{\text{percentage}}$.

(b) Back-transformations to mean % fertilization and mean % D-shape larvae can be made using Appendix A 16 in Snedecor and Cochran (1967).

(c) Factor U = dilution ratio (cryoprotective solution:sperm-SW solution):
1 = 1:1
2 = 1:2
3 = 2:1

(d) Factor E = freezing rate
1 = -30°C/min
2 = - 5°C/min

(e) Factor T = thawing temperature
1 = 22°C air bath
2 = 22°C water bath
3 = 4°C water bath

(f) Factor L = glycerol concentration (v/v)
1 = 0%
2 = 5%
3 = 10%
4 = 20%

(g) Factor N = glycine concentration (1.0 M, v/v)
1 = 0%
2 = 5%
3 = 10%
4 = 20%

I. Solutions containing 20% DMSO

A. Initial sperm concentration = 2.7×10^9/ml

1. Means of fertilization

Grand mean: 11.5

Factor U:

(1)	16.1	(2)	6.6	(3)	11.8		

Factor E:

(1)	11.4	(2)	11.5				

Factor T

(1)	6.9	(2)	15.9	(3)	11.6		

Factor L

(1)	19.0	(2)	14.3	(3)	8.6	(4)	4.0

Factor N

(1)	13.4	(2)	11.6	(3)	10.5	(4)	10.4

Factor U vs Factor E

(1, 1)	15.8	(1, 2)	16.4				
(2, 1)	6.9	(2, 2)	6.3				
(3, 1)	11.6	(3, 2)	12.0				

Factor U vs Factor T

(1, 1)	10.0	(1, 2)	20.5	(1, 3)	17.8		
(2, 1)	4.4	(2, 2)	9.3	(2, 3)	6.0		
(3, 1)	6.3	(2, 2)	18.0	(3, 3)	11.1		

Factor U vs Factor L

(1, 1)	23.1	(1, 2)	21.8	(1, 3)	14.0	(1, 4)	5.4
(2, 1)	13.2	(2, 2)	6.2	(2, 3)	3.8	(2, 4)	3.1
(3, 1)	20.8	(3, 2)	14.8	(3, 3)	7.9	(3, 4)	3.6

Factor U vs Factor N

(1, 1)	17.0	(1, 2)	15.6	(1, 3)	15.0	(1, 4)	16.8
(2, 1)	8.9	(2, 2)	6.9	(2, 3)	6.0	(2, 4)	4.5
(3, 1)	14.3	(3, 2)	12.2	(3, 3)	10.5	(3, 4)	10.1

Factor E vs Factor T

(1, 1)	7.8	(1, 2)	15.5	(1, 3)	11.0		
(2, 1)	6.0	(2, 2)	16.4	(2, 3)	12.2		

Factor E vs Factor L

(1, 1)	19.6	(1, 2)	14.6	(1, 3)	9.0	(1, 4)	2.7
(2, 1)	18.5	(2, 2)	14.0	(2, 3)	8.2	(2, 4)	5.4

Factor E vs Factor N

(1, 1)	13.5	(1, 2)	11.7	(1, 3)	10.7	(1, 4)	9.9
(2, 1)	13.3	(2, 2)	11.5	(2, 3)	10.3	(2, 4)	11.0

(Continued on next page)

Factor T vs Factor L

(1, 1)	12.4	(1, 2)	10.3	(1, 3)	5.0	(1, 4)	0
(2, 1)	26.0	(2, 2)	17.1	(2, 3)	11.8	(2.4)	8.8
(3, 1)	18.8	(3, 2)	15.5	(3, 3)	8.9	(3, 4)	3.3

Factor T vs Factor N

(1, 1)	7.4	(1, 2)	7.2	(1, 3)	6.9	(1, 4)	6.1
(2, 1)	19.2	(2, 2)	16.5	(2, 3)	13.9	(2, 4)	14.0
(3, 1)	13.5	(3, 2)	11.1	(3, 3)	10.6	(3, 4)	11.2

