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Cryobiology

PROPERTIES OF TENCH – *TINCA TINCA* L. SPERM AND EXPERIMENTS WITH FREEZING IT AT –196°C

WŁAŚCIWOŚCI NASIENIA LINA *TINCA TINCA* L. I PRÓBY ZAMRAŻANIA TEGO NASIENIA W TEMPERATURZE –196°C

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Properties of tench sperm were studied by means of the following indices: sperm volume, spermatozoa concentration, total number of spermatozoa, spermatocrit, and sperm motility. A series of diluting media was tested in order to find optimal conditions for tench sperm freezing; phosphate buffer (BF) enriched by a 15% addition of ethylene glycol (EG) was found to be the most suitable diluting medium.

INTRODUCTION

A crucial step for progress in animal sperm preservation was made when Polge et al. (1949) applied glycerol to protect bull spermatozoa from detrimental effects of freezing and thawing. Glycerol acts both by preventing large ice crystals to form and by slowing down an excessive increase in salt concentration in cells (Wierzbowski, 1972).

Studies aimed at implementing the practically tested methods of fish sperm cryo-preservation have been carried out only recently. The task is being made more

complicated by the specific nature of fertilisation in fishes. Spermatozoa, immobile while in the vas deferens, become motile on contact with water, their movement lasting for several minutes depending on a species. As opposed to domestic animals, the fishes of the moderate climatic zone as a rule exhibit one reproductive cycle per year, i.e., sperm is available over a limited period only. The amount of sperm in a single ejaculate is very small in some species, e.g., pike (*Esox lucius* L.), tench (*Tinca tinca* L.), or gryaling (*Thymallus thymallus* (L.)).

Most studies carried out so far have been made in USA and Canada on commercially important species yielding sufficient amounts of sperm. Thus the studies have dealt mostly with salmonid genera *Oncorhynchus* and *Coregonus* (Ridgeway and Hodgins, 1964; Ott and Horton, 1971) as well as with *Salmo gairdneri* Richardson (Graybill and Horton, 1969; Ott and Horton, 1969). Sneed and Clemens (1956) experimented with carp sperm preservation and obtained o 20% spermatozoa survival rate. Kossman (1973) was freezing carp sperm and evaluated the thawed motile spermatozoa activity as 100%; the sperm, however, had lost its fertilising properties. Moczarski (1976) obtained a carp hatch, from eggs fertilised with previously frozen sperm, of up to 16.2%.

In the present paper the quality of tench sperm is assessed and conditions of preservation of this sperm at the liquid nitrogen temperature described.

MATERIAL AND METHODS

The experiment was carried out in June 1979.

The sperm was obtained from mature tench males of 0.5–0.8 kg body weight. For a couple of days prior to the scheduled injection the fishes had been kept at about 23°C. The males were injected with 2 mg carp pituitary homogenate/kg fish weight.

The sperm properties studied are:

- volume of sperm obtained at a time,
- spermatozoa concentration as calculated (to $0.01 \times 10^6/mm^3$) by means of the hemacytometric technique in Bürker's cell,
- total number of spermatozoa, i.e., the product of sperm volume times spermatozoa concentration,
- spermatocrit, a volume of spermatozoa per unit sperm volume; the technique involves centrifuging the sperm microhematocrit capillaries (12 000 rpm, 5 min.) and finding a percentage of spermatozoa in the sperm,
- spermatozoa motility, measured in fresh water as the duration (min.) of each movement phase: I – progressive movement, II – circulating movement, III – oscillating movement.

Having found the movement to be of phase I and II, the sperm was frozen as in Forgasson et al. (1961), i.e., by a gradual freezing of the sperm placed in vials over the surface of liquid nitrogen. Vials were kept 5 cm over the liquid nitrogen surface for 5 min. and then submerged.

