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Development and evaluation of sperm diluents for the artificial insemination of rainbow trout (*Oncorhynchus mykiss*)

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Abstract

In this study an attempt was made to develop a suitable diluent based on the characteristics of good quality ovarian fluid of *Oncorhynchus mykiss*. The resulting simulated ovarian fluid (SOF) was compared with Borax-boric acid buffer diluent and adjusted versions thereof. The composition of all these diluents are given in this paper. Maximum fertilization success (81.76%) was achieved when ovarian fluid was employed as a sperm diluent. Spermatozoa showed maximum (34 seconds) forward motility after dilution with Borax 3. The use of diluents in artificial fertilization techniques by trout farmers can help them to increase the fertilization success and also facilitates hatchery procedures and broodstock management.

Keywords: Trout, diluents, ovarian fluid, fertilization success, sperm motility.

Développement et évaluation de dilueurs de sperme pour l'insémination artificielle de la truite arc-en-ciel (Oncorhynchus mykiss).

Résumé

Dans cette étude, nous avons tenté de développer un dilueur approprié, basé sur les caractéristiques d'un fluide ovarien de qualité chez *Oncorhynchus mykiss*. Le fluide ovarien simulé (SOF) a été comparé au dilueur tampon Borax-acide borique et à des versions ajustées. La composition de ces dilueurs est donnée. Le taux maximum de fertilisation (81,76 %) a été obtenu lorsque le fluide ovarien était utilisé comme dilueur de sperme. Les spermatozoïdes eurent une motilité maximum (34 secondes) après dilution au moyen du Borax 3. L'utilisation de dilueurs pour les techniques de fécondation artificielle par les aquaculteurs peuvent accroître le succès de la fécondation et faciliter également les procédures dans les écloseries et la gestion des stocks.

Mots-clés : Truite, dilueurs, fertilisation, motilité du sperme.

INTRODUCTION

One of the most common problems associated with artificial insemination of rainbow trout, (*Oncorhynchus mykiss*), is fertilization success. Previously crude artificial insemination techniques, merely involved mixing eggs and sperm together in water (Billard, 1991). Later refinements consisted of mixing sperm and ova together, with or without ovarian fluid with the addition of water afterwards (Billard, 1991).

The diluent technique was proposed by Nomura (1964) in Japan and was later perfected by Billard *et al.* (1974) in France. Sperm dilution, which was

performed in saline (Baynes *et al.*, 1981; Billard *et al.*, 1974; 1987); Tris-buffer (Billard *et al.*, 1974; Billard, 1975; 1977 *a*; *b*); ovarian fluid (Yoshida and Nomura, 1972; Holtz *et al.*, 1977; Benau and Terner, 1980; Billard, 1983); MIX-saline (Benau and Terner, 1980); Borax-boric acid buffer (Steyn *et al.*, 1989) improves the fertilization of eggs when compared with results obtained following fertilization in water (Poon and Johnson, 1970; Plosida *et al.*, 1972; Billard, 1977 *b*).

Sperm motility is used as a variable to determine sperm quality and is usually estimated in fish on an arbitrary scale of intensity ranging from 0 to 5 (Hoyle *et al.*, 1968; Sanchez-Rodriguez and Billard, 1977), and duration of motility (Carpentier and Billard, 1978), or by a combination of these two parameters (Baynes *et al.*, 1981). Sperm motility varies widely in individual males and also between the beginning and the end of the reproductive season (Billard *et al.*, 1977). Motility time is generally short in salmonids and decreases progressively within 30 seconds. Motility time is slightly longer in a saline solution (Billard, 1978; Steyn *et al.*, 1989) and Borax-boric acid buffer diluent (Steyn *et al.*, 1989) than in freshwater, consequently fertilization success is improved in such solutions. Furthermore, it has been claimed that the duration and intensity of sperm motility and the proportion of spermatozoa activated are higher in ovarian fluid than in artificial diluents (Dorier, 1949; Yoshida and Nomura, 1972; Holtz *et al.*, 1977).

An enhancing effect of the ovarian fluid in rainbow trout on the sperm motility has been well known amongst fish culturists (Yoshida and Nomura, 1972). It is, however difficult to obtain a large volume of clear ovarian fluid completely free of broken eggs, blood and urine, which are all inhibitory to the sperm motility. This is a phenomenon obstacle for the practical implementation of ovarian fluid in the artificial fertilization of rainbow trout (Yoshida and Nomura, 1972). This study was conducted to develop a suitable diluent based on the characteristics of good quality ovarian fluid. The resulting simulated ovarian fluid (SOF) was compared with Borax-boric acid diluent and adjusted versions thereof.