Factor L vs Factor N

(1, 1)	21.0	(1, 2)	17.8	(1, 3)	19.0	(1, 4)	18.4
(2, 1)	16.0	(2, 2)	14.8	(2, 3)	14.5	(2, 4)	12.0
(3, 1)	11.9	(3, 2)	9.5	(3, 3)	5.5	(3, 4)	7.4
(4, 1)	4.7	(4, 2)	4.3	(4, 3)	3.0	(4, 4)	4.1

2. Means of larvae developing to D-shape

Grand mean = 8.6

Factor U

(1)	12.7	(2)	4.4	(3)	8.9		

Factor E

(1)	8.6	(2)	8.7				

Factor T

(1)	4.7	(2)	12.4	(3)	8.9		

Factor L

(1)	14.9	(2)	11.0	(3)	6.1	(4)	2.6

Factor N

(1)	11.0	(2)	8.4	(3)	7.5	(4)	7.7

Factor U vs Factor E

(1, 1)	12.3	(1, 2)	13.1				
(2, 1)	4.9	(2, 2)	3.8				
(3, 1)	8.7	(3, 2)	9.1				

Factor U vs Factor T

(1, 1)	7.4	(1, 2)	16.6	(1, 3)	14.0		
(2, 1)	2.5	(2, 2)	6.6	(2, 3)	4.0		
(3, 1)	4.0	(3, 2)	13.9	(3, 3)	8.7		

Factor U vs Factor L

(1, 1)	18.9	(1, 2)	18.1	(1, 3)	10.2	(1, 4)	3.6
(2, 1)	9.1	(2, 2)	4.1	(2, 3)	2.6	(2, 4)	1.6
(3, 1)	16.6	(3, 2)	10.7	(3, 3)	5.5	(3, 4)	2.7

Factor U vs Factor N

(1, 1)	14.2	(1, 2)	12.0	(1, 3)	11.5	(1, 4)	13.1
(2, 1)	6.9	(2, 2)	4.2	(2, 3)	3.6	(2, 4)	2.8
(3, 1)	11.8	(3, 2)	9.1	(3, 3)	7.3	(3, 4)	7.3

(Continued on next page)

Factor E vs Factor T

(1, 1)	5.4	(1, 2)	12.1	(1, 3)	8.4		
(2, 1)	3.9	(2, 2)	12.6	(2, 3)	9.4		

Factor E vs Factor L

(1, 1)	15.5	(1, 2)	11.0	(1, 3)	6.4	(1, 4)	1.6
(2, 1)	14.2	(2, 2)	11.0	(2, 3)	5.8	(2, 4)	3.6

Factor E vs Factor N

(1, 1)	11.1	(1, 2)	8.4	(1, 3)	7.6	(1, 4)	7.4
(2, 1)	10.9	(2, 4)	8.4	(2, 3)	7.3	(2, 4)	8.1

Factor T vs Factor L

(1, 1)	8.5	(1, 2)	7.3	(1, 3)	2.9	(1, 4)	0
(2, 1)	21.5	(2, 2)	13.6	(2, 3)	8.5	(2, 4)	5.9
(3, 1)	14.6	(3, 2)	12.1	(3, 3)	7.0	(3, 4)	1.9

Factor T vs Factor N

(1, 1)	5.7	(1, 2)	4.5	(1, 3)	4.8	(1, 4)	3.6
(2, 1)	16.1	(2, 2)	12.6	(2, 3)	9.8	(2, 4)	11.0
(3, 1)	11.1	(3, 2)	8.1	(3, 3)	7.8	(3, 4)	8.6

Factor L vs Factor N

(1, 1)	17.7	(1, 2)	13.7	(1, 3)	14.7	(1, 4)	13.3
(2, 1)	13.5	(2, 2)	10.7	(2, 3)	10.5	(2, 4)	9.2
(3, 1)	9.3	(3, 2)	6.6	(3, 3)	3.3	(3, 4)	5.3
(4, 1)	3.4	(4, 2)	2.6	(4, 3)	1.4	(4, 4)	3.1

