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Control of Reproduction in Northern Pike

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Abstract

Several methods to control gamete availability in northern pike were investigated. Precocious induction of ovulation was achieved with a single injection of partially purified salmon gonadotropin, but the treatment should be administered soon after capture in order to avoid ovarian atresia. Ovulated oocytes should be inseminated within 24 hours after ovulation. Loss of fertility due to aging occurred 1-3 days after ovulation. High doses of progesterone induced a threefold increase of sperm release. Cryopreservation of diluted sperm at -196°C led to variable fertilizing capacities after thawing, depending on the donor male. A diluent originally tested for trout was used successfully for artificial insemination.

In France, the main purpose of northern pike breeding is the stocking of natural waters. However, the amount of professional fishing is far from being sufficient to gather ripe breeders in adequate numbers and obtain fry or fingerlings in quantities that would meet the national needs.

The knowledge of spawning migration habits improved the catch of ripe females (Chimits 1947, 1956; Cointat and Darley 1953, Preudhomme 1968). However, the results were very irregular from year to year, depending notably on weather conditions. Therefore, some attempts were made to control reproduction, mostly the phases of maturation and ovulation.

Ovulation does not occur spontaneously under confined conditions. Moreover, most confined females quickly undergo ovarian atresia and may die after a few weeks (Portal 1947; Chimits 1947, 1956; de Montalembert et al. unpublished observations).

In small ponds, ripening can be attained, but a certain range of temperature must be maintained despite the atmospheric conditions (Cointat and Darley 1953; Huet 1972). Natural spawning and fertilization may then also take place (Schaperclaus 1961; Kausch 1976); yet the real efficiency (i.e. the number of fry collected compared to the potential number of eggs) of such an extensive method has not been specified.

Hypophysation with carp pituitaries, performed in one or several injections, can induce ovulation (Sorenson et al. 1966) but precise data concerning the state of matur-

ity of recipient females, as well as the ovulation and hatching rates, are lacking.

Another difficulty commonly experienced lies in the low volume of sperm usually delivered by the males, especially at the end of the spawning season (Portal 1947; Chimits 1956; Sorenson et al. 1966; Preudhomme 1975; Huet 1976).

Salmon pituitary extracts, HCG (human chorionic gonadotropin), and various steroids induce sperm release in goldfish (Yamazaki and Donaldson 1969; Billard 1976). Progesterone is more potent than androgens (Billard 1976). In northern pike, sperm release was stimulated within 24 hours following hypophysation (Anwand 1963), but no steroid—a much cheaper treatment—was tested.

Cryopreservation of viable spermatozoa would allow the ova of belated females to be fertilized with semen collected from ripe males early in the spawning season. With the sperm of the northern pike, the only trials reported to date resulted in an average fertilizing capacity of 25% (Stein 1977).

Finally, with a given quantity of semen, the best way to increase the efficiency of artificial insemination is to lengthen the life of spermatozoa and thus greatly reduce the number of spermatozoa needed per ovulated oocyte. Since a buffered and osmotically balanced diluent has been used with success with trout to obtain high fertilization rates with minute quantities of sperm (Petit et al. 1973; Billard 1977), it seemed particularly worthy to examine its possible effectiveness with northern pike.

The present investigation was carried out in order to develop methodologies for (1) hormonal induction of maturation and ovulation in submature females, (2) hormonal stimulation of sperm release, (3) sperm cryopreservation, and (4) artificial insemination with the aid of a diluent.

Methods

The experiments were performed in March-April 1976 and 1977. Northern pike breeders (males: 1–2 yr old, weighing 0.2–0.4 kg; females: 2–3 yr old, weighing 0.8–2 kg) were collected from natural ponds and kept in full darkness at a temperature of about 12 C. Before each handling (injection, gamete sampling), the fishes were anesthetized in a solution of 2-phenoxyethanol (Merck) (0.5 ml/liter).

Induction of Ovulation

The nature of the injected material as well as the determination of oocyte stages, ovulation, and fertilization rates have been described previously (de Montalembert et al. 1978). In brief: the efficiency of various hormonal treatments (gonadotropic preparation and/or steroid) on *in vivo* maturation and ovulation was investigated in submature females; the germinal vesicle in non-peripheral position was the criterion for initial oocyte stage. Two gonadotropic preparations (partially purified salmon gonadotropin and HCG) and two steroids (17 α -hydroxy-20 β -dihydroprogesterone and 17 α -hydroxyprogesterone) were employed.

