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SUPERCOOLED STORAGE OF SALMONID OVA

by

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INTRODUCTION

Most of the problems associated with cryopreservation of salmonid spermatozoa are close to solution (see review by Scott and Baynes, 1980); preservation of unfertilized ova, on the other hand, remains a formidable challenge. Many workers hold that the great size of teleost ova renders them unlikely candidates for successful freeze-thawing, and that success is more likely to be had in short-term preservation at temperatures around 0°C (Harvey 1982; Stoss and Donaldson 1983). Storage has been attempted by holding salmonid ova in ovarian fluid at 0°C; by incorporating antibiotics in the fluid, Pueschel (1979) was able to maintain 70% fertility in rainbow trout ova for 20 days (compare also Stoss and Donaldson, 1982). Recent reviews on short-term preservation of fish ova can be found in Stoss (1983) and Jensen and Alderdice (1984).

Our previous attempts at cryopreservation of salmonid ova brought to light the ability of the salmonid egg to undergo a modest degree of supercooling - as low as -20°C for several hours - and remain fertile. Supercooling of fertilized salmonid ova has also been reported by Haga (1982). We undertook the present study to test the supercooled storage of ova as a means of medium-term preservation (30-60 d), with emphasis on the development of appropriate media to replace ovarian fluid.

MATERIALS AND METHODS

Ova were stripped from various species (coho salmon, Oncorhynchus kisutch; chum salmon, O. keta; and rainbow trout, Salmo gairdneri) and were used interchangeably. Ova pooled from several fish were held in oxygenated plastic bags on ice for up to 2 h before storage.

1. Storage conditions

Unsealed 250 mL glass jars were used to store ova for 24 h; in all other cases ova were held in 75 mL plastic tissue culture flasks. Eggs formed a single layer on the bottom of the storage containers and were covered with medium to a depth of 5 mm (egg to medium ratio 1:1.5 v/v). Since ovarian fluid is not available in large quantities, egg: ovarian fluid ratios were kept at 1:0.1-1:0.4 in those experiments in which ovarian fluid alone was added.

Capped flasks were laid flat in ducted styrofoam containers (approx. 90 x 60 x 20 cm) placed within an 18 ft^3 commercial freezer at -7°C. Airflow was provided by a continuously operating fan within the styrofoam box; storage temperature was maintained by variable output from a temperature-controlled 75-watt heating element mounted in the airflow. Individual flasks were separated by plastic or wooden spacers to allow free circulation of air. For most experiments the temperature was held at -1°C (-0.75 to -1.25°C); several experiments were also done at -4°C (-3.75 to -4.25°C).

2. Media

Ovarian fluid was routinely separated and ova re-suspended in one of the following:

a) Medium HM. This medium is based on the synthetic mammalian tissue-culture medium "199", with the following additions:

Hepes buffer	25 mM
Kanamycin sulfate	50 µg ml^{-1}
Sodium pyruvate	0.86 mM
$NaHCO_3$	0.08% mM
Calf serum	13.0% v/v
Ficoll	2.5% w/v

pH adjusted to 8.3 with NaOH at room temperature.

b) Medium SM. This medium is based on the mineral composition of rainbow trout ovarian fluid and has been used earlier without antibiotic in supercooling trials (Stoss and Donaldson 1983):

NaCl	145.4 mM
KCl	3.4 mM
$MgSO_4$	0.5 mM
$CaCl_2$	3.0 mM
Kanamycin sulfate	50 µg mL^{-1}
Tris (hydroxymethyl) aminomethane	20 mM
Bovine serum albumin	10 mg mL^{-1}

pH 8.4 adjusted with citric acid at 0°C

These two basic media were altered by addition or variation of the following components, according to the needs of the experiment:

- Ficoll, 2.5-7%
- Dimethylsulfoxide (DMSO), 1.7 - 7%
- Dextrose, 5-15%
- Methanol, 1-5%
- Sucrose, 5-30%
- Polyvinylpyrollidone (PVP), 5-25%
- Carboxymethylcellulose, 0.5-4%

3. General storage and fertilization procedures

All glassware, media, and eggs were held on ice (0°C) until use. Batches of eggs were measured volumetrically and added to storage containers that were already partly filled with medium.

At the end of the storage period eggs and medium were allowed to warm to 10°C before insemination with fresh or frozed milt. Prior to insemination with fresh milt, eggs were rinsed in 1.3% $NaHCO_3$ (10°C) (Wilcox et al., in prep.). Milt was kept for up to 3 days in oxygenated polyethylene bags on ice (0°C) (Stoss and Holtz, 1983). Only those pooled samples with high motility (> 70%) were used.