B. Initial sperm concentration = 3.0×10^8/ml

1. Means of fertilization

Grand mean = 9.2

Factor U

(1)	14.0	(2)	4.1	(3)	9.4		

Factor E

(1)	9.6	(2)	8.8				

Factor T

(1)	5.6	(2)	13.0	(3)	8.9		

Factor L

(1)	16.8	(2)	12.5	(3)	5.7	(4)	1.7

Factor N

(1)	11.2	(2)	9.6	(3)	8.2	(4)	7.6

Factor U vs Factor E

(1, 1)	14.0	(1, 2)	14.0
(2, 1)	4.8	(2, 2)	3.4
(3, 1)	9.9	(3, 2)	9.0

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Factor U vs Factor T							
(1, 1)	8.4	(1, 2)	18.3	(1, 3)	15.3		
(2, 1)	3.1	(2, 2)	6.1	(2, 3)	3.2		
(3, 1)	5.4	(3, 2)	14.7	(3, 3)	8.3		
Factor U vs Factor L							
(1, 1)	22.5	(1, 2)	19.8	(1, 3)	9.6	(1, 4)	4.0
(2, 1)	9.4	(2, 2)	4.7	(2, 3)	2.2	(2, 4)	0.2
(3, 1)	18.4	(3, 2)	13.0	(3, 3)	5.4	(3, 4)	0.9
Factor U vs Factor N							
(1, 1)	16.5	(1, 2)	13.9	(1, 3)	13.0	(1, 4)	12.5
(2, 1)	6.1	(2, 2)	4.7	(2, 3)	3.4	(2, 4)	2.3
(3, 1)	11.0	(3, 2)	10.3	(3, 3)	8.4	(3, 4)	8.1
Factor E vs Factor T							
(1, 1)	6.5	(1, 2)	13.1	(1, 3)	9.1		
(2, 1)	4.7	(2, 2)	12.9	(2, 3)	8.8		
Factor E vs Factor L							
(1, 1)	17.4	(1, 2)	13.0	(1, 3)	6.7	(1, 4)	1.2
(2, 1)	16.2	(2, 2)	12.0	(2, 3)	4.8	(2, 4)	2.2
Factor E vs Factor N							
(1, 1)	11.5	(1, 2)	10.6	(1, 3)	8.6	(1, 4)	7.5
(2, 1)	10.9	(2, 2)	8.6	(2, 3)	7.9	(2, 4)	7.7
Factor T vs Factor L							
(1, 1)	10.7	(1, 2)	8.7	(1, 3)	3.0	(1, 4)	0
(2, 1)	24.5	(2, 2)	15.4	(2, 3)	8.6	(2, 4)	3.4
(3, 1)	15.2	(3, 2)	13.4	(3, 3)	5.5	(3, 4)	1.6
Factor T vs Factor N							
(1, 1)	6.6	(1, 2)	6.4	(1, 3)	5.0	(1, 4)	4.4
(2, 1)	16.3	(2, 2)	13.7	(2, 3)	11.5	(2, 4)	10.5
(3, 1)	10.6	(3, 2)	8.8	(3, 3)	8.2	(3, 4)	8.0
Factor L vs Factor N							
(1, 1)	18.2	(1, 2)	16.8	(1, 3)	16.4	(1, 4)	15.8
(2, 1)	15.0	(2, 2)	14.5	(2, 3)	12.6	(2, 4)	7.9
(3, 1)	9.4	(3, 2)	5.4	(3, 3)	2.9	(3, 4)	5.1
(4, 1)	2.2	(4, 2)	1.8	(4, 3)	1.1	(4, 4)	1.7

2. Means of larvae developing to D-shape

Grand mean = 6.8

Factor U					
(1)	10.8	(2)	2.5	(3)	6.9
Factor E					
(1)	7.1	(2)	6.4		
Factor T					
(1)	3.8	(2)	9.9	(3)	6.6