Retention of Ova

In the first of two experiments, 5 groups of 2 females were used. Ovulation was induced by salmon gonadotropic extracts (0.1 mg/kg). One thousand ova were sampled from each female of group 1 on the day of ovulation and inseminated with sperm pooled from 10 males, by means of the dilution technique described below (dilution rate: 1/1,000). The eggs were incubated in a fine mesh trough at about 12 C. The same procedure was applied to group 2 one day after ovulation, to group

3 two days after ovulation, and so on until day 4. Fertilization rates were expressed as the percentage of embryos on the third day following insemination (i.e. at about 40 degree-days of development).

The second experiment was carried out with 7 females kept at a constant temperature of 13 C. Ovulation was obtained with a low priming dose of trout gonadotropic extracts followed by 3 mg/kg of 17 α -hydroxy-20 β -dihydroprogesterone (17 α -20 β P) 24 hours later. Four hundred ova were sampled from each of the 7 females on the day of ovulation and on the five following days, and inseminated with sperm pooled from 10 males. The fertility of the ova was assessed at the time of hatching.

Stimulation of Sperm Release

HCG was dissolved in saline (0.8% NaCl). Progesterone and testosterone were dissolved in pure ethanol, then precipitated in saline just before injection (20% ethanol in the injected product). The injected volume always was 1 ml/kg.

Every two days, sperm was expelled by gentle handstripping and the volume of sperm was measured with a precision of 10 μ l. In a preliminary experiment the efficiencies of HCG (1,000 IU/kg), progesterone (10 mg/kg), and testosterone (10 mg/kg) (2 injections 24 hours apart) were compared in groups of 7 fish by measuring the volume of sperm release until exhaustion. In a second experiment, 3 doses of progesterone (100, 10, 1 mg/kg, a single injection each) were tested on 3 groups of 10 fish. A control group was treated with saline. The volume of sperm and the spermatocrit (volume of spermatozoa in %) were measured. The fertilizing capacity of the sperm was checked 2 days after treatment (peak of the response) by insemination of a 300-egg sample with 10 μ l of sperm (dilution rate: 1/1,000).

Cryopreservation of the Sperm

A drop of handstripped sperm was mixed with the insemination diluent (DIA_{TR}; see below) and the motility classified as one of the 5 stages described by Emmens (1947)

and Jaspers (1972). The activity of the spermatozoa was defined as the duration (in minutes) of the most active stage following the dilution. The fertilizing capacity of the fresh semen was tested on 300 ova using the dilution technique (dilution rate: 1/100). The fertilization rate was the percentage of embryos at 40 degree days.

For cryopreservation, 50- μ l aliquots of sperm were mixed with the V₂ extender described by Stein (1977): 750 mg NaCl; 200 mg NaHCO₃; 53 mg Na₂HPO₄·2H₂O; 23 mg MgSO₄·7H₂O; 38 mg KCl; 46 mg CaCl₂·2H₂O; 100 mg glucose; 500 mg glycine; 20% egg yolk; 10% dimethyl sulfoxide; 100 ml H₂O.

In a preliminary experiment, four dilution ratios (volume of sperm: volume of extender: 1:2; 1:3; 1:5; 1:10) were tested with sperm pooled from 10 males. The diluted sperm was equilibrated in an ice water bath during 5 min and the absence of motility of the spermatozoa was checked. The samples were then dropped onto dry ice (-79 C) and stored in liquid nitrogen (-196 C). After a 7-day storage, the motility and activity were assessed in the DIA_{TG} diluent. In order to estimate the fertilizing capacity, the sperm was thawed directly in DIA_{TG} containing the ova.

In a second experiment, the semens of 10 males were individually cryopreserved (dilution ratio 1:2) and checked for fertility according to the same experimental procedure.

Artificial Insemination by a Dilution Technique

Ovulation was induced by a single injection of partially purified salmon gonadotropin (de Montalembert et al. 1978). The ova and the sperm were collected carefully by handstripping, in dry condition. Ova from several females were mixed and divided into batches of 300 eggs. Sperm was sampled from 10 males, examined to assess motility, and pooled. The control lots were inseminated according to the classical dry method.