Eggs were also inseminated with cryopreserved sperm in some trials.

Freezing was performed according to Stoss and Holtz (1981) with subsequent storage in liquid nitrogen. A 300 mM glucose solution containing 9.1% (v/v) dimethylsulfoxide (DMSO) served as extender (Stoss and Refstie, 1983). Sperm pellets were thawed in 1% $NaHCO_3$ (30°C; 1 pellet per mL) and one pellet was used to inseminate 10 salmon eggs or 15 trout eggs. Untreated eggs from the same batch were inseminated with unstored sperm and served as controls. Eggs were incubated in Techna Heath Trays and fertility checked by clearing embryonated eggs (0.9 NaCl + 5% acetic acid) at day 7 to 10 of incubation (10°C) or at the eyed stage.

RESULTS AND DISCUSSION

1. Effect of medium volume

The volume of ovarian fluid expressed along with ripe ova varies between individuals, and it was important first to establish whether stored eggs were sensitive to the amount of synthetic medium surrounding them. The data below demonstrate no such effect after 24 h storage at -1.0°C in HM:

Table 1 - Fertility of coho salmon ova (day 8) stored for 24 h at -1°C with HM-medium. 2 replicates per treatment, 65 eggs per replicate.

Egg to medium ratio v/v	Fertility (%)
Untreated control	94.6
1/8	59.7
1/4	50.7
1/2	60.6
1	60.4
2	54.8
4	60.6
8	45.0

It seems clear that ova need not be immersed in medium; condensation on the inner walls of the flasks after 24 hr indicated that, at all volumes used, the relative humidity within the flask was 100%. The fertility in all treatments was lower than in the unstored, control eggs.

2. Osmolality of medium

The osmotic pressure of ovarian fluid from salmonid fishes maturing in freshwater is roughly 300 mOsm (Hirano et al. 1978; Stoss and Fagerlund 1982; Lam et al. 1982; Stoss and Holtz, unpublished). To investigate the sensitivity of ova to osmotic pressure during storage coho salmon eggs were exposed to HM-medium adjusted to various osmolalities by dilution or

concentration of the basic medium. The results (Table 2) indicate that media of 200-450 mOsm are equally tolerated for 24 h.

Table 2 - Fertility of coho salmon ova (day 8) stored for 24 hr at -1°C in media of varying osmotic pressure. 2 replicates, 65 eggs per replicate.

Osmotic pressure of HM-medium (mOsm kg^{-1})	Fertility (%)
untreated control	80
200	73.1
250	73.5
300	62.8
350	71.1
400	71.9
450	74.9

It is, of course, entirely possible that longer storage at 'incompatible' tonicities will prove detrimental; our experiment was not designed to test this. In subsequent experiments, osmolality was maintained at 300 mOsm kg^{-1}.

3. Effect of antibiotics

Pueschel (1979) has demonstrated a prolongation of storage of unfertilized salmonid ova through addition of antibiotics to the ovarian fluid in which the ova were kept. We have routinely incorporated kanamycin sulfate (50 µg ml^{-1}) in our storage media, although there is little reason to expect bacterial contamination to be limiting within 24 h. A short-term test, reported in Table 3, demonstrated that kanamycin had no adverse effect on egg fertility.

Table 3 - Effect of kanamycin in storage medium on fertility (day 8) of coho salmon ova after 24 h storage at -1°C. 2 replicates, 73 eggs per replicate.

Medium	Fertility (%)
unstored control	95.2
ovarian fluid	95.1
ovarian fluid + kanamycin	89.9
HM-medium	60.0
HM-medium + kanamycin	63.9

These data clearly show, however, that 24 hr exposure to HM-medium is detrimental whereas storage in ovarian fluid under identical conditions maintains fertility. This observation was also made in following experiments and will be discussed below.

4. Interaction between calf serum and Ficoll in HM-medium

Calf serum is a standard component in many tissue-culture systems and was included in HM simply because low-temperature storage of salmonid ova is a cell maintenance system in its own right. Unpublished observations on unfertilized zebrafish embryos (Harvey 1982) have shown Ficoll, a high molecular weight polymer, to be effective in delaying egg activation, and various concentrations of this compound were incorporated in HM as well. There seems, however, to be an interaction between these extracellular agents: either calf serum or Ficoll in HM elevated fertility of coho eggs after 24 h storage at -1°C, while a combination of the two was less effective. We also noted a decline in fertility with increasing amounts of Ficoll added to HM + calf serum.