Composition of diluting media (g) 1000 cm^3 H_2O/ after various authors

Medium	1	2	3	4	5	6	7	8	9	10
	BF	Al	mC	E	FRS	H	NR 1	NR 2	JAL	KOS
NaCl		4.0	1.88	6.80	6.50	8.00	10.77	7.50	7.30	
KCl			7.20	0.40	0.14	0.40	0.38	0.38	0.18	
$NaHCO_3$			1.00	2.20	0.20	0.35		2.00	0.98	
$NaH_2PO_4 \cdot H_2O$			0.41	0.14	0.01					1%
$Na_2HPO_4 \cdot 2H_2O$	20.00					0.045	0.53	0.53		
$MgSO_4 \cdot 7H_2O$			0.23	0.20		0.10	0.23	0.23	0.07	
$MgCl_2 \cdot 6H_2O$						0.10			0.18	
KH_2PO_4	2.00					0.05				
$CaCl_2 \cdot 2H_2O$			0.23	0.27	0.12	0.19	0.46	0.46	0.35	
Natrium citrate		8.00								1%
Glucose		20.50	1.00	1.00	2.00	1.00	1.00	1.00	1.00	
Glycine							5.00	5.00		
Egg yolk							20%	20%		
Citric acid										0.7%
TRIS										1.0–2.5%

Diluting media composition qouted from the following sources: 1,5 – Sneed a. Clemens, 1956; 2 – Hodgins a. Ridgeway, 1964; 3 – Truscott a.al., 1968; 4,6 – Wolf, 1963; 7,8 – Stein, 1975; 9 – Jalabert a.al., 1974; 10 – Kossmann, 1973.

The sperm was dilued (1:1 and 1:2) with different diluting media, their composition being given in Table 1.

The utility of three protective factors was tested: glycerol (G), ethylene glycol (EG), and dimethylsulphoxide (DMSO). After diluting, the sperm was equilibrated for 5 min. in order to balance the osmotic pressures by allowing the diffusion of a protective substance inside the spermatozoa from the serum.

Sperm samples were thawed in 30–40° water bath, 6 h after freezing. The thawed spermatozoa motility was studied, while no test was performed for their ability to fertilise.

RESULTS

The sperm was obtained 12–18 h after injection, depending on an individual reaction of the fishes. Not every male responded to the hormonal injection by producing sperm.

The highest single sperm portion obtained from a male was 2.5 cm^3; the mean calculated for all the males was 1.58 cm^3 (Table 2).

The spermatozoa concentration ranged from 11.5–27.5 million/mm^3 with the mean of 19.67 million/mm^3 (Table 2).

Table 2

Properties of tench (*Tinca tinca* L.) sperm

Property	Ejaculate volume (cm^3)	Spermatoza concentration ($\times 10^6$) (mm^3)	Total number of spermatozoa (bilion)	Spermatocrit (%)	Duration of motility (min.)		
					phase I	phase I+II	phase I+II+III
Mean	1.58	19.67	27.07	33.55	0.41	0.68	3.58
Minimum	0.50	11.50	13.75	29.67			
Maximum	2.50	27.50	61.25	38.60			

The total number of spermatozoa per male ranged from 13.75 billion to 61.25 billion. The mean total number of spermatozoa as calculated for all males was 27.07 billion (Table 2).

The spermatocrit value ranged from 29.67 to 38.60%, the mean for all the spermatocrit values being 33.55% (Table 2).

Phase I in the spermatozoa motility (progressive movements) lasted, on the average, for 0.41 min., while the mean duration of phase I and II combined and of phase I, II, and

III combined was 0.68 and 3.58 min., respectively (Table 2). The longest duration of the tench spermatozoa motility observed in the experiment described was 6.20 min.

After thawing the sperm, the diluting media tested were arranged in order reflecting their decreasing utility for sperm freezing: BF, Al, mC, E, RS, H, NR1, NR2, JAL (Table 1). The KOS proved totally unsuitable.

The optimum concentration of ethylene glycol (EG), the best protective factor used in freezing, was 15%.

DISCUSSION

Different fish species have been found to produce widely varying amounts of sperm at a time. The carp (*Cyprinus carpio* L.) yield up to 6.5 cm^3 of sperm (Moczarski, 1976). The mean volume of tench ejaculate obtained during the present study, 1.58 cm^3, is close to a mean pike ejaculate volume of about 1 cm^3 (Stein and Lamina, 1975) and to a mean for the eel (*Anguilla anguilla* L.) ranging within 0.57–1.12 cm^3 (Bieniarz and Epler, 1977).