Each experimental lot (300 ova) was placed in 10 ml of DIA_{TG} diluent and immediately inseminated. The composition of DIA_{TG} (Billard 1977) was the following: solution of NaCl buffered with 0.02 M Tris

and 0.05 M glycine to an osmotic pressure of 250 mosmol and pH 9. Various amounts of sperm (100 μ l, 10 μ l, 1 μ l, 0.1 μ l; dilution rates: 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵) were used in a first experiment. The eggs were allowed to stay for 15 min following insemination, then poured into a fine meshed trough where incubation was carried out at about 12 C until day 4 following insemination. The eggs were then cleared in Stockard's solution and the fertilization rates were determined as the percentage of embryos at 40 degree days.

In a second experiment, sperm was pre-diluted in DIA_{TG} (dilution ratio 1:100 or 1:1,000) or in fresh water (dilution ratio 1:1,000) during various periods of time (0, 1, 2, 4, 8, 16 min) and then mixed with ova.

Statistical Analysis

The mean values of the ovulation, fertilization, and hatching rates and their confidence limits at the 95% level of probability were computed from the angular (arc-sine) transformation of the percentages. The levels of significance were determined by the *F* test in the induction-of-ovulation experiment, and by the *t*-test on paired data in the retention-of-ova experiment.

Unless otherwise indicated, differences noted as significant in the text correspond to *P* < 0.05.

Results

Induction of Ovulation

The detailed results concerning induction of ovulation have been described in a previous paper (de Montalembert et al. 1978). Therefore we will only mention here the most important experimental facts.

A single injection of partially purified salmon gonadotropin (0.1 mg/kg) induced maturation and ovulation in submature females (initial stage of the oocytes: nucleus in nonperipheral position). Ovulation occurred 4 days after the treatment (water temperature: 12 C).

In 6 females out of 14 the ovulation was total. In the remaining 8 females the ovulation was only partial but was always over 50% (general mean: 89%). In all cases, the

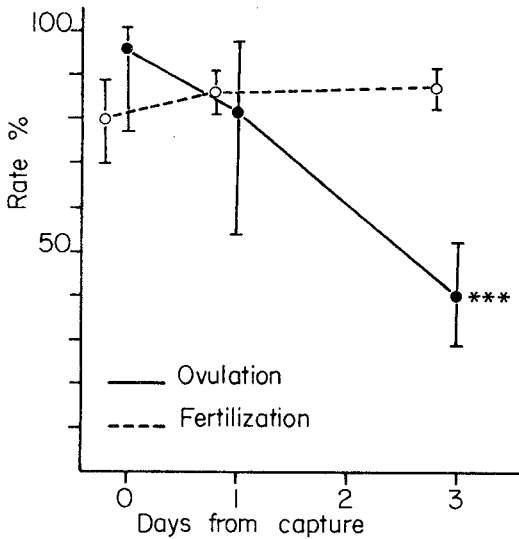


FIGURE 1.—Effects of time between capture and treatment (partially purified salmon gonadotropin: 0.1 mg/kg) on ovulation and fertilization rates. Vertical bars show confidence limits at the 95% level of probability. *** indicates statistical difference from initial rate ($P < 0.001$).

fertilization rates were high (mean 83%). Figure 1 shows the effect of various time intervals between capture and treatment. When the treatment was performed within 24 hours after capture no significant changes could be noted in ovulation and fertilization rates but when the females were held under confined conditions of captivity during 3 days before treatment, ovulation rate dropped to 40% ($P < 0.001$). However, the fertility of ovulated oocytes was not affected.

Effect of the Retention of Ova on Fertility

In the first experiment where each female was sampled once only, no significant alteration of fertility occurred during 48 hours after ovulation (fertilization rates at 40 degree-days: 90 and 85%). On the third day following ovulation, the fertilization rate sharply dropped to 30% (Fig. 2).

In the second experiment, where each female was sampled every day during 6 days, a significant alteration occurred one day earlier (day 2; Fig. 2). The medium fertility (50%) obtained on day 0 results from the nature of the hormonal treatment

to induce ovulation and from the moment fertility was checked (at hatching).

