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ZEMĚDĚLSKÁ FAKULTA

DISERTAČNÍ PRÁCE

Kryokonzervace rybích spermií k ochraně genofondu

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Obsah:

1. Souhrn

Tato práce je věnována problematice zmražování spermatu, především pro účely uchování genofondu některých sladkovodních druhů ryb chovaných v České republice. Zahrnuje jednak práce týkající se přímo zmražování spermatu kapra obecného (*Cyprinus carpio*), lína obecného (*Tinca tinca*), sumce velkého (Silurus glanis) a zástupce chrupavčitých ryb veslonosa amerického (*Polyodon spathula*), a dále práce týkající se postupů před kryokonzervací (odběr a krátkodobé uchování spermatu) a postupů nutných pro použití zmraženého spermatu a zjišťování úspěšnosti kryokonzervace.

U kapra byla pozornost zaměřena na: parametry pohyblivosti rozmraženého spermatu, na oplozenost a líhnivost dosaženou při použití zmraženého spermatu, na ověření dávky spermií na 1 jikru, na vhodný aktivační roztok a jeho množství na objem jiker. Dále bylo testováno odlepkování jiker pomocí enzymů.

Více v publikacích :

Linhart O., Rodina M., and Cosson J., 2000: Cryopreservation of Sperm in Common Carp Cyprinus carpio: Sperm Motility and Hatching Success of Embryos, Cryobiology 41, 241-250

Linhart O., Rodina M., Gela D., Kocour M. and M. Rodriguez, 2003. Improvement of common carp artificial reproduction using enzyme for elimination of eggs stickiness. Aquat.Liv.Res., 16, 450-456

U lína byla nejprve pozornost zaměřena na problematiku kontaminace spermatu močí, složení moče a spermiální plazmy a vlivu na pohyblivost spermií. Následovalo testování modifikací roztoku podle Kurokury jako imobilizačního roztoku. Dalším krokem bylo zmražování spermatu při použití různých kombinací kryopotektantů, imobilizačních roztoků, mrazících programů a mražených objemů. Nejen pro potřeby kryokonzervace byl revidován postup výtěru gamet, oplození a odlepování jiker.

Více v publikacích :

Linhart O., Rodina M., Bastl J. and Cosson J.,2003: Urinary bladder, ionic composition of seminal fluid and urine with charakterization of sperm motility in tench (Tinca tinca L.) Journal of Appl. Ichthyology 19: 177-181,

Rodina M., Cosson J., Gela D. and Linhart O. 2004: Kurokura solution as immobilizing medium for spermatozoa of tench (Tinca tinca L.) Aquaculture International 12: 119-131,

Rodina M, Gela D., Kocour M., Alavi S.M.H., Hulak M. and Linhart O: Cryopreservation of Tench Tinca tinca Sperm: Sperm Motility and Hatching Success of Embryos Theriogenology (přijato do tisku)

Linhart O., Rodina M., Kocour M. and Gela D. 2006: Insemination, fertilization and gamete management in tench, Tinca tinca (L.) Aquaculture International 14: 61-73

Testy kryokonzervace spermatu sumce zahrnovaly testy různých kryoprotektantů, jejich kombinací a koncentrací, dále test doby equilibrace a objemu zmražené dávky. Nejen pro potřeby kryokonzervace byl optimalizován potup umělé reprodukce sumce, především: složení imobilizačního roztoku pro spermie, aktivačního roztoku, dávka spermií na 1 jikru, poměr objemu aktivačního roztoku a hmotnosti jiker.

Více v publikacích:

Linhart O., Rodina M., Flajšhams M., Gela D., Kocour M.: Cryopreservation of European catfish Silurus glanis sperm: Sperm motility, viability and hatching succes of embryo, Cryobiology 51: 250-261 (2005)

Linhart O., Rodina M., Gela D., Kocour M., 2004. Optimalization of artificial propagation in European catfish, Silurus glanis L. Aquaculture, 235, 619-632 (IF 1,5)

Při zmražování spermatu představitele chrupavčitých ryb veslonosa amerického byly úspěšně testovány různé extendery a kryoprotektory.

Více v publikaci:

Linhart O., Mims S.D., Gomelsky B., Cvetkova L., Cosson J., Rodina M., Horvath A. and Urbanyi B. 2006 : Effect of cryoprotectant and male on motility parameters and fertilization rate in paddlefish (Polyodon spathula) frozen-thawed spermatozoa, J.Appl. Ichtyol. In press

2. Summary

This study concerns sperm cryopreservation especially for purposes of gene pool protection in some freshwater fish species reared in the Czech Republic. It includes firstly own methodologies of sperm freezing in common carp, common tench, European catfish as members of bony fish species, American paddlefish as member of cartilaginous fish species, secondly procedures prior to sperm freezing (collection and short-term storage of sperm) and finally procedures for effective using of frozen sperm and assignment of success of sperm freezing.