The tench individuals did not show any large differences between their spermatozoa concentrations. The tench mean of 19.67 million per mm^3 is closest to that of the pike (20 million/mm^3) (Grodziński, 1971). On the other hand, the carp sperm shows higher concentrations ranging within 23.8–25.6 million/mm^3 (Clemens and Grant, 1965).

The total spermatozoa number is an indicator of a potential male fertility; its values tend to vary depending on a species. According to Smirnov (1963), males of the coho salmon (*Oncorhynchus kisutch*) produce 115 billion spermatozoa, (Table 3), while the male chum (*O. keta*) yield 93 billion. The total spermatozoa number in the pollan (*Coregonus lavaretus*) is 84 billion (Pliszka, 1964); the carp are less productive with their 58 billion (Moczarski, 1976).

One of biological methods of sperm evaluation involves assessing spermatozoa concentration from spermatocrit, which allows a fast and precise determination of the spermatozoa number per unit sperm volume to be made. According to Winnicki and Tomasik (1976), the trout spermatocrit ranges within 20–53%. (Table 3). Bouck and Jacobson (1976) who determined sperm concentration by means of the microhematocrit technique obtained values of 25.63% and 27.10% for the coho salmon and steelhead trout (*Salmo gairdneri*), respectively. The tench spermatocrit determined in the present study was slightly higher compared to those species and amounted to 33.55%. Markedly higher values have been determined for the carp (77.42%) (Table 3) and eel (69.03%) (unpublished data).

A characteristic feature of fish spermatozoa is their motility commencing rapidly on their contact with water. The absence of any movement in water points to a lack of viability and in consequence to spermatozoa inability to fertilise or their death. The mean duration of tench spermatozoa movement including the oscillatory phase was found to be 3.58 min. The total motility duration of carp spermatozoa is 5–6 min. (Haempel, 1913).

Table 3

Comparison between sperm properties of tench and other fish species

Species	Spermatoza concentration ($\times 10^6$) (mm^3)	Author	Total number of spermatozoa (billion)	Author	Spermatocrit %	Author
Tench	19.67		29.07		33.55	
Carp	25	Stein a. Lamina, 1975	58	Moczarski, 1976	77.42	unpublished data
Steelhead trout	18.73	Bouck a. Jacobson, 1976			27.095	Bouck a. Jacobson 1976
Trout	8.8–20.16	Winnicki a. Tomasik, 1976			20–53	Winnicki, a. Tomasik, 1976
Coho salmon	16.87	Bouck a. Jacobson, 1976	115	Smirnov,	25.63	Bouck. a. Jacobson, 1976

Spermatozoa of the pike and trout show a lower viability: up to 2 min. (Lindroth, 1947) and 1 min. (Pliszka, 1964), respectively. The progressive movement and its fastest form – a torpedo-like movement is the phase crucial to the fertilisation. It is, however, of a very short duration in all fish species and gradually evolves into the other phases. The progressive movements of tench spermatozoa proceeded, on the average, for 0.41 min., 0.46 min. being an average found for the carp (unpublished data).

Preliminary studies on the optimum tench sperm freezing regime at $-196^{\circ}C$ confirmed the applicability of ethylene glycol as a protective substance known for its properties in freezing sperm of other fish species. The best surival of thawed spermatozoa was observed for a 15% protective compound concentration relative to a diluting medium. When using ethylene glycol, Truscott and Idler (1969) obtained their best results in freezing the Atlantic salmon (*Salmo salar*) sperm. It was Mounib et al. (1968) who also succeeded in applying ethylene glycol to cod (*Gadus morhua*) sperm freezing.

Of the 10 diluting media tested, phosphate buffer (BF) proved the best one, Alsewer's medium (Al) being also suitable; the latter is known as the best medium for the pre-freezing dilution of carp sperm (Moczarski, 1977).

