DEVELOPMENTS IN FISH CULTURE

THE USE OF VARIOUS FERTILIZATION MEDIA AND THEIR EFFECTS ON RAINBOW TROUT GAMETES

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ABSTRACT

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Artificial insemination of salmonids is a common, though unperfected, fish cultural practice well over 100 years old. Over the years much of the effort to improve insemination methodology has focused on the fertilization process.

This study evaluates egg survival to the eyed stage using 5 different fertilization diluents at dilution rates of 10^{-2} and 10^{-3}. It also examines the duration of forward and static sperm motility achieved with each diluent.

There was no significant difference in percent eye-up when saline or modified saline diluents were administered at either dilution rate. However, when water was used as a diluent percent eye-up was significantly and adversely affected at both dilution rates ($P<0.01$).

There was a significant effect of diluent on both forward and total sperm motion ($P<.0001$). Water chemistry and pH at different hatcheries or laboratories can influence the efficacy of any fertilization medium. With this in mind we recommend testing simple saline solutions in place of water for their possible value as fertilization mediums.

INTRODUCTION:

Artificial insemination of salmonids is a common, though unperfected, fish cultural practice. Much of the research effort to improve embryo

survival has focused on the fertilization process. Sperm dilution in saline solutions similar in composition to physiological saline levels (0.89%) has been reported to improve fertilization rate (Nomura 1964). Billard et al. (1974) studied the effects of various fertilization diluents on eggs and sperm and reported that a modified saline diluent (organically buffered to pH 9$\pm$0.5) allowed for use of a minimum amount of sperm to obtain a maximum rate of fertilization in rainbow trout eggs. Billard (1980) reported that sperm motility was intensified and prolonged by adding theophylline to the saline diluent. Scheer and Thorgaard (1989) reported the addition of theophylline to the fertilization media improved fertilization rate with cryopreserved RBT sperm. Billard and Cosson (1989) reported that sperm motility was prolonged beyond 30 s when 1 mM Ca++ was added to a modified saline diluent. Petit et al. (1973) reported that pH and osmotic pressure were important factors in determining the success of a fertilization diluent. At Ennis NFH, using a 0.75% saline fertilization diluent in place of water resulted in a 15% increase in egg survival to the eyed stage (Orr & Shrable unpublished 1988). The literature reports wide variation in sperm:egg ratios for optimum fertilization. Wharton (1957) and Plosila & Keller (1974) recommend about 10,000,000 sperm/egg for maximum fertilization.

Billard et al. (1974), reports the minimum number of spermatozoa per egg for fertilization to be 200,000, and later (1977) recommends providing 500,000 to 1,000,000 sperm per egg for optimum fertilization success. Moccia et al.(1987), reported that when sperm:egg ratios were > 200,000:1 neither sperm motility nor sperm density were major factors in determining the percent of eggs reaching the eyed stage. They also reported that late

in the reproductive season there was a significant correlation between sperm density and fertilization rate, but no significant relationship between sperm motility and fertilization rate.

Spawning seasons at Ennis NFH are characterized by low fertilization rates and few spermiating males at first, high fertilization rates with many spermiating males during the peak, and low fertilization rates with many spermiating males at the end of the spawning season. The cause of lowered egg survival at the beginning and end of the spawning cycle is unknown.

Objectives were 1) evaluate several fertilization mediums for their effect on survival to the eyed stage of development,
2) examine the effect of sperm dilution on eyeup, and 3) determine if there is a correlation between sperm motion and egg survival using the different diluents described.

MATERIALS AND METHODS:

PART I. Diluents and Dilution Rate Procedures:

Five different fertilization media: 1) Hatchery spring water, 2) 0.75% saline, 3) D532 (a commercial diluent composed of 0.75% saline, 20 mM Tris, and 50 mM Glycine), 4) D532 + 5 mM Theophylline and 5) D532 + 10 mM $CaCl_2$ were prepared in one liter quantities and refrigerated at 48° F.