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Factor L							
(1)	12.7	(2)	9.6	(3)	3.8	(4)	0.9
Factor N							
(1)	8.3	(2)	7.2	(3)	6.1	(4)	5.5
Factor U vs Factor E							
(1, 1)	10.8	(1, 2)	10.8				
(2, 1)	3.1	(2, 2)	1.9				
(3, 1)	7.3	(3, 2)	6.5				
Factor U vs Factor T							
(1, 1)	6.1	(1, 2)	14.3	(1, 3)	12.0		
(2, 1)	1.7	(2, 2)	4.1	(2, 3)	1.8		
(3, 1)	3.6	(3, 2)	11.3	(3, 3)	5.9		
Factor U vs Factor L							
(1, 1)	17.9	(1, 2)	16.3	(1, 3)	6.8	(1, 4)	2.4
(2, 1)	6.1	(2, 2)	2.9	(2, 3)	1.1	(2, 4)	0
(3, 1)	14.2	(3, 2)	9.7	(3, 3)	3.6	(3, 4)	0.4
Factor U vs Factor N							
(1, 1)	13.0	(1, 2)	10.9	(1, 3)	10.1	(1, 4)	9.3
(2, 1)	3.8	(2, 2)	3.1	(2, 3)	1.8	(2, 4)	1.3
(3, 1)	8.1	(3, 2)	7.6	(3, 3)	6.2	(3, 4)	5.8
Factor E vs Factor T							
(1, 1)	4.4	(1, 2)	9.9	(1, 3)	7.0		
(2, 1)	3.2	(2, 2)	10.0	(2, 3)	6.1		
Factor E vs Factor L							
(1, 1)	13.4	(1, 2)	10.0	(1, 3)	4.7	(1, 4)	0.3
(2, 1)	12.0	(2, 2)	9.2	(2, 3)	3.0	(2, 4)	1.5
Factor E vs Factor N							
(1, 1)	8.9	(1, 2)	8.0	(1, 3)	6.3	(1, 4)	5.3
(2, 1)	7.7	(2, 2)	6.5	(2, 3)	5.9	(2, 4)	5.6
Factor T vs Factor L							
(1, 1)	7.1	(1, 2)	6.6	(1, 3)	1.5	(1, 4)	0
(2, 1)	19.8	(2, 2)	12.0	(2, 3)	6.1	(2, 4)	1.7
(3, 1)	11.2	(3, 2)	10.2	(3, 3)	3.9	(3, 4)	1.0
Factor T vs Factor N							
(1, 1)	4.2	(1, 2)	4.7	(1, 3)	3.4	(1, 4)	2.9
(2, 1)	12.8	(2, 2)	10.3	(2, 3)	8.8	(2, 4)	7.7
(3, 1)	7.8	(3, 2)	6.7	(3, 3)	6.0	(3, 4)	5.8
Factor L vs Factor N							
(1, 1)	14.7	(1, 2)	12.7	(1, 3)	12.1	(1, 4)	11.4
(2, 1)	12.3	(2, 2)	11.6	(2, 3)	9.4	(2, 4)	6.1
(3, 1)	5.8	(3, 2)	3.8	(3, 3)	2.1	(3, 4)	3.6
(4, 1)	1.4	(4, 2)	0.9	(4, 3)	0.7	(4, 4)	0.7

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II. Solutions containing 10% DMSO

A. Initial sperm concentration = 2.7×10^9/ml

1. Means of fertilization

Grand mean = 2.3

Factor U							
(1)	3.9	(2)	0	(3)	3.1		
Factor E							
(1)	2.4	(2)	2.3				
Factor T							
(1)	2.9	(2)	2.2	(3)	2.0		
Factor L							
(1)	3.2	(2)	2.6	(3)	2.5	(4)	1.0
Factor N							
(1)	2.2	(2)	2.5	(3)	2.2	(4)	2.4
Factor U vs Factor E							
(1, 1)	3.8	(1, 2)	4.0				
(2, 1)	0	(2, 2)	0				
(3, 1)	3.3	(3, 2)	2.9				
Factor U vs Factor T							
(1, 1)	4.9	(1, 2)	3.4	(1, 3)	3.4		
(2, 1)	0	(2, 2)	0	(2, 3)	0		
(3, 1)	3.7	(3, 2)	3.1	(3, 3)	2.5		
Factor U vs Factor L							
(1, 1)	5.0	(1, 2)	4.7	(1, 3)	4.0	(1, 4)	1.7
(2, 1)	0	(2, 2)	0	(2, 3)	0	(2, 4)	0
(3, 1)	4.6	(3, 2)	3.1	(3, 3)	3.6	(3, 4)	1.2
Factor U vs Factor N							
(1, 1)	3.9	(1, 2)	4.1	(1, 3)	3.8	(1, 4)	3.7
(2, 1)	0	(2, 2)	0	(2, 3)	0	(2, 4)	0
(3, 1)	2.8	(3, 2)	3.4	(3, 3)	2.9	(3, 4)	3.4
Factor E vs Factor T							
(1, 1)	3.0	(1, 2)	2.0	(1, 3)	2.1		
(2, 1)	2.7	(2, 2)	2.3	(2, 3)	1.8		
Factor E vs Factor L							
(1, 1)	3.3	(1, 2)	2.7	(1, 3)	2.6	(1, 4)	0.9
(2, 1)	3.2	(2, 2)	2.5	(2, 3)	2.5	(2, 4)	1.0
Factor E vs Factor N							
(1, 1)	2.2	(1, 2)	2.6	(1, 3)	2.2	(1, 4)	2.5
(2, 1)	2.2	(2, 2)	2.5	(2, 3)	2.2	(2, 4)	2.2

