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Effects of coelomic and seminal fluids and various saline diluents on the fertilizing ability of spermatozoa in the rainbow trout, *Salmo gairdneri*

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Summary. When trout (*Salmo gairdneri*) spermatozoa were diluted in coelomic fluid or saline diluents at high dilution rates of 10^{-3} and 10^{-2} for increasing periods of time before insemination, there was a rapid decline and loss of fertilizing ability. At a lower dilution rate of 10^{-1}, there was partial or no loss of fertility. Dilution in a KCl-enriched saline diluent to inhibit sperm motility produced a slight decrease in fertility at a 10^{-3} dilution rate, indicating that the spermatozoa, although sensitive to dilution, were less so when they were kept immotile. A partial loss of fertility was observed after the spermatozoa or eggs had been washed with saline diluents. The loss of fertility was total when both gametes were washed. Removing the seminal fluid by centrifugation led to a significant decrease in the fertilizing ability of the spermatozoa when insemination was carried out in saline diluent but not in coelomic fluid. Adding BSA at high doses (10 mg BSA/ml) into the diluent led to longer survival of the diluted spermatozoa. We conclude that (1) sperm dilution rate is a major factor in the maintenance of fertilizing ability of diluted salmonid spermatozoa, (2) as reported in the literature, coelomic fluid is superior to mineral diluents only when the gametes have been washed, and (3) some substances (possibly proteins) present in seminal and coelomic fluids play a role in gamete protection. These findings may explain the discrepancies in the literature concerning the duration of motility and fertilizing ability of salmonid spermatozoa.

Introduction

Although many studies have shown that salmonid spermatozoa apparently lose their motility and fertilizing ability within a few minutes after activation in saline diluent or coelomic fluid (see reviews by Ginsburg, 1963; Scott & Baynes, 1980), several workers have recorded much longer durations of motility ranging from several hours (Nomura, 1964) to 7 days (Ellis & Jones, 1939). It has also been demonstrated several times that spermatozoa which have apparently lost their motility or fertilizing ability after 2–5 min can be reactivated 1 or 2 h later by further dilution (Ginsburg, 1963, 1968; Terner & Korsh, 1963). Furthermore, it has been claimed that the duration and intensity of sperm motility and the proportion of spermatozoa activated are higher when spermatozoa are in coelomic fluid (which surrounds the eggs) than in artificial diluents (Dorier, 1949; Yoshida & Nomura, 1972; Holtz, Stoss & Büyükhatipoglu, 1977). Scott & Baynes (1980) ascribe the variability in motility estimates to differences in subjective assessments of motility and to technical problems. As examples of the latter, the use of a cover-slip appears to reduce artificially the duration of sperm motility (Terner & Korsh, 1963), and trace amounts of calcium ions in the diluents can prolong duration (Baynes, Scott & Dawson, 1981). While these and several other differences in techniques (e.g. temperature, pH) may explain small differences in motility time,

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they do not account for very prolonged durations of motility, the phenomenon of sperm reactivation, or observations on enhanced sperm motility in coelomic fluid.

The dilution rate, i.e. the volume of diluent/volume of spermatozoa, is not often quoted in research papers and in this paper we have examined whether different dilution rates could provide an explanation for some of the anomalous results.

To obtain more information on the problem of enhanced sperm motility in coelomic fluid, all dilution rate experiments in our study were carried out in coelomic fluid and/or in a variety of diluents with mineral and protein compositions approximating those of coelomic and seminal fluids.

Materials and Methods

Just before spawning, adult fish were taken from a private local fish farm and brought to our laboratory rearing facilities which are supplied with recycled water (Petit, 1974); they were not fed after arrival in the laboratory. The eggs were taken within 1 week after ovulation and the spermatozoa within 1 month after the males had shown the first signs of sperm release. The eggs of several females were collected by the incision method to avoid polluting the coelomic fluid with broken eggs; the coelomic fluid was filtered on a millipore membrane (1·2 μm) before use. Females with overripe eggs were discarded. The milt of several males was pooled and its motility checked microscopically (× 100) on a slide without a cover-slip before and after dilution or insemination. Sperm concentration was determined by counting on a haemocytometer. The experimental procedure for testing sperm survival has been described previously by Billard & Jalabert (1974). Briefly, the spermatozoa to be tested were mixed with the diluent at various dilution rates, and the mixture was poured over the fresh eggs after increasing intervals of time. The percentage of fertilized eggs, measured from the percentage of embryos after 10–15 days of incubation, gave a good estimate of sperm survival.