CONCLUSIONS

1. The mean tench sperm volume obtained was 1.58 cm^3.
2. The tench spermatozoa concentration ranged from 11.5 to 27.5 million/mm^3 (the mean of 19.67 million/mm^3).
3. The total number of tench spermatozoa ranged from 13.75 to 61.25 billion (the mean of 29.07 billion).
4. The spermatocrit ranged from 29.67 to 38.60% (the mean of 33.55%).
5. The mean duration of each motility stage was 0.41 min. for phase I, 0.68 min. for phase I and II combined, and 3.58 min. for phase I, II, and III combined.
6. The optimum conditions for tench sperm freezing are as follows: phosphate buffer as a diluting medium with a 15% addition of ethylene glycol (1:1), 5 sec. equilibration.

REFERENCES

Bieniarz K., P. Epler, 1977: Investigations on inducing sexuel maturity in the male eel Anguilla anguilla L. – Journal of Fish Biology, **10:** 555–559.

Bouck G.R., J. Jacobson 1976: Estimation of salmonid sperm concentration by microhematocrit technique. – Transactions of the American Fisheries Society, **105, 4:** 534–535.

Clemens H.P., Grant F.B., 1965: The seminal thinning response of carp (Cyprinus carpio L.) and rainbow trout (Salmo gairdneri) after injections of pituitary extracts. – Copeia, **2:** 174–177.

Forgason J.L., W.T. Jr. Berry, B.E. Goodwin, 1961: Freezing bull semen in liquid nitrogen vapor without instrumentation. – Journal of Animal Science, **20:** 970–971.

Graybill J.R., H.F. Horton, 1969: Limited fertilization of steelhead trout eggs with cryo-preserved sperm. – Journal of the Fisheries Research Board of Canada, 5: 1400–1404.

Grodziński Z., 1971: Anatomia i embriologia ryb [Fish anatomy and embryology]. Państw. Wyd. Roln. i Leśne. Warszawa.

Haempel O., 1913: Fische. I – Allgemeine anatomish – physiologische übersicht. Handbuch der Biologie der Wirbeltiere. Stuttgart.

Hodgins H.O., G.J. Ridgeway, 1964: Recovery of viable salmon spermatoza after fast – freezing. Progressive Fish – Culturist, **26:** 95.

Kossmann H., 1973: Versuch zur Konserwierung des Karpfenspermas (Cyprinus carpio L.) – Arch. Fisch. Wiss., **24,** 1–3: 125–128.

Lindroth A., 1947: Time of activity of freshwater fish spermatozoa in relation to temperature. – Zool. Bidrag., **25:** 165–168.

Moczarski M., 1976: Badania nad jakością nasienia karpia i wpływ na nią głębokiego mrożenia. [Studies on carp sperm quality and effect of deep freezing on it]. Akademia Rolnicza, Kraków. (typescript).

Moczarski M., 1977: Deep freezing of carp (Cyprinus carpio L.) sperm. Bulletin de L'Academie Polonaise des Sciences, Cl. V, **XXV,** 3.

Mounib M.S., P.C. Hwang, D.R. Idler, 1968: Cryogenic preservation of Atlantic cod (Gadus morhua L.) sperm. – Journal of the Fisheries Research Board of Canada, **25,** 12: 2623–2632.

Ott A.G., H.F. Horton, 1969: Developments in cryo-preservation of salmonid sperm. – Proc. 49-th Annu. Conf. Western Ass. State Game Fish Comm. pp. 249–250.

Ott A.G. H.F. Horton, 1971: Fertilization of chinook and coho salmon eggs with cryo-preserved sperm. – Journal of the Fisheries Research Board of Canada, **28,** 745–748.

Ott A.G., H.F. Horton, 1971: Fertilization of steelhead trout eggs with cryo-preserved sperm. – Journal of the Fisheries Research Board of Canada, 28, 12: 1915–1918.

Pliszka F., 1964: Biologia ryb. [Fish biology]. Państw. Wyd. Nauk. Rol. i Leś., Warszawa.

Polge C., A.U. Smith, A.S. Parkes, 1949: Revival of spermatozoa after vitrification and dehydration at low temperatures. – Nature, 164, 4172: 666.