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Factor T vs Factor L

(1, 1)	3.3	(1, 2)	3.5	(1, 3)	3.3	(1, 4)	1.3
(2, 1)	3.2	(2, 2)	1.9	(2, 3)	2.5	(2, 4)	1.0
(3, 1)	3.1	(3, 2)	2.4	(3, 3)	1.8	(3, 4)	0.6

Factor T vs Factor N

(1, 1)	2.1	(1, 2)	3.0	(1, 3)	3.1	(1, 4)	3.2
(2, 1)	2.4	(2, 2)	2.3	(2, 3)	1.9	(2, 4)	2.1
(3, 1)	2.2	(3, 2)	2.2	(3, 3)	1.7	(3, 4)	1.8

Factor L vs Factor N

(1, 1)	4.4	(1, 2)	3.9	(1, 3)	2.1	(1, 4)	2.4
(2, 1)	3.2	(2, 2)	2.3	(2, 3)	2.1	(2, 4)	2.8
(3, 1)	1.3	(3, 2)	2.4	(3, 3)	3.2	(3, 4)	3.2
(4, 1)	0	(4, 2)	1.4	(4, 3)	1.5	(4, 4)	1.1

2. Means of larvae developing to D-shape

Grand mean = 1.1

Factor U

(1)	1.8	(2)	0	(3)	1.6		

Factor E

(1)	1.1	(2)	1.2				

Factor T

(1)	1.4	(2)	1.0	(3)	1.0		

Factor L

(1)	1.7	(2)	1.2	(3)	1.2	(4)	0.4

Factor N

(1)	1.3	(2)	1.2	(3)	1.0	(4)	1.0

Factor U vs Factor E

(1, 1)	1.7	(1, 2)	1.9				
(2, 1)	0	(2, 2)	0				
(3, 1)	1.5	(3, 2)	1.7				

Factor U vs Factor T

(1, 1)	2.3	(1, 2)	1.5	(1, 3)	1.6		
(2, 1)	0	(2, 2)	0	(2, 3)	0		
(3, 1)	1.9	(3, 2)	1.6	(3, 3)	1.3		

Factor U vs Factor L

(1, 1)	2.3	(1, 2)	2.2	(1, 3)	1.8	(1, 4)	0.8
(2, 1)	0	(2, 2)	0	(2, 3)	0	(2, 4)	0
(3, 1)	2.9	(3, 2)	1.3	(3, 3)	1.8	(3, 4)	0.5

Factor U vs Factor N

(1, 1)	2.5	(1, 2)	1.8	(1, 3)	1.6	(1, 4)	1.3
(2, 1)	0	(2, 2)	0	(2, 3)	0	(2, 4)	0
(3, 1)	1.4	(3, 2)	2.0	(3, 3)	1.4	(3, 4)	1.7

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Factor E vs Factor T

(1, 1)	1.5	(1, 2)	0.9	(1, 3)	0.8		
(2, 1)	1.3	(2, 2)	1.2	(2, 3)	1.2		

Factor E vs Factor L

(1, 1)	1.7	(1, 2)	1.0	(1, 3)	1.1	(1, 4)	0.4
(2, 1)	1.8	(2, 2)	1.4	(2, 3)	1.3	(2, 4)	0.5