Sperm survival after dilution

Batches of 200 eggs each were mixed with 10 ml of a diluent and 1000, 100 or 10 μl of a sperm suspension (dilution rates: 10^{-1}, 10^{-2}, 10^{-3}) were added. The dilution time before insemination varied from 2 to 180 min. The diluents tested were: (i) coelomic fluid of the same origin as the eggs, (ii) diluent ID for artificial insemination (NaCl solution, 250 mosmol, pH 9·0, 0·02 M-Tris-buffer, 0·05 M-glycine) (Billard, 1977), (iii) balanced saline diluents reproducing either the mineral composition of the coelomic fluid (CFMM) (in mmol/l: NaCl, 155; KCl, 3·1; $MgSO_4.(7H_2O)$, 1·3; $CaCl_2.(2H_2O)$, 3·4) or that of seminal fluid (SFMM) (NaCl, 110; KCl, 28·3; $MgSO_4.(7H_2O)$, 1·1; $CaCl_2.(2H_2O)$, 1·8); both these diluents were buffered at pH 9 with 0·02 M-Tris–HCl (Billard & Jalabert, 1974). Unlike Diluent CFMM, Diluent SFMM with a high potassium content (28·3 mmol/l) does not initiate sperm motility after dilution (Schlenk & Kahmann, 1938). We therefore employed the double-dilution technique (Billard & Jalabert, 1974) when using Diluent SFMM because sperm motility had to be initiated at insemination. With this method, the eggs were diluted in 10 ml Diluent ID and motility was initiated when Diluent SFMM was added and the potassium concentration dropped. To keep a constant egg–dilution rate, the number of eggs per batch was doubled.

Effect of washing on sperm survival

Using the following protocol in an additional experiment, the eggs and spermatozoa were washed before insemination to eliminate most of the seminal or coelomic fluid.

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(1) Three types of sperm suspension were prepared: (i) intact milt of known sperm concentration; (ii) spermatozoa deprived of seminal fluid by centrifugation at 1500 *g* for 30 min at 4°C; the seminal fluid was replaced by the same amount of Diluent SFMM, and control spermatozoa were kept for the same time at 4°C; (iii) washed spermatozoa; as above, the centrifuged spermatozoa were separated from the seminal plasma and diluted to 10 times the volume of the pellet for 5 min in a NaCl diluent (final osmotic pressure: 250 mosmol, pH 9·0, 0·02 M-Tris, 0·05 M-glycine) including KCl (28·3 mmol/l). The ionic composition of this diluent was simpler than that of Diluent SFMM but the KCl composition was identical, thus preventing sperm motility. After centrifugation at 1500 *g* for 30 min, the supernatant was discarded and replaced by the same amount of Diluent SFMM as the discarded seminal fluid volume to restore the initial sperm concentration ($14{\cdot}6 \times 10^9$ spermatozoa/ml).

(2) The sperm suspension was used at 10^{-3} dilution to fertilize eggs which had been treated as follows: (A) left in 10 ml normal coelomic fluid (control eggs; about 200 per batch); (B) strained and diluted in 10 ml Diluent ID; (C) washed in 50 ml Diluent CFMM for 5 sec while stirred, then strained and immediately diluted in 10 ml Diluent ID and inseminated; (D) as for (C) but washed in distilled water only briefly to prevent activation. Egg fertility decreases after 10–15 sec in distilled water (R. Billard, unpublished) but is maintained for 20 min in Diluent CFMM (Billard & Jalabert, 1974) and so Exp. C constituted a control for the activation in Exp. D.

(3) After insemination with intact spermatozoa, the protein content (albumin reference) of the diluent in all four experiments was determined by the Lowry method as modified by Hartree (1972).