In common carp, the attention was focused on: 1) parameters of motility in melted sperm, 2) fertilization and hatching rate using frozen/melted sperm, 3) determination of the best egg : spermatozoa rate, 4) composition of suitable activation solution and its volume per dose of eggs. Consequently, the procedures of egg stickiness elimination were tested.

The details are described in publications:

Linhart O., Rodina M., and Cosson J., 2000: Cryopreservation of Sperm in Common Carp Cyprinus carpio: Sperm Motility and Hatching Success of Embryos, Cryobiology 41, 241-250

Linhart O., Rodina M., Gela D., Kocour M. and M. Rodriguez, 2003. Improvement of common carp artificial reproduction using enzyme for elimination of eggs stickiness. Aquat.Liv.Res., 16, 450-456

In common tench, the attention was focused firstly on problem with contamination of sperm with urine, composition of urine and seminal plasma and their affect on sperm motility. Other step concerned testing of Kurokura solution modifications for immobilizing of sperm, followed investigation on freezing of tench sperm using various cryoprotectant mixtures, immobilizing solutions, procedures of sperm freezing and volumes of sperm for freezing. Methodology of artificial spawning, fertilization and elimination of egg

stickiness were updated not only for purposes of tench sperm cryopreservation.

The details are described in publications:

Linhart O., Rodina M., Bastl J. and Cosson J.,2003: Urinary bladder, ionic composition of seminal fluid and urine with charakterizatio of sperm motility in tench (Tinca tinca L.) Journal of Appl. Ichthyology 19: 177-181,

Rodina M., Cosson J., Gela D. and Linhart O. 2004: Kurokura solution as immobilizing medium for spermatozoa of tench (Tinca tinca L.) Aquaculture International 12: 119-131,

Rodina M., Gela D., Kocour M, Alavi S.M. H, Hulak M. and Linhart O.: Cryopreservation of Tench Tinca tinca Sperm: Sperm Motility and Hatching Success of Embryos Theriogenology (in press)

Linhart O., Rodina M., Kocour M. and Gela D. 2006: Insemination, fertilization and gamete management in tench, Tinca tinca (L.) Aquaculture International 14: 61-73

In European catfish the work on sperm cryopreservation included tests with various cryoprotectants, their mutual combinations, duration of equilibration and volume of frozen dose. Procedure of artificial reproduction, not only for purposes of cryopreservation, was modified mainly with regard to 1) composition of immobilizing solution for sperm, 2) activation solution, 3) number of sperm per one egg and 4) rate of activation solution volume to weight of eggs.

For details see:

Linhart O., Rodina M., Flajšhams M., Gela D., Kocour M. 2005: Cryopreservation of European catfish Silurus glanis sperm: Sperm motility, viability and hatching succes of embryo, Cryobiology 51: 250-261

Linhart O., Rodina M., Gela D., Kocour M., 2004. Optimalization of artificial propagation in European catfish, Silurus glanis L. Aquaculture, 235, 619-632

In American paddlefish, the member of cartilaginous fish species, various extenders and cryoprotectants were successfully tested.

The details are described in publication:

Linhart O., Mims S.D., Gomelsky B., Cvetkova L., Cosson J., Rodina M., Horvath A. and Urbanyi B. 2006 : Effect of cryoprotectant and male on motility parameters and fertilization rate in paddlefish (Polyodon spathula) frozen-thawed spermatozoa, J.Appl. Ichtyol. In press

3. Úvod

Rybářství má v českých zemích staletou tradici a především rybníkářství, jehož zlatý věk byl v 16. století, kdy bylo v Čechách 180 000 ha rybníků (Čítek a kol.1993). I když je dnes tato rozloha nižší (v bývalé ČSFR více než 53 200 ha), produkce ryb z jednotky plochy se značně zvýšila. Tento vývoj byl podmíněn rozvojem znalostí z biologie ryb a jejich přenesením do rybářské praxe, které se projevilo zdokonalením technologie chovu vedoucím ke zkrácení výrobního turnusu, zvýšení produkce z jednotky plochy a její kvality.

Základní otázkou úspěšného chovu bylo zvládnutí reprodukce ryb. Od počátku používaná technologie prosté přirozené reprodukce byla zpočátku zdokonalována zařazením přesazování. Dalším stupněm byl tzv. poloumělý výtěr, kdy docházelo ještě k přirozenému rozmnožování, ale již v prostředí připraveném člověkem ("Dubraviova" metoda výtěru kapra,"Šustova" metoda výtěru štiky). Mezníkem v reprodukci ryb bylo provedení umělého výtěru a jeho metodické propracování. První umělý výtěr na našem území byl proveden již v roce 1784 v Horažďovicích u lososa obecného (*Salmo salar*). Naopak mezi poslední úspěšně propracované metody umělého výtěru patří umělý výtěr sumce velkého (*Silurus glanis*), lína obecného (*Tinca tinca*) bolena dravého (*Aspius aspius*) a dalších.