Factor E vs Factor N

(1, 1)	1.2	(1, 2)	1.2	(1, 3)	0.8	(1, 4)	1.0
(2, 1)	1.4	(2, 2)	1.2	(2, 3)	1.2	(2, 4)	1.0

Factor T vs Factor L

(1, 1)	1.6	(1, 2)	1.8	(1, 3)	1.5	(1, 4)	0.7
(2, 1)	1.8	(2, 2)	0.7	(2, 3)	1.3	(2, 4)	0.2
(3, 1)	1.8	(3, 2)	1.0	(3, 3)	0.8	(3, 4)	0.4

Factor T vs Factor N

(1, 1)	1.1	(1, 2)	1.3	(1, 3)	1.7	(1, 4)	1.5
(2, 1)	1.4	(2, 2)	1.3	(2, 3)	0.6	(2, 4)	0.8
(3, 1)	1.4	(3, 2)	1.1	(3, 3)	0.7	(3, 4)	0.7

Factor L vs Factor N

(1, 1)	2.5	(1, 2)	2.4	(1, 3)	0.8	(1, 4)	1.2
(2, 1)	1.9	(2, 2)	1.1	(2, 3)	0.9	(2, 4)	0.9
(3, 1)	0.8	(3, 2)	0.9	(3, 3)	1.8	(3, 4)	1.4
(4, 1)	0	(4, 2)	0.7	(4, 3)	0.5	(4, 4)	0.5

B. Initial sperm concentration = 3.0×10^8/ml

1. Means of fertilization

Grand mean = 2.5

Factor U

(1)	4.1	(2)	0	(3)	3.4		

Factor E

(1)	2.7	(2)	2.3				

Factor T

(1)	2.6	(2)	2.4	(3)	2.4		

Factor L

(1)	2.9	(2)	2.7	(3)	2.7	(4)	1.6

Factor N

(1)	3.0	(2)	2.5	(3)	2.2	(4)	2.2

Factor U vs Factor E

(1, 1)	4.4	(1, 2)	3.7
(2, 1)	0	(2, 2)	0
(3, 1)	3.6	(3, 2)	3.2

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Factor U vs Factor T

(1, 1)	4.2	(1, 2)	3.9	(1, 3)	4.1		
(2, 1)	0	(2, 2)	0	(2, 3)	0		
(3, 1)	3.5	(3, 2)	3.4	(3, 3)	3.2		

Factor U vs Factor L

(1, 1)	4.7	(1, 2)	4.8	(1, 3)	4.3	(1, 4)	2.5
(2, 1)	0	(2, 2)	0	(2, 3)	0	(2, 4)	0
(3, 1)	3.9	(3, 2)	3.3	(3, 3)	3.9	(3, 4)	2.4

Factor U vs Factor N

(1, 1)	4.4	(1, 2)	4.4	(1, 3)	4.0	(1, 4)	3.4
(2, 1)	0	(2, 2)	0	(2, 3)	0	(2, 4)	0
(3, 1)	4.5	(3, 2)	3.2	(3, 3)	2.6	(3, 4)	3.2

Factor E vs Factor T

(1, 1)	3.1	(1, 2)	2.4	(1, 3)	2.6		
(2, 1)	2.1	(2, 2)	2.5	(2, 3)	2.3		

Factor E vs Factor L

(1, 1)	3.0	(1, 2)	3.1	(1, 3)	2.8	(1, 4)	1.8
(2, 1)	2.8	(2, 2)	2.3	(2, 3)	2.6	(2, 4)	1.5

Factor E vs Factor N

(1, 1)	3.7	(1, 2)	2.5	(1, 3)	2.6	(1, 4)	1.9
(2, 1)	2.3	(2, 2)	2.6	(2, 3)	1.8	(2, 4)	2.5

Factor T vs Factor L

(1, 1)	3.3	(1, 2)	2.7	(1, 3)	2.5	(1, 4)	1.8
(2, 1)	2.7	(2, 2)	2.5	(2, 3)	3.0	(2, 4)	1.5
(3, 1)	2.6	(3, 2)	2.9	(3, 3)	2.6	(3, 4)	1.6