(4) At 15 min after insemination, the eggs were placed in recycled temperature-controlled (10 ± 1°C) fresh water and incubated for 10–15 days.

(5) The eggs were then fixed in a modified Stockard solution (formol 5%, acetic acid 5%, glycerol 5%, water 85%) and the eggs with eyes were counted.

Sperm survival in a protein-enriched diluent

In an additional experiment, BSA (NBC grade IV) was added to Diluent ID at doses of 0·1, 1 and 10 g/l; in the control group, Diluent ID alone was used. The spermatozoa were suspended in these diluents (10^{-3} and 10^{-4}) and exposed for 0 (control) to 16 min before insemination (diluted spermatozoa added to eggs deprived of coelomic fluid).

The χ^2 test and analysis of variance were used for statistical comparison after angular transformation of the fertilization percentages for the analysis of variance.

Results

Sperm survival after dilution in coelomic fluid

The fertilizing ability of spermatozoa diluted in coelomic fluid (Text-fig. 1) was not maintained for long with a high dilution rate (10^{-3}, 10^{-2}). At the lower dilution rate of 10^{-1}, the spermatozoa retained some fertilizing ability, showing a loss of about 30% within 4 min ($P < 0{\cdot}01$). The percentage of fertilization remained at that level for about 60 min, then rose slightly. The protein content of the coelomic fluid was 2·2 g/l.

Comparison of sperm survival in coelomic fluid and mineral diluents

Using the same pool of eggs, this experiment tested the fertilizing ability of spermatozoa diluted in three different mineral diluents. Dilution in coelomic fluid resulted in a decline of fertility with time at dilution rates of 10^{-2} and 10^{-3} but not at 10^{-1} (Text-fig. 2a). After dilution for 30 min in Diluent ID, a significant decline in fertility was observed at all dilution rates. After 60 min, the fertilization rate rose slightly at 10^{-1} dilution, but continued to decrease at 10^{-2} and 10^{-3} dilutions

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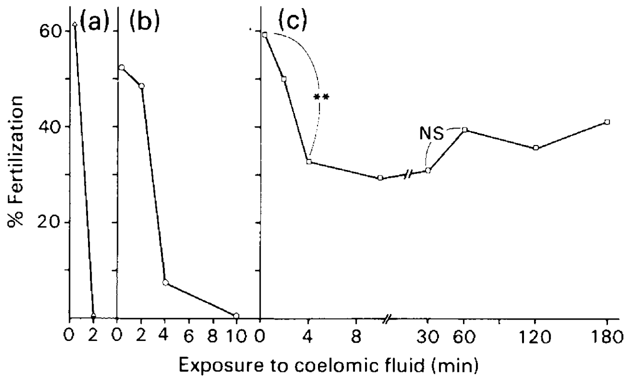
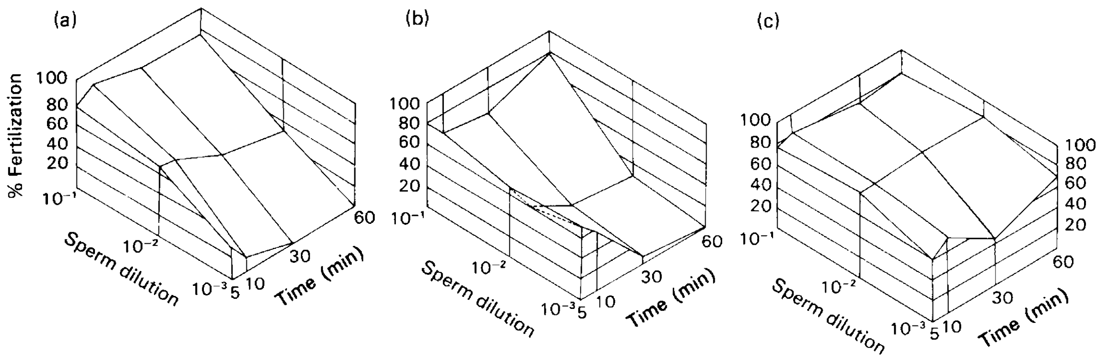


Text-fig. 1. Conservation of the fertilizing ability of trout spermatozoa diluted with coelomic fluid at dilution ratios of (a) 10^{-1}, (b) 10^{-2}, and (c) 10^{-3}. The sperm concentration was $8{\cdot}5 \times 10^9$/ml. $^{**}P < 0{\cdot}01$; NS, not significant.