Twelve spermiating Erwin strain rainbow trout were stripped, and the milt pooled into a plastic container. The milt was mixed gently by swirling, then placed in an insulated box cooled with ice. The eggs from 24 ripe Erwin females were air stripped, gently but thoroughly mixed together in a plastic container and placed in an insulated container cooled

with ice. All gametes were transferred indoors. Thirty, 1 gallon buckets were labeled for 3 replicates each of the 5 diluents at both dilution rates of 10^{-2} and 10^{-3}. One hundred ml of eggs (approximately 2700) were randomly added to each of these buckets from the pooled egg lot. Six buckets of eggs received 50 ml of the first diluent, 6 buckets received 50 ml of the second diluent, etc.

Using a serological pipette, 0.05 ml of milt (10^{-3} dilution) was added to each of 15 egg/diluent mixtures (3 replicates of 5 diluents), and 0.50 ml of milt (10^{-2} dilution) was added to the remaining 15 replicates (Table 1). All replicates were gently swirled after milt addition to mix the contents. Each replicate was allowed to stand undisturbed for 1 min, decanted, and rinsed with fresh water. Eggs in each replicate were then water hardened in 500 ml of a 50 mg/L iodophor solution. After 30 minutes the eggs from each replicate were carefully poured into separate 1 L capacity upwelling incubators (54° F). Eggs were treated for 15 minutes daily with 1200 mg/L formalin to prevent fungus, and were exposed to weekly iodophor treatments of 40 mg/L active iodine for 10 minutes to prevent soft shell disease. They were mechanically shocked on day 15 of incubation (330 TU), and sorted on day 16 with an electronic egg picker (Jensorter Model JX-4). Numbers of eyed and non-viable eggs were enumerated with an electronic egg counter (Jensorter Model BC). Data was statistically analyzed with the Statistical Analysis System (SAS 1988).

PART II. Sperm Motility Procedures:

Using a small eye dropper, 1 drop of diluent was expressed onto a clean glass slide and aligned under the microscope. The point of a regular dissecting (teasing) needle was inserted vertically into a container of

milt 1/8" thick. The tip of the needle was then inserted into the drop of diluent on the slide and gently stirred in a circular motion 10 times. This method was calculated to be a sperm dilution of 1:500, intermediate between 10^{-2} and 10^{-3}.The needle was cleaned and dried after each application. Using low power, a phase contrast setting of 10 and a fiber optic light intensity setting of 2, motility was easily observed. A stop watch was used to record the time from activation to when forward motion (a period of intense flagellar activity which propels the sperm forward) ceased in 95% of the sperm cells in the field of view. A second timed reading recorded the total time from activation to when no further sperm motion (static sperm movement caused by slight flagellar activity) could be seen in the field of view. This was repeated 5 times for each diluent.

RESULTS AND DISCUSSION:

Of the five diluents tested, water was the only diluent that caused a significant difference in egg survival ($P<0.05$). There was also an effect of dilution rate when water was used. When the milt dilution rate was 10^{-2} percent eyeup was 51.4. Using a dilution rate of 10^{-3} egg survival dropped to 22.4%.

Using an average sperm concentration of 10^{10}/ml and an egg size of 20,000/L the dilution rate of 10^{-3} provided about 185,000 sperm per egg while the dilution of 10^{-2} provided an estimated 1,850,000 per egg. It appears that the number of sperm cells provided for fertilization were adequate at both dilution rates in every medium except water. The sperm dilution rate routinely used at Ennis is $2(10^{-2})$ (20ml sperm: 1L diluent: 2L

eggs) or an estimated 5,000,000 sperm cells/egg. Does Ennis use more milt than necessary? If so, then the operation could be more efficient if less milt was used and the number of males on hand were reduced. However, experience has shown that gametes may be inferior in quality at the beginning and end of the reproductive season, so a dilution rate providing a higher sperm to egg ratio may be beneficial to eyeup success.

The motility test showed a significant correlation between the diluent used and both forward and total motion. ($P<.0001$). It was interesting that the increased forward motion gained with D532 + Theophylline, and the extra static motion gained with 0.75% saline made no difference in percent eyeup at either dilution rate.

This investigation seems to have generated more questions than answers but based on our results in naturally buffered spring water at Ennis, we recommend testing a saline fertilization medium as a means of increasing egg survival. Water chemistry and pH may influence the result so we emphasize "testing" before implementation!

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Table 1. Fertilization media used, pH, and dilution rates tested.