Stimulation of Sperm Release

The greatest sperm release was obtained with progesterone (10 mg/kg) and occurred three days after the second injection; the average volume delivered was twice as much as in the controls and all 7 animals responded (Fig. 3). With testosterone (10 mg/kg) no change could be noted in 3 males and the response was heterogeneous in the remaining 4 individuals. HCG (1,000 IU/kg) seemed to have a delayed effect after 10 days.

In the second experiment very high doses of progesterone (100 mg/kg) were the most efficient; all the fish were stimulated and the maximum response (a three-fold increase $P < 0.001$) was recorded 48 hours after treatment. The spermatocrit was not affected by the hormonal treatment, and insemination performed at the 1:1,000 dilution ratio led to high fertilization rates (mean: 70%); there was no significant difference from the fertilizing

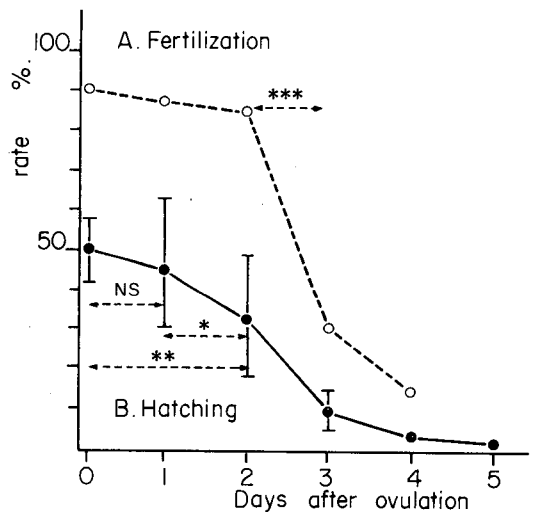


FIGURE 2.—Effect of the in vivo retention of ova on fertilization and hatching. Curve A: each female was sampled only once. Each point is the mean fertility of ova from 2 females. Fertility was checked at 40 degree-days. Curve B: each of the 7 females was sampled once a day and fertility was checked at hatching time. Vertical bars show confidence limits at the 95% level of probability. Levels of significance: NS: $P > 0.05$; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

capacity of the control sperm. With the intermediate dose of progesterone (10 mg/kg) only 5 males out of 10 showed a significant response (2 to 3 times increase). With the lowest dose (1 mg/kg) there was a 2 to 3 times increase in 4 animals (Fig. 4).

Cryopreservation of the Sperm

Among the dilution ratios tested during cryopreservation tests, 1:2 gave the best results (Fig. 5).

The motility, activity, and fertilizing capacity of the ten sperm samples cryopreserved individually appear in Table 1. Eight of the sperm samples showed variable fertilizing capacities after cryostorage (1% to 68%), while two had lost all viability (no motility and no fertilizing ability).

Artificial Insemination by a Dilution Technique

The fertilization rate (Fig. 6) stayed at its maximum value (94–98%) with the 10^{-1} to 10^{-3} dilution ratios and dropped at 10^{-4} (41%) and 10^{-5} (10%). The control (dry insemination) was fertilized at 75%.

When fresh sperm was diluted in DIA_{TC} prior to insemination, fertilization was still 80% after 2 min and 15–25% after 8 min (Fig. 7). In fresh water, fertilization dropped to 25% after 1 min and was no longer possible after 2.5 min.

Discussion

Induction of Ovulation

Our data on induced ovulation clearly demonstrate the efficiency of an adequate hormonal treatment (a single injection of partially purified salmon gonadotropin) and bring out the drastic effects of captivity which have been discussed previously (de Montalembert et al. 1978).

Hormonal control of ovulation offers the obvious advantage of independence with regard to such restraints as temperature and space. Moreover, the females become synchronized, since most ovulations occur on day 4 after treatment. However, before a widespread use of the technique can be considered, progress should be made towards economic production of standardized fish gonadotropic extracts.