Table 4 - The effect of calf serum and Ficoll on fertility (day 8) of coho salmon eggs. 24 hr storage at -1°C, 2 replicates.

Medium	Fertility (%)
unstored control	94.9
100% calf serum	0.0
HM-medium (no Ficoll, no calf serum)	30.5
HM-medium + 13% calf serum	63.9
HM-medium + 2.5% Ficoll	60.3
HM-medium + 5.0% Ficoll	45.6
HM-medium + 7.0% Ficoll	32.5
HM-medium + 13% calf serum + 5% Ficoll	45.6

5. Effects of cryoprotectants at -4°C

Unprotected salmonid ova held at -4°C will remain supercooled for several hours, but intracellular freezing (and consequent death) is inevitable (Harvey and Ashwood-Smith 1982). Can cryoprotectants play a role in preventing freezing and loss of fertility at this temperature? We tested the effect of 24 h exposure of coho eggs to the intracellular cryoprotectants DMSO (dimethylsulfoxide) and methanol, and to the extracellular cryoprotectants polyvinylpyrrolidone (PVP), sucrose and dextrose. Current cryobiological theory holds that penetrating protectants displace cell water and so act colligatively; the action of extracellular protectants has yet to be satisfactorily accounted for. Our results show that, while considerable protection from freezing is afforded by these protectants, fertility declines precipitously after 24 h at -4°C.

Sucrose at 5 and 10% did not prevent the medium from feezing during

storage for 24 hr at -4°C. At 15 to 30%, ice seeding was observed in half the samples; however, eggs from non-frozen samples had swollen and become almost transparent. Few of these eggs water-hardened upon transfer to water. PVP between 5 and 15% did not prevent freezing of the medium. Similarly, ice crystallization was observed in the presence of 5-25% dextrose and eggs from unfrozen samples with $\geq$15% dextrose resembled those treated with sucrose.

One of the two replicates with 1.7% and 3.5% DMSO was frozen. Eggs stored in DMSO were partially collapsed, suggesting osmotic stress due to slow permeation of DMSO. Only methanol prevented freezing at all concentrations tested. There is little doubt that all cryoprotectants tested are directly toxic to salmonid ova, since fertility was extremely low in all cases. Of note, however, is the relative high fertility observed with 1% methanol. This accords with previous findings that methanol penetrates fish ova more rapidly than do the conventional cryoprotectants DMSO and glycerol (Harvey and Ashwood-Smith 1982).

Table 5 - The effects of various cryoprotectants on fertility (day 8) of coho salmon ova. Storage for 24 hr at -4°C, 2 replicates, 101 eggs per replicate. HM-medium with 13% calf serum and 2.5% Ficoll.

% cryoprotectant in HM-medium	Osmotic pressure (mOsm kg^{-1})	Fertility (%)
untreated control	-	98.6
PVP (5% - 20%)	359 - 471	0
15% sucrose*	718	0.66
20% sucrose*	828	0
1% methanol	*	48.5
2% methanol	*	15.2
3% methanol	*	12.0
4% methanol	*	2.6
5% methanol	*	2.7
1.7% DMSO*	638	15.3
3.5% DMSO*	916	14.3
5.1% DMSO	1100	1.4
7% DMSO	1298	0
5% dextrose	619	0.68
10% dextrose	954	0

* 1 replicate; osmolality not recorded.

6. Effect of carboxymethyl cellulose

Laird and Wilson (1979) reported a protective effect when fertilized salmonid ova were bottled in a solution of methylcellulose for transportation, and suggested that the viscous fluid served to attenuate mechanical shock. We found a protective effect with this compound at 0.5 and 1% v/v in SM medium; this finding may reflect structural similarities between methylcellulose and Ficoll, another high molecular weight polymer.

Table 6 - Carboxymethyl-cellulose as medium component for coho salmon eggs stored at -2°C for 24 hr. 2 replicates, 53 eggs per replicate.

% carboxymethyl cellulose in SM-medium	Eyed embryos (%)
untreated control	93.4
0	77.3
0.5	93.4
1.0	85.4
2.0	17.0
4.0	15.1

7. Long-term experiments

We have already noted that all of the artificial media used in this study produced a pronounced decline in fertility after 24 h storage at 1.0°C, and it is not surprising that success in storage at -1°C for periods greater than 24 h was limited. For example, although ovarian fluid is clearly superior to both artificial media, fertility in all three treatments declined severely by 20 d. Figure 1 shows this decline in fertility in coho salmon ova stored at 1°C in ovarian fluid + kanamycin. If supercooled storage of ova is to be pursued and if ova are to be stored in large numbers, it is of paramount importance to develop an artificial saline whose performance is at least as good as ovarian fluid. At present, we recommend storing ova in ovarian fluid at temperatures around 0°C for up to 10 days. We also recommend that, in future work on this problem, ova from as many females as possible be used in each experiment, to eliminate the possibility that individual variation may be reflected in the viability of stored ova. This is particularly important when ova from females nearing the end of the spawning season are used.