TREATMENT#	pH	MEDIA COMPOSITION	DILUTION RATIO*	REPLICATIONS
1	7.8	Water	0.001:1:2 or 10^{-3} (186,000 sperm/egg)	3
2	7.8	Water + NaCL	"	3
3	8.5	Water + D532**	"	3
4	8.4	Water + D532 + 0.05 M Theo.***	"	3
5	8.4	Water + 1mM Ca++ ****	"	3
6	7.8	Water alone	0.01:1:2 or 10^{-2} (1,860,000 sperm/egg)	3
7	7.8	Water + 0.75% NaCl	"	3
8	8.5	Water + D532	"	3
9	8.4	Water + D532 + 5mM Theo.	"	3
10	8.4	Water + D532 + 10mM Ca ++	"	3

* Volume of Milt:diluent:eggs.
** Commercial Diluent: 0.75% NaCl, 20mM Tris, and 50mM glycine at pH 9.0
*** Theophylline
**** Calcium Chloride

Table 3.

	Motility Evaluation		
	Single Slide Sample Rep # ($5X10^{-2}$)	E.T. (min:sec) Cessation of Forward Movement <5%	E.T. (min:sec) Cessation of All Motion
H_2O	1	00:21	00:50
H_2O	2	00:24	00:51
H_2O	3	00:21	00:40
H_2O	4	00:27	01:10
H_2O	5	00:22	01:06
0.75% Saline	1	00:29	10:+
0.75% Saline	2	00:29	10:+
0.75% Saline	3	00:27	10:+
0.75% Saline	4	00:24	10:+
0.75% Saline	5	00:33	10:+
D532	1	00:27	01:53
D532	2	00:26	02:27
D532	3	00:27	01:28
D532	4	00:30	02:23
D532	5	00:24	02:24
D532+Theophylline	1	01:01	04:50
D532+Theophylline	2	00:42	04:28
D532+Theophylline	3	00:48	03:36
D532+Theophylline	4	N/A	N/A
D532+Theophylline	5	00:59	03:57
D532+$CACL_2$	1	00:30	02:05
D532+$CACL_2$	2	N/A	N/A
D532+$CACL_2$	3	00:35	02:42
D532+$CACL_2$	4	00:34	02:36
C532+$CACL_2$	5	00:39	02:31

Table 4

Use of Various Fertilization Media
Effect on Egg Viability

Diluent/Dilution	Jar #	TOTAL EGGS	EYED EGGS	BAD EGGS
D532/10^{-3}	1	2739	1851	324
D532/10^{-3}	2	2730	1826	348
D532/10^{-3}	3	2660	1724	378
D532+Theo/10^{-3}	4	2557	1675	319
D532+Theo/10^{-3}	5	2673	1747	333
D532+Theo/10^{-3}	6	2466	1692	231
Saline/10^{-3}	7	2720	1776	289
Saline/10^{-3}	8	2514	1482	183
Saline/10^{-3}	9	2547	1609	378
Water/10^{-3}	10	2601	688	94
Water/10^{-3}	11	2571	170	26
Water/10^{-3}	12	2437	834	125
D532+$CACL_2$/10^{-3}	13	2644	1653	446
D532+$CACL_2$/10^{-3}	14	2684	1818	321
D532+$CACL_2$/10^{-3}	15	2617	1613	403
Water/10^{-2}	16	2529	1438	274
Water/10^{-2}	17	2527	1112	212
Water/10^{-2}	18	2617	1394	172
D532+Theo/10^{-2}	19	2770	1830	323
D532+Theo/10^{-2}	20	2649	1528	536
D532+Theo/10^{-2}	21	2732	1702	426
Saline/10^{-2}	22	1685	2710	448
Saline/10^{-2}	23	1687	2763	275
Saline/10^{-2}	24	1666	2667	469
D532+$CACL_2$/10^{-2}	25	1699	2680	373
D532+$CACL_2$/10^{-2}	26	1743	2797	491
D532+$CACL_2$/10^{-2}	27	1596	2564	424
D532/10^{-2}	28	1743	2781	409
D532/10^{-2}	29	1651	2618	439
D532/10^{-2}	30	1791	2654	342