MATERIALS AND METHODS

Development of new diluents

For the development of the simulated ovarian fluid (SOF) diluent, the experiments were conducted on three-year old female rainbow trout, *Oncorhynchus mykiss*. Twenty rainbow trout females were stripped into strainers within plastic bowls. The eggs and ovarian fluid of each female were kept separately. Each batch of eggs were fertilized with Borax-boric acid buffer diluent (Steyn *et al.*, 1989) and incubated in separate incubation trays, until the eggs were eyed. The fertilization success for each batch of eggs were then determined. The different ovarian fluid samples were analysed for the following characteristics: pH with a Radiometer BMS MK 3 blood-gas analyser; osmotic pressure by means of a osmomat 030 osmometer; sodium and potassium with a radiometer FLM 3 flame photometer; calcium by means of a corning 940 calcium analyser and chloride with a corning 925 chloride analyser.

Only 7 of the fish analysed had a fertilization success higher than 50% in comparison to the 13 that had less than 50%. The characteristics of the ovarian fluid samples differed greatly. The results obtained concerning the composition of the ovarian fluid of the 3 females which gave fertilization success higher than 70% was used to formulate a diluent which theoretically should give the best fertilization success. The characteristics of this ovarian fluid were as follows:

$$\begin{gathered} \text{pH} = 6.957 \\ \text{Osmolality} = 289\ \text{mOsm.kg}^{-1} \\ \text{Sodium} = 144\ \text{mmol.l}^{-1} \\ \text{Potassium} = 2.97\ \text{mmol.l}^{-1} \\ \text{Calcium} = 8.22\ \text{mg\%} \\ \text{Chloride} = 143.50\ \text{mmol.l}^{-1} \end{gathered}$$

The procedures to prepare simulated ovarian fluid (SOF), Borax 2 and 3 are given in *table* 1. The Borax 2 and 3 diluents are based on the original Borax-boric acid buffer (Borax 1), with slight changes in composition. The chemical characteristics of all these diluents are compared in *table* 2.

Table 1. – Composition of diluents.

Simulated ovarian fluid (SOF) Diluent:	
To make up a stock solution, prepare the following:	
Solution A:	12.4 g boric acid + 1 l distilled water
Solution B:	19.05 g disodium tetraborate + 1 l distilled water
Stock solution:	combine the following:
	1. 500 ml solution A
	2. 20 ml solution B
	3. 1480 ml distilled water
	Total volume 2000 ml
Activator solution:	combine the following:
	1. 500 ml stock solution
	2. 1500 ml distilled water
	3. 16.66 g sodium chloride
	4. 0.477 g potassium chloride
	5. 1.354 g calcium lactate
The activator solution should have an osmolality value of $\pm 290\ \text{mOsm.kg}^{-1}$ and a pH value in the region of 7.0.	
Borax-boric acid buffer (Borax 1) (Steyn *et al.*, 1989).	
Borax 2:	
The same composition as Borax 1, but calcium chloride was added to achieve a calcium concentration of 4.19 mg% and chloride concentration of $64.80\ \text{mmol.l}^{-1}$.	
Borax 3:	
The same composition as Borax 1, but calcium was added to achieve a calcium – and chloride concentration of 7.72 mg% and $69.40\ \text{mmol.l}^{-1}$ respectively.	

Table 2. – Characteristics of newly formulated diluents.

	Borax 1	Borax 2	Borax 3	SOF
pH	9.07	9.05	9.08	7.25
Osmolality mOsm.kg^{-1}	0.246	0.250	0.246	0.291
Sodium mmol.l^{-1}	109	109	109	141
Potassium mmol.l^{-1}	0	0	0	3.1
Calcium mg%	0.49	4.19	7.72	8.02
Chloride mmol.l^{-1}	63.67	64.80	69.40	145.25

Comparison between different sperm diluents

During this experiment, six diluents were compared with each other for their suitability as a fertilising medium. The diluents were: ovarian fluid; Borax 1; 2 and 3; SOF and streamwater. Experiments were conducted from 1989 to 1991 with one to two-year old male and two to four-year old female rainbow trout, *Oncorhynchus mykiss*. Inspection of the fish for readiness to spawn was carried out every three to four days. Care was taken that fish was stressed as little as possible. Before stripping, the fish were dried with towels to prevent contamination of the gametes with water. Thereafter the fish were put into a cloth bag to ease the handling of the fish during the stripping process. For each series of experiments, the milt of at least five males were collected into separate 50 ml clean, dry glass holders. The duration of sperm motility from each milt sample was assessed microscopically. Only samples with high cell counts and high motility values ($\geqslant$80%; 5$^+$ motility) were pooled (Chao, 1982; Steyn and Van Vuren, 1987) and the samples having low motility were discarded. The milt was pooled into a 100 ml glass beaker to compensate for individual variation which may have occurred. After collection, the gametes awaiting insemination were kept at 10 ± 1°C.