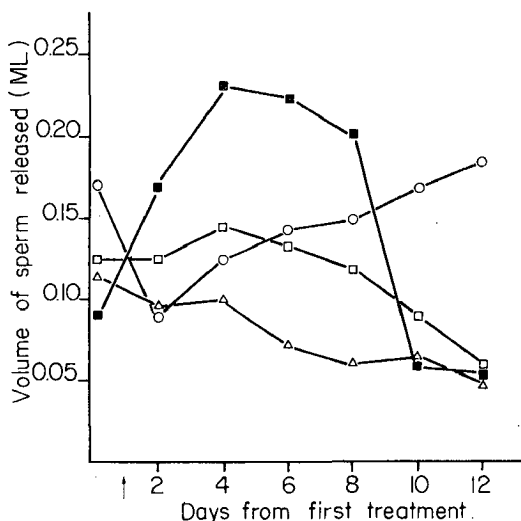


FIGURE 3.—Effect of 2 injections of hormones administered 24 hours apart (arrows) on the volume of sperm released. ○: human chorionic gonadotropin (1,000 IU/kg); ■: progesterone (10 mg/kg); □: testosterone (10 mg/kg); △: controls.

In Vivo Retention of the Ova

The drop in fertility occurred on the third day after ovulation in our first experiment and on day 2 in the second experiment. A slightly higher water temperature in the latter case, as well as the repeated handling of the females (once a day during 6 days), and individual variability might account for such a difference.

The in vivo aging of ova appears to be a rapid phenomenon in northern pike when compared to that in rainbow trout, in which ova fertility is preserved for at least 8 days after ovulation (Sakai et al. 1975; Escaffre et al. 1976; Bry, unpublished data).

Stimulation of Sperm Release

The highest and most efficient dose of progesterone tested was 100 mg/kg. In the goldfish, doses of 10 mg/kg (progesterone) and 100 to 1,000 mg/kg (methyltestosterone) were necessary for a maximum response (Yamazaki and Donaldson 1969; Billard 1976). The requirement for such considerable amounts might result from a gradual absorption of the steroid as Yamazaki and Donaldson (1969) pointed out, and from biological inactivation at the