(Text-fig. 2b). When dilution was carried out in Diluent SFMM, fertilizing ability was unchanged at 10^{-2} and 10^{-1}, and a slight but significant ($P < 0{\cdot}05$) decrease occurred at 10^{-3} (Text-fig. 2c).

Microscopic examination showed that the spermatozoa became totally immotile after a few minutes of dilution. They were experimentally reactivated at a low dilution rate (10^{-1}) when rediluted with coelomic fluid or Diluent ID. The spermatozoa diluted at 10^{-1} were also seen to move when mixed with the strained eggs during insemination; this was obviously due to redilution with the coelomic fluid still present around the eggs. After dilution in Diluent SFMM, the spermatozoa remained immotile, except for a very slight agitation at 10^{-2} and 10^{-3} dilution rates.



Text-fig. 2. Changes in the fertilizing ability of spermatozoa diluted in (a) coelomic fluid, (b) Diluent ID, and (c) Diluent SFMM with increasing dilution ratios and exposure times. The same pool of eggs was used for the 3 treatments. The sperm concentration was $12{\cdot}4 \times 10^9$/ml.

Experiments with washed gametes

When intact spermatozoa were used to fertilize eggs washed in saline or distilled water, a significant ($P < 0{\cdot}01$) drop in fertility was observed compared with the controls (Text-fig. 3). When the seminal fluid was discarded and the spermatozoa were resuspended in Diluent SFMM, fertilizing ability was unchanged if the eggs were kept in the coelomic fluid, but it declined considerably ($P < 0{\cdot}001$) after insemination of eggs with spermatozoa diluted in Diluent ID; there was almost no fertilization when the eggs were washed. Washed spermatozoa partly fertilized eggs diluted in coelomic fluid or Diluent ID but did not fertilize washed eggs. After insemination, the protein content of the coelomic fluid (Exp. A) was 3·9 mg/ml; it was nearly undetectable in Exp. **B** and not detectable at all in Exps C and D (see legend Text-fig. 3).

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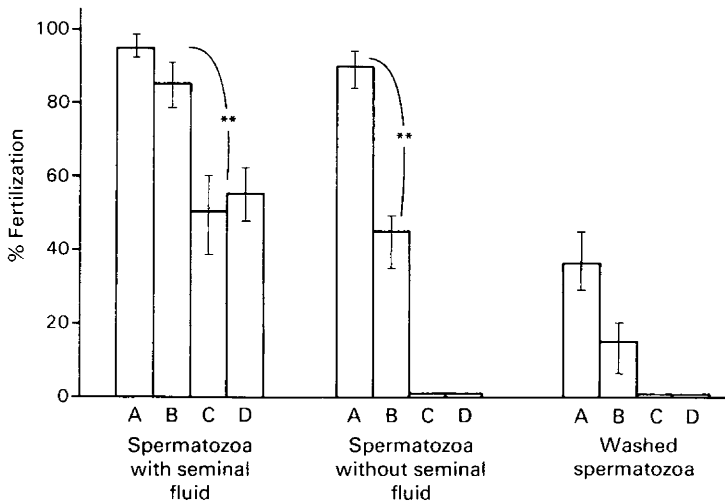

Text-fig. 3. Percentage of fertilization following insemination of batches of 200 eggs kept in 10 ml coelomic fluid (A) or Diluent ID (B) or washed in 50 ml Diluent CFMM (C) or distilled water (D) with intact milt, with spermatozoa resuspended in Diluent SFMM or with spermatozoa washed in the same medium. The same pool of eggs, different from that in Text-fig. 2, was used for all combinations. The sperm concentration was 14·6 × 10^9/ml; protein content (Folin) in the mixture after insemination was 3·9 mg/ml in A and < 50 μg/ml in B, C and D. $^{**}P < 0{\cdot}01$.