Factor T vs Factor N

(1, 1)	2.8	(1, 2)	2.3	(1, 3)	2.4	(1, 4)	2.8
(2, 1)	3.0	(2, 2)	2.6	(2, 3)	2.4	(2, 4)	1.7
(3, 1)	3.1	(3, 2)	2.6	(3, 3)	1.9	(3, 4)	2.1

Factor L vs Factor N

(1, 1)	3.3	(1, 2)	3.0	(1, 3)	2.5	(1, 4)	2.6
(2, 1)	3.2	(2, 2)	2.6	(2, 3)	2.4	(2, 4)	2.5
(3, 1)	3.2	(3, 2)	2.5	(3, 3)	2.3	(3, 4)	3.0
(4, 1)	2.1	(4, 2)	2.0	(4, 3)	1.6	(4, 4)	0.7

2. Means of larvae developing to D-shape

Grand mean = 1.2

Factor U

(1)	2.1	(2)	0	(3)	1.4

Factor E

(1)	1.4	(2)	1.0

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Factor T							
(1)	1.3	(2)	1.3	(3)	1.0		
Factor L							
(1)	1.7	(2)	1.3	(3)	1.4	(4)	0.5
Factor N							
(1)	1.6	(2)	1.3	(3)	1.0	(4)	0.9
Factor U vs Factor E							
(1, 1)	2.5	(1, 2)	1.8				
(2, 1)	0	(2, 2)	0				
(3, 1)	1.7	(3, 2)	1.2				
Factor U vs Factor T							
(1, 1)	2.4	(1, 2)	2.2	(1, 3)	2.0		
(2, 1)	0	(2, 2)	0	(2, 3)	0		
(3, 1)	1.6	(3, 2)	1.7	(3, 3)	1.1		
Factor U vs Factor L							
(1, 1)	3.0	(1, 2)	2.5	(1, 3)	2.3	(1, 4)	0.8
(2, 1)	0	(2, 2)	0	(2, 3)	0	(2, 4)	0
(3, 1)	2.0	(3, 2)	1.3	(3, 3)	1.7	(3, 4)	0.7
Factor U vs Factor N							
(1, 1)	2.9	(1, 2)	2.5	(1, 3)	2.0	(1, 4)	1.3
(2, 1)	0	(2, 2)	0	(2, 3)	0	(2, 4)	0
(3, 1)	2.0	(3, 2)	1.5	(3, 3)	0.9	(3, 4)	1.4
Factor E vs Factor T							
(1, 1)	1.7	(1, 2)	1.3	(1, 3)	1.3		
(2, 1)	1.0	(2, 2)	1.3	(2, 3)	0.8		
Factor E vs Factor L							
(1, 1)	1.9	(1, 2)	1.6	(1, 3)	1.4	(1, 4)	0.7
(2, 1)	1.4	(2, 2)	1.0	(2, 3)	1.3	(2, 4)	0.4
Factor E vs Factor N							
(1, 1)	2.3	(1, 2)	1.2	(1, 3)	1.1	(1, 4)	1.0
(2, 1)	0.9	(2, 2)	1.5	(2, 3)	0.8	(2, 4)	0.9
Factor T vs Factor L							
(1, 1)	1.9	(1, 2)	1.6	(1, 3)	1.3	(1, 4)	0.6
(2, 1)	1.8	(2, 2)	1.0	(2, 3)	1.7	(2, 4)	0.7
(3, 1)	1.3	(3, 2)	1.3	(3, 3)	1.0	(3, 4)	0.4
Factor T vs Factor N							
(1, 1)	1.8	(1, 2)	1.2	(1, 3)	1.1	(1, 4)	1.3
(2, 1)	1.6	(2, 2)	1.6	(2, 3)	1.2	(2, 4)	0.6
(3, 1)	1.5	(3, 2)	1.2	(3, 3)	0.6	(3, 4)	0.8
Factor L vs Factor N							
(1, 1)	2.1	(1, 2)	1.8	(1, 3)	1.3	(1, 4)	1.5
(2, 1)	2.1	(2, 2)	1.2	(2, 3)	0.9	(2, 4)	1.1
(3, 1)	1.7	(3, 2)	1.5	(3, 3)	1.1	(3, 4)	1.1
(4, 1)	0.6	(4, 2)	0.8	(4, 3)	0.6	(4, 4)	0