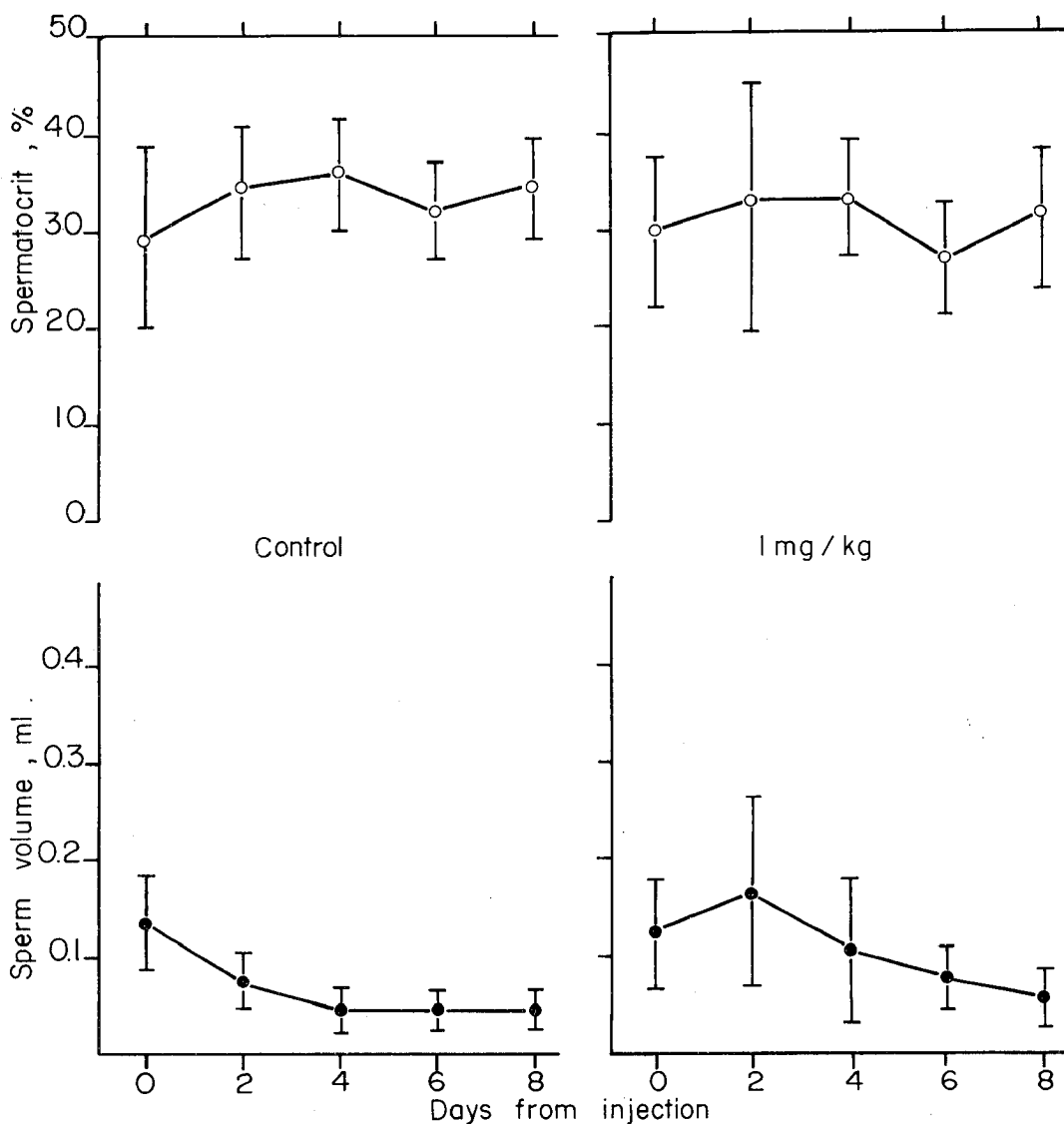


FIGURE 4.—Effect of a single injection of progesterone (1, 10, 100 mg/kg) on sperm release and spermatocrit. Vertical bars show confidence limits at the 95% level of probability.

plasma level by protein binding as suggested by the existence of two steroid binding systems with high capacities in female trout plasma (Fostier and Breton 1975).

Furthermore, a lack of responsiveness might occur at the testicular level, because progesterone must probably be metabolized to activate sperm release.

Our stimulation experiments were carried out on males that delivered 100–150 μ l

of sperm at the first sampling, prior to treatment. The question of the possible reinitiation of sperm release at the end of the reproductive season, when most males cease to give milt, would certainly be worthy of investigation.

Cryopreservation of the Sperm

We obtained considerable variability (0–68%) in the fertilizing capacity of the sperm