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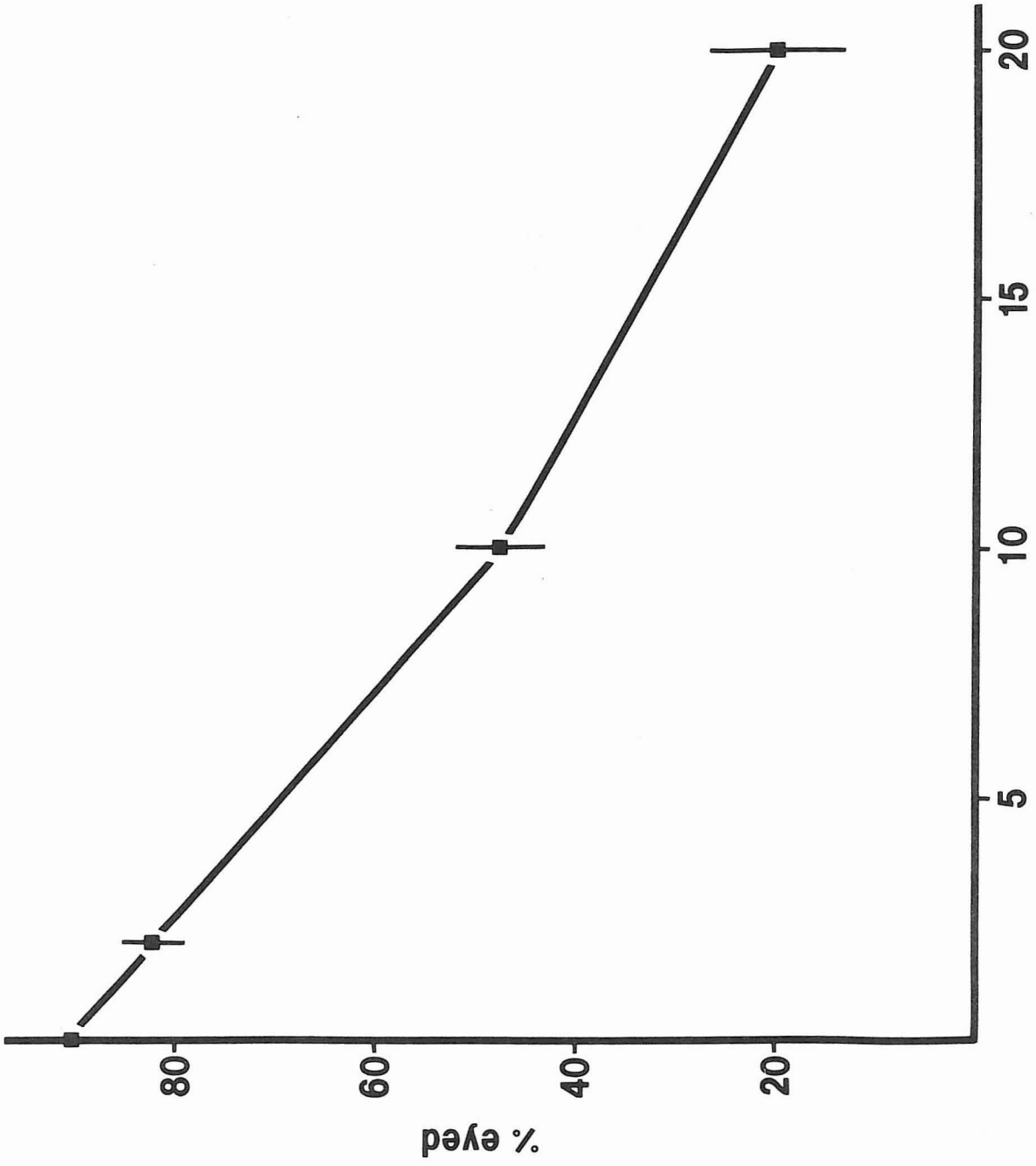

Figure 1. Fertility of coho salmon ova stored at -1°C in ovarian fluid. Y ± SD.