Sperm survival in a protein-enriched diluent

When spermatozoa were diluted in Diluent ID enriched with increasing amounts of BSA, survival increased accordingly (Table 1). In the control (Diluent ID alone) or with the lowest dose of BSA (0·1 mg/ml), sperm survival did not exceed 1 min, but at a dose of 10 mg/ml, 15% fertilization was realized after 16-min exposure at 10^{-3} dilution; at 10^{-4} dilution, survival did not exceed 4 min.

Table 1. Fertilizing capacity (% of fertilization) of trout spermatozoa mixed with a saline solution including various amounts of BSA

Exposure to diluent before insemination (min)	BSA added (mg/ml) 0		0·1		1		10	
	10^{-3}	10^{-4}	10^{-3}	10^{-4}	10^{-3}	10^{-4}	10^{-3}	10^{-4}
0	66	60	64	66	74	54	66	67
1	16	8	29	6	47	—	47	20
2	0	0	0	0	25	—	44	6
4	0	0	0	0	1	0	22	5
8	0	0	0	0	0	0	15	0
16	0	0	0	0	0	0	15	0

The sperm concentration was 12·5 × 10^9/ml, and dilution rates of 10^{-3} and 10^{-4} were studied.

Discussion

Effect of dilution rate on sperm fertilizing ability

The survival of spermatozoa diluted for some time in coelomic fluid and in a saline diluent before insemination depended mainly on the dilution rate; fertilizing ability declined more rapidly

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when the dilution rate increased (Text-figs 1 & 2). At t_0, when the gametes were mixed together directly without previous exposure of the spermatozoa to the diluent, the rates of fertilization were roughly similar at 10^{-1}, 10^{-2} and 10^{-3} dilution (Text-fig. 1). Therefore, the number of spermatozoa was sufficient to fertilize most of the fertilizable eggs, and the subsequent differences observed between the fertilization rates could not be attributed to a different initial sperm–egg ratio but must have resulted from dilution. Microscopic examination indicated that spermatozoa in Diluent ID were activated at all dilution rates. This phenomenon of improved maintenance of sperm fertilizing ability at 10^{-1} dilution was probably due to two factors: (i) some spermatozoa retained a low motility and (ii) the motility of the others was reactivated by further dilution.

At dilutions of 10^{-2} and 10^{-3}, the duration of motility is very short (30 sec or a few min, depending on the male) but a few of the spermatozoa often remain slightly active at 10^{-1}; according to Sanchez-Rodriguez & Billard (1977), such motility can last for up to 10 min or more. This fact might account for a small part of the percentage of fertilization which occurs for at least a few minutes after dilution since spermatozoa of very low motility, if provided in sufficient numbers, can fertilize eggs at the time of insemination (Legendre & Billard, 1980).

It has already been shown that spermatozoa can retain their motility for some time at a low dilution rate (7 h at 10^{-2}: Ellis & Jones, 1939; 17 h at 10^{-1}: Nomura, 1964). However, most of the maintenance of fertilizing ability at 10^{-1} is probably due to the reactivation process that we observed in the remnants of coelomic fluid surrounding the eggs or after redilution in Diluents CFMM or ID. The protein residues found in the diluent after insemination of strained eggs clearly indicate that some coelomic fluid was still present round the eggs. This process of diluted sperm reactivation, shown previously in salmonids by Ellis & Jones (1939), Terner & Korsh (1963), Ginsburg (1963) and Nomura (1964), seems to occur only at low dilution rates (10^{-1}, 10^{-2}, depending on the male) but not at 10^{-3} or 10^{-4} (R. Billard, unpublished). One possible explanation is that all the spermatozoa are not activated after the first dilution or are not entirely 'exhausted' and can move again after a second dilution; this reactivation would be due to redilution and not specifically to the coelomic fluid or "egg factor" as suggested by Benau & Terner (1980), especially since reactivation also occurs in a mineral diluent such as Diluent ID.