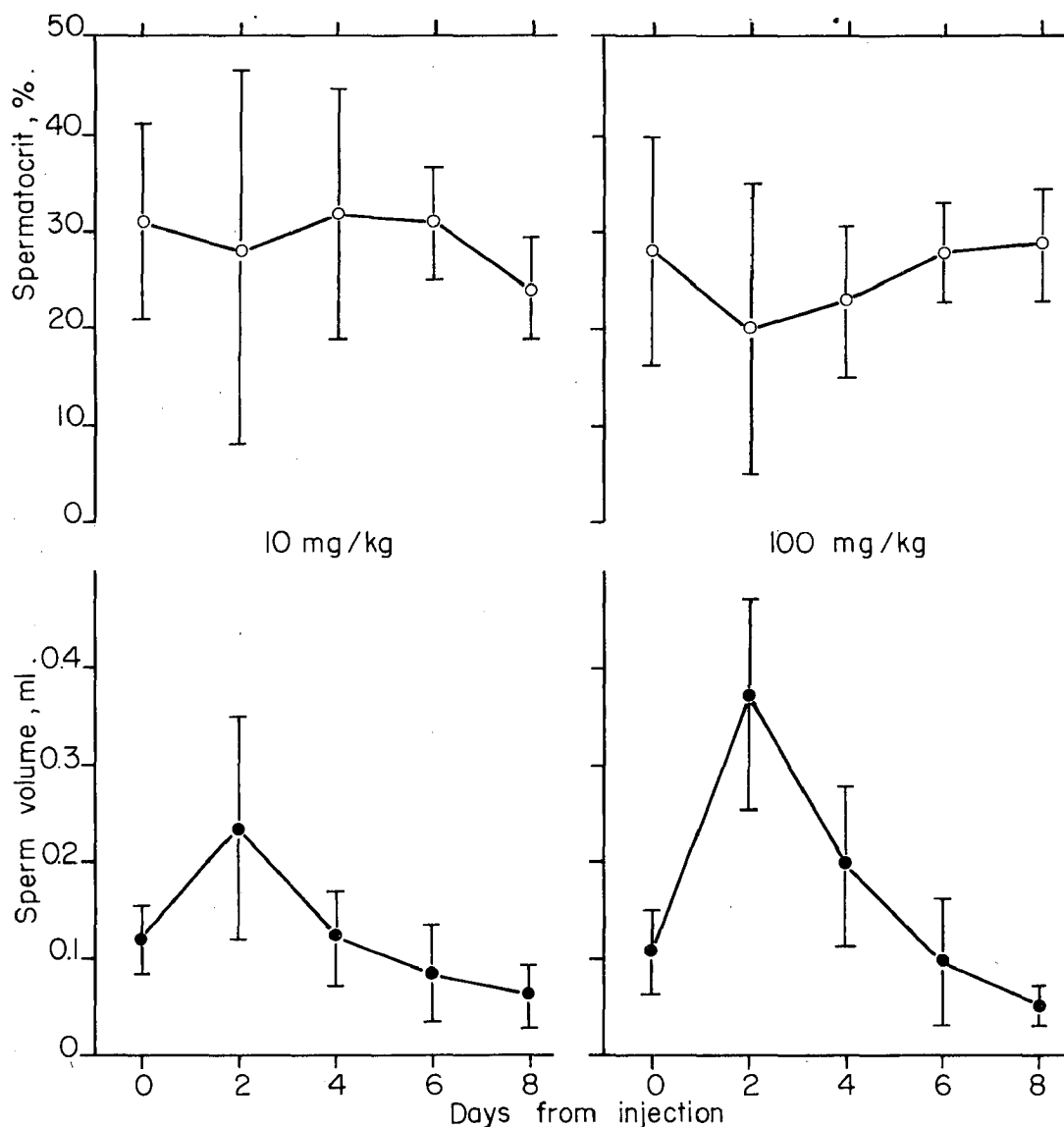


FIGURE 4.—Continued.

after cryopreservation, depending on the donor male, as Mounib et al. (1968) did with Atlantic cod sperm. Some steps of the freezing, freezing-storage, and thawing procedures cause irreversible modifications in some spermatozoa which lose their fertilizing ability, while others retain it. A careful adjustment of each step should allow progress toward the achievement of the ideal conditions. It might also be of interest to examine whether or not the

degree of fertility of semen from a given male following cryopreservation is a reproducible feature.

Regarding the estimation of fertility after cryostorage, no rigorous protocol has ever been defined to the authors' knowledge. In order to determine what is the actual rate of fertility preserved after cryostorage one should work in slightly saturating conditions, i.e., inseminate with the minimum amount of fresh sperm required to obtain

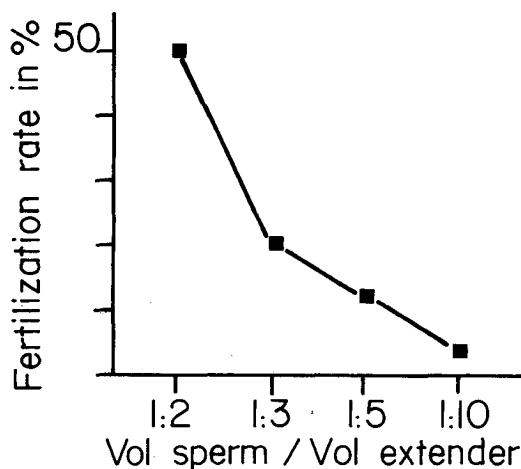


FIGURE 5.—Effect of the dilution ratio (vol sperm/vol cryo-extender) on the fertilizing capacity of sperm after thawing. Sperm was pooled from 10 males. Each dilution was tested with 2 samples.

the highest fertilization rate. The fertilizing ability after storage could then be expressed as a valid percentage of the initial level.

Such an approach could not be realized in our reported experiments, since the influence of high dilutions (10^{-3} , 10^{-4}) on fertilization had not been tested at the time of our cryopreservation attempts.