Twenty four females (or the number females needed for 1.5 litres of eggs) were stripped into a strainer within a plastic bowl. Overripe eggs and blood contaminated ovarian fluid samples were discarded, while good quality ovarian fluid samples were pooled for fertilization experiments. The eggs without ovarian fluid from all females were thoroughly mixed by gently pouring over four times from one container to another to ensure even distribution of different quality eggs. The eggs were then separated into 6 batches of 200 ml and then gently poured into separate round bottomed plastic bowls. The samples (200 ml) contained approximately 2800 or 4428 depending on the age of the donor fish.

The wet fertilization technique, where the sperm were first activated in the diluent before insemination, was used to fertilize the eggs. The proposed wet fertilization technique involves the following: 2 ml of milt added to 25 ml of each of the 6 different diluents respectively. It was then mixed very quickly and poured over each of the 200 ml of egg samples. The inseminated egg samples were mixed immediately, but with delicate handling. The best method to mix the egg and sperm-diluent mixture is by simply pouring the sperm, eggs and diluent into another container two to three times or just by swirling the bowl. All this was done within 30 to 35 s. After insemination this mixture was left for 3 min to allow maximal fertilization. After fertilization, 100 ml water was added to each bowl and allowed to stand for another 3 min. Eggs were then transferred in strainers into clean oxygenated river water to allow water hardening. After an hour the green eggs were transferred to vertical drip incubation trays, where they were left until the eyes of the embryo fish became visible. The eggs were treated on a weekly basis with malachite green.

This series of experiments was repeated ten times during a time span of three years.

Determination of fertilization success

The eggs were not picked during the incubation period. The number of eyed –; dead – and unfertilized eggs were counted approximately 22 to 29 days (depending on the temperature) after fertilization and the subsequent percentages of embryos were calculated. The arithmetic mean was calculated for each of the samples. Test samples were exceptionally large. The mean egg population size was 51 530. The statistical significance and sequence of difference were therefore calculated according to the null hypothesis test for two proportional values and differences were accepted at the 5% significance ($p<0.05$) level (Ferguson, 1976).

Assessment of sperm motility in different diluents

The duration of sperm motility in each of the diluents was assessed microscopically in order to support the findings which were obtained during the fertilization experiments. Spermatozoa were activated by mixing a minute volume of milt by means of the tip of a toothpick into a drop of each of the diluents (10 ± 1°C) previously employed in this investigation. Motility was then evaluated at 400 × magnification. The sperm motility duration was taken as the time at which 50% of the activated spermatozoa ceased forward movement. This was obtained using a stopwatch. This procedure was repeated five times for each of eight males and only good quality milt, *i.e.* $\geqslant$80%; 5$^+$ motility (Steyn *et al.*, 1989), was employed.

RESULTS

Comparison between different sperm diluents

Maximum fertilization success (81.76%) was achieved when ovarian fluid was employed as a sperm diluent. The fertilization success obtained with ovarian fluid were significantly better ($p<0.05$) than the results obtained with SOF (81.12%); Borax 2 (80.03%); Borax 3 (79.03%); Borax 1 (78.98%) and water (69.71%) (*fig.* 1). SOF and Borax 2 showed the second and third best fertilization success respectively and were both significantly ($p<0.05$) higher than the fertilization successes obtained with Borax 3; Borax 1 and water. There was no significant difference ($p<0.05$) between results obtained with Borax 3 and Borax 1, but both of these diluents showed significantly ($p<0.05$) higher fertilization success than water

Assessment of sperm motility

Spermatozoa showed maximum (34 s) forward motility after dilution with Borax 3 (*fig.* 1), and was

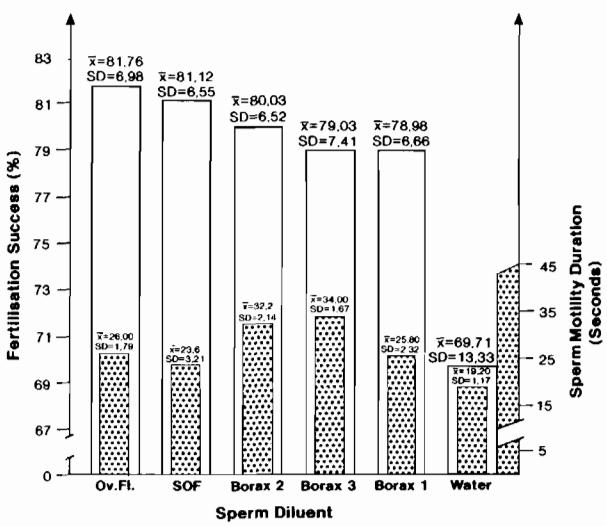

Fig. 1. – Fertilization success obtained as well as duration of sperm motility achieved after dilution in different sperm diluents. ($n=10$; Mean egg population size = 51 530).