Ridgeway G.J., H.O. Hodgins, 1964: Recovary of viable salmon spermatozoa after fast freezing. – Progressive Fish-Culturist, 26, 2: 95.

Smirnov A.I., 1963: Producirovanie spermy tihookeanskimi lososjami roda Oncorhynchus. – Vopr. Icht., 3, 26: 84–98. [in Russian].

Sneed K.E., H.D. Clemens, 1956: Survival of fish sperm after freezing and storage at low temperatures. – Progressive Fish Culturist, 17: 99–103.

Stein H., J. Lamina, 1975' Die Spermkonservierung bei Fischen – ein Instrument zur Erleichterung und Verbesserung der Praktischen und Wissenschaftlischen Arbeit. – AFZ – Fischweid, 100: 507–510.

Truscott.B., D.R. Idler, 1969: An improved extender for freezing Atlantic Salmon spermatozoa. – Journal of the Fisheries Research Board of Canada, 26, 12: 3254–3258.

Wierzbowski S., 1972: Zagadnienia kriobiologii w sztucznym unasienianiu zwierząt. [Problems of cryo-biology in artificial insemination of animals] – Zeszyty Problemowe Postępu Nauk Rolniczych, 124.

Winnicki A.,L. Tomasik, 1976: Spermatocrit as a method for biological evaluation of fish sperm. – Acta Ichthyologica et Piscatoria, VI, 2: 3–8.

Wolf K., 1967: Physiological salines for freshwater teleosts. Progressive Fish Culturist, 25: 135–140.

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WŁAŚCIWOŚCI NASIENIA LINA TINCA L. I PRÓBY ZAMRAŻANIA NASIENIA W TEMPERATURZE –196°C

STRESZCZENIE

W wyniku iniekcji samców lina hormonami gonadotropowymi otrzymano nasienie, którego średnia objętość wynosiła 1,58 cm^3. Przeprowadzono charakterystykę nasienia lina uzyskując średnią wartość koncentracji 19,67 mln/mm^3, ogólną liczbę plemników 29.07 mld i wartość spermatokrytu 33,55%. Wyróżniono trzy fazy ruchliwości plemników lina, a czas ich trwania wynosił: I faza (ruch postępowy) – 0.41 min., I i II faza (ruch postępowy i ruch krążący) – 0,68 min., I, II i III faza (ruch postępowy, krążący i drgający) – 3,58 min.

Maksymalny czas ruchu plemników zaobserwowany w tym doświadczeniu wynosił 6,20 min.

Stwierdzono, że proces zamrażania nasienia lina w temperaturze –196°C wymaga następujących warunków: rozcieńczalnik w postaci buforu fosforanowego, glikol etylenowy jako substancja ochraniająca w ilości 15%, stosunek rozcieńczenia 1:1, czas ekwilibracji 5 sek.

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СВОЙСТВА СПЕРМЫ ЛИНИЯ Tinca tinca L. И ПОПЫТКИ ЗАМОРАЖИВАНИЯ ЭТОЙ СПЕРМЫ ПРИ ТЕМПЕРАТУРЕ - 196° С

Р е з ю м е

В результате иньекции самцам линия гонадотропных гормонов получили сперму, которой объём в среднем составлял 1,58 $см^3$. Произвели характеристику спермы линия, получая среднее значение концентрации 19,67 млн/$м^3$ общее количество спермиев 29,07 млд и значение сперматокрита 33,55%. Нашли 3 стадии подвижности спермиев линия. Время движения составляло: 1 стадия (прогрессивное движение) - 0,41 мин., I и II стадия (прогрессивное движение и вращение) - 0,68 мин., I,II и III стадия (прогрессивное движение, вращение и дрожание) - 3,58 мин. Максимальное время движения спермиев наблюдаемое в этом опыте составляло 6,20 мин. Нашли, что процесс замораживания спермы линия при температуре -196°С требует следующих условий; разбавитель в виде фосфатного буффера, этиленгликол как жидкость охраняющая в количестве 15%, соотношение разбовления 1:1, время эквилибрации - 5 секунд.

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