Artificial Insemination with a Diluent

According to our results the sperm dilution should not exceed 1:1,000. This is a safety measure to reduce the influence of individual variations in the sperm concen-

TABLE 1.—Effect of cryostorage on the motility, activity, and fertilizing capacity of northern pike sperm.

Male no.	Fresh sperm			Thawed sperm		
	Motility in DIA_{TG}^a	Activity min	Fertilizing capability ^b %	Motility in DIA_{TG}^a	Activity min	Fertilizing capability ^b %
1	4	1.50	76	3	0.50	50
2	4	1.75	50	2	0.75	48
3	4	2.25	75	0	0	0
4	3	2.25	72	1	0.15	1
5	4	1.75	80	2	0.80	66
6	3	2.50	70	2	0.35	14
7	4	2.75	84	0	0	0
8	5	2.50	82	3	0.75	68
9	4	1.75	72	1	0.25	5
10	5	2.25	74	2	0.15	11

^a Motility scale: 0 = non motile; 5 = fully motile.

^b Volume sperm/volume diluent (DIA_{TG}) = 10^{-2} .

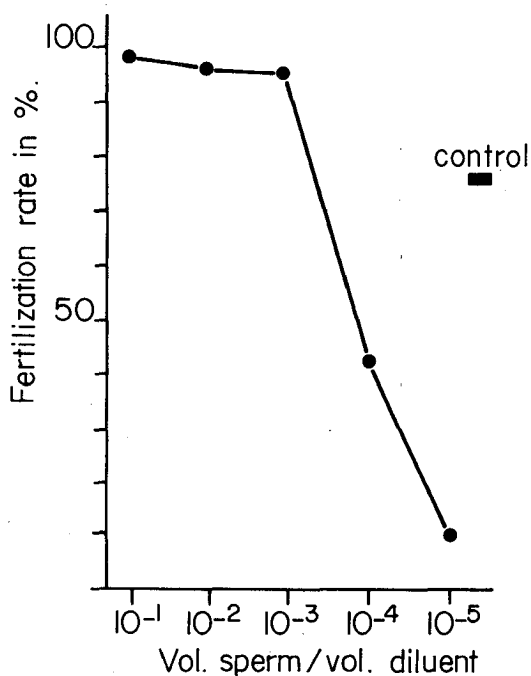


FIGURE 6.—Effect of the dilution ratio (vol fresh sperm/vol diluent) on fertilization.

trations and quality as mentioned by Billard (1975) for trout. In the northern pike, sperm concentration has been found to vary in the range of $18-30 \times 10^9$ spermatozoa/ml (Lindroth 1946; de Montalembert unpublished data). Such concentrations correspond to 800,000 spermatozoa per fertilized egg when the dilution ratio is 1:1,000. An insemination diluent elaborated for northern pike with specific values of osmotic pressure and pH might extend

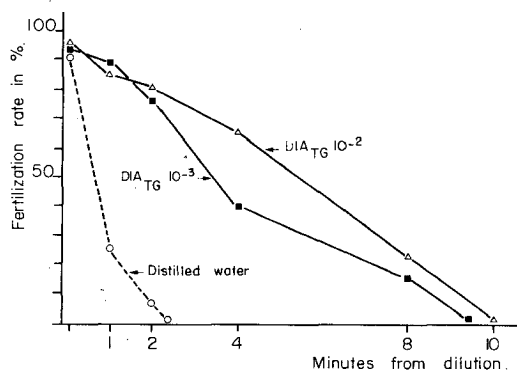


FIGURE 7.—The fertilizing capacity of the sperm when diluted in DIA_{TG} or water before insemination.

the viability of the sperm and consequently allow greater dilution rates. The ratio between the number of ova and the volume of diluent is also an important parameter in artificial insemination (Billard 1975). In the present study, we have only worked with one ratio (300 ova/10 ml diluent) and the optimum value of this parameter remains to be determined.

Conclusions

Due to the timing and synchronization of induced ovulations, artificial insemination can be planned in advance and hormonal activation of sperm release performed in the males, if necessary, 2 days before insemination. Insemination should be carried out within 24 hours of ovulation. Handling of the breeders can be reduced to a minimum: once for hormonal treatment and once for gamete sampling. Fish need to be held in captivity only for a few days. The use of DIA_{TG} as an insemination diluent allows reproducible results with minimum amounts of sperm.

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