significantly ($p<0.05$) better than results obtained with Borax 2 (32.2 seconds); ovarian fluid (26 s); Borax 1 (25.80 s); SOF (23.6 s) and fresh streamwater (19.20 s). Borax 2 showed the second best motility duration after dilution and was also significantly better ($p<0.05$) than ovarian fluid, Borax 1; SOF and water. There was no significant difference ($p>0.05$) between the motility duration obtained with ovarian fluid, Borax 1 and SOF, but these diluents provided significantly ($p<0.05$) higher results than water. Activation with fresh streamwater resulted in the shortest motility duration.

DISCUSSION

In this investigation, the fertilization success obtained when ovarian fluid was employed as a sperm diluent was significantly better ($p<0.05$) than the fertilization successes obtained with all the other diluents tested. Billard (1983; 1988) suggests it is possible that ovarian fluid plays a protective role in the process of natural fertilization occurring in rivers where the gametes are indeed highly diluted. It is therefore very likely that the protein fraction of the biological fluids helps to protect against dilution and washing, as shown in mammals (Bredderman and Foote, 1971; Harrison *et al.*, 1978). Differences in the protein content of the ovarian fluid might explain the high variability of successful artificial insemination when the eggs of different females are inseminated in water (Fredrich, 1981).

The quality of ovarian fluid can vary from one female to another (Dorier, 1949) and is dependent on pH value (pH 8.0; Inaba *et al.*, 1958). Ovarian fluid can also be contaminated with broken eggs and blood during the stripping process. The fertilizing ability of trout eggs is adversely affected by contamination of yolk from broken eggs and blood (Grobler *et al.*, 1992). The quality of ovarian fluid can also change during the reproductive season, because of the stripping time post ovulation. It was therefore decided to develop a simulated ovarian fluid (SOF) that closely resembles ovarian fluid. During these experiments significantly ($p<0.05$) higher fertilization successes were obtained when SOF was employed as a sperm diluent. Bad quality ovarian fluid should be discarded and replaced by SOF or one of the other diluents.

Calcium was added to Borax 2 and 3 in different concentrations as a measure to improve the fertilization successes obtained with Borax 1 diluent. Billard *et al.* (1987) came to the conclusion that if calcium is added, motility is slightly prolonged. In this investigation, Borax 2 (with low calcium chloride concentration) showed significantly ($p<0.05$) higher fertilization success than the fertilization successes obtained with Borax 3 (with high calcium chloride concentration) and Borax 1. There was no significant difference between the fertilization successes obtained with Borax 3 and Borax 1. Borax-boric acid buffer (Steyn *et al.*, 1989) showed improved fertilization with a low calcium chloride content.

Traditionally, freshwater is used as the medium in which the male and female gametes are mixed. It has been shown that freshwater appears to be an unsuitable medium for carrying out artificial fertilization,

since trout spermatozoa swell with dilution in freshwater (Winnicki and Tomasik, 1976) and undergo considerable morphological changes, including the swelling, and even bursting of the plasmatic membrane (Billard, 1978). Water used as a diluent for insemination resulted in the lowest fertilization success.

The maximum (34 s) forward motility obtained after dilution with Borax 3, and Borax 2 (second best) is probably caused by the stimulation of calcium chloride which was added to these diluents. Doroshev (1967) showed that excess Ca^{2+} could be harmful, but in salmonids low amounts of Ca^{2+} are necessary and motility is initiated by Ca^{2+} entry into the cell (Billard *et al.*, 1986).

No significant difference could be observed between the motility duration obtained with ovarian fluid, SOF and Borax 1. Billard (1977*b*) also reported that ovarian fluid did not improve motility duration of rainbow trout spermatozoa when compared to other buffered diluents. The lower mean motility duration (25.8 s) obtained with Borax 1 diluent compared to the results presented by Steyn *et al.* (1989) for the same diluent (35 s) is probably the result of the change in sperm quality towards the end of the reproductive season. Benau and Terner (1980) for instance noticed that motility lasted 30 to 55 s at the peak of the reproductive season and only 15 s at the end of the season.

Activation with fresh streamwater resulted in the shortest motility duration, possibly because of the hypotonic shock which causes the sperm cell structure to deteriorate within several minutes. It is well known that fertility in salmonids declines very sharply in water (Billard *et al.*, 1974).

In conclusion, the use of diluents like SOF, Borax 1, 2 and 3 in artificial fertilization techniques increases the fertilization success and also facilitates hatchery procedures and broodstock management, since semen can be utilised more efficiently.

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