The importance of coelomic and seminal fluids on sperm fertilizing ability

The reported duration of sperm motility after dilution in total fluid, or a fraction of it, is quite variable (30 sec: Holtz *et al.*, 1977; ~1 min: Dorier, 1949; Benau & Terner, 1980; 9 min: Yoshida & Nomura, 1972; 1 h: Rucker, Conrad & Dickeson, 1960; 17h: Nomura, 1964; 5–7 days: Ellis & Jones, 1939). Contrary to these contradictory reports, the present work shows that coelomic fluid does not extend the life-span of rainbow trout spermatozoa any more than do properly buffered diluents (Billard, 1977). Nevertheless, the superiority of coelomic fluid over mineral diluents was demonstrated in the washing experiments, especially when the spermatozoa were washed and deprived of seminal fluid (Text-fig. 3). In addition, sperm survival was enhanced by adding a high dose (10 mg/ml) of BSA into Diluent ID. The fact that this amount was higher than that in the normal coelomic fluid used in the present experiments (Text-fig. 3)—which did not exceed 4 mg/ml and had no noticeable effect on sperm survival—suggests that the frequently reported beneficial effect of the coelomic fluid results from a high level of protein that can reach 20 mg/ml (B. Breton & R. Billard, unpublished). BSA has already been shown to have a beneficial effect in the deep-freezing of trout spermatozoa (Legendre & Billard, 1980). Differences in the protein content of the coelomic fluid might explain the high variability of successful artificial insemination when the eggs of different females are inseminated in water (Fredrich, 1981). The favourable effect of the coelomic fluid on washed gametes may also be due to the presence of Ca^{2+} which is essential to fertilization (Yamamoto, 1961; Ginsburg, 1963; Gilkey, 1981) but washing the eggs in a balanced mineral diluent (Diluent CFMM) did not improve the fertilization rate compared to washing in distilled water; the protein environment seems to be more important than the mineral one,

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although the ionic composition of the diluent has been shown to be essential to egg fertility (Billard, 1981). 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 & Foote, 1971; Harrison, Dott & Foster, 1978). Proteins may also act by complexing heavy metals.

Other variables, such as pH and osmotic pressure (Petit, Jalabert, Chevassus & Billard, 1973; Baynes *et al.*, 1981), could explain the discrepancies in the literature, although Stoss, Büyükhatipoglu & Holtz (1977) found that pH and osmotic pressure had no great impact on trout sperm motility. Another important factor is the wide individual variation in the quality of the spermatozoa (see differences in fertilization rate at 10^{-1} dilution; Text-figs 1 & 2a). Sperm quality changes during the reproductive season; for instance, Benau & Terner (1980) noticed that motility lasted 30–55 sec at the height of the reproductive season and only 15 sec at the end with a decrease in sperm cAMP concentration. The storage temperature is also important; Benau & Terner (1980) reported that Atlantic salmon spermatozoa diluted in coelomic fluid were motile for 3 min at 11°C and for several hours if kept on ice. Van Der Horst, Dott & Foster (1980) reported that in a diluent such as sucrose, which inhibits motility at 220–300 mosmol/kg, the blocking effect was overcome by increasing the dilution rate. Although this was not found by Billard (1980) for sucrose, such a phenomenon (a very slight agitation of the spermatozoa at 10^{-3} and 10^{-2} but not at 10^{-1}) did seem to occur with Diluent SFMM, and might account for the drop in fertility observed at 10^{-3} dilution in Diluent SFMM. It could be attributed to a larger amount of Ca^{2+} antagonizing the inhibitory effect of K^+ (Baynes *et al.*, 1981). However, the sperm–egg ratio is slightly lower in the experiments with Diluent SFMM. The decline in fertility after dilution is much less in Diluent SFMM than in Diluent ID, suggesting that immotile spermatozoa are far less sensitive to dilution.

In conclusion, while coelomic fluid, even at high dilution rates, does not appear to be better than saline diluent when used in artificial insemination with intact gametes, it does seem to have a protective effect when the gametes are washed. A similar protective effect can also be attributed to seminal fluid. When these companion fluids are removed, gamete fertility is considerably decreased. It is therefore possible that they play a protective role in the process of natural fertilization occurring in rivers where the gametes are indeed highly diluted.

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