A právě v souvislosti se širokým zavedením umělého výtěru ryb, nebo lépe řečeno řízené reprodukce ryb je třeba brát v úvahu původ, vlastnosti a určení rozmnožovaného materiálu a jeho místo v systematické plemenářské práci.

V současné době se plemenářská práce v chovu ryb provádí především u kapra obecného, lína obecného, sumce velkého a pstruha duhového a zahrnuje:

- uchování a rozmnožování čistých druhů (jeseter malý, síhové), plemen (kapr obecný, lín obecný, sumec obecný, pstruh duhový), či populací ryb(pstruh obecný),
- novošlechtění (kapr),

- produkci finálních hybridů a jejich testování (kapr, lín).

Do Národního programu ochrany genových zdrojů byly ryby zahrnuty formou in situ , tedy celé chovné skupiny ryb vyjmenovaných druhů, plemen a populací, a dále formou ex situ, tedy kryobankou pohlavních buněk- spermií.

Předkládaná disertační práce **Kryokonzervace rybích spermií k ochraně genofondu** zahrnuje jednak práce týkající se přímo kryokonzervace spermií kapra, lína, sumce a jeseterovitých, a dále práce týkající se postupů před kryokonzervací (odběr a krátkodobé uchování spermatu) a postupů nutných pro použití mraženého spermatu a zjišťování úspěšnosti kryokonzervace.

Pro přehlednost je práce členěna podle druhů a dále podle kroků během kryokonzervace.

4. Teorie kryokonzervace

KRYOKONZERVACE – metoda uchování živých buněk a tkání při velmi nízkých teplotách (dosahovaných obvykle pomocí tekutého dusíku nebo suchého ledu) při použití ochranných látek – tzv. kryoprotektantů. Úspěšnost kryokonzervace spermií je dána: zachováním integrity buňky, zachováním pohyblivosti buňky a zachováním oplozeníschopnosti buňky.

Při kryokonzervaci dochází ke tvorbě intra a extracelulárních krystalů vody, (přičemž je důležitá velikost krystalů a jejich uspořádání), dále ke změnám iontové koncentrace (intracelulární i extracelulární) (vedoucím k lokálnímu zvýšení koncentrace elektrolytů) a ke změnám struktury buněčné membrány.

Pro eliminaci uvedených vlivů na buněčnou membránu jsou používány ochranné látky - KRYOPROTEKTORY (kryoprotektiva, kryoprotektanty). Nejčastěji používanými kryoprotektory jsou GLYCEROL, DMSO, METANOL, PROPANDIOL, ETYLENGLYKOL. Kryoprotektanty tedy omezují tvorbu, velikost a polarizaci (uspořádání) krystalů ledu (exitacelulárně i intracelulárně), chrání membránu před lokálmím působením zvýšené koncentrace iontů a před strukturálními změnami.

Technologie kryokonzervace spermatu ryb zahrnuje následující kroky: Výběr a příprava vhodných ryb: hlediskem výběru je jednak známý původ - plemeno, chovná skupina, (zákon o šlechtění, plemenitbě a evidenci hospodářských zvířat č. 154/2000 Sb. Vyhl. 357/2001),dále aktuální zdravotní stav a kondice. Příprava (předvýtěrová příprava) spočívá především v úpravě teplotního režimu a hormonální stimulaci mlíčáků.

Odběr spermatu je specifický podle druhu, cílem je získat nekontaminované sperma, popř. eliminovat účinek kontaminantů, hlavně moče, použitím vhodných postupů a prostředků.

Kontrola kvality spermatu zahrnuje makroskopickou kontrolu (barva, konzistence a přítomnost přimísenin) a mikroskopickou kontrolu (koncentrace spermatu, a stanovení či odhad motility – podílu pohyblivých buněk).

Příprava k vlastnímu mražení spočívá ve vytemperování spermatu, (veškerá manipulace probíhá na ledu při teplotě do +4°C), úpravě koncentrace (ředění nebo naopak zkoncentrování spermatu), přidání kryoprotektiva a distribuce do kryozkumavek či pejet (slámek) o různém objemu.

Vlastní zmražení je postupné zchlazování dávek pomocí tekutého dusíku v programovatelném zmrazovacím přístroji (podle zvoleného zmrazovacího programu), nebo pouze v parách nad hladinou tekutého dusíku.

Uchování zmražených dávek se provádí v kontejnerech pod hladinou tekutého dusíku při teplotě -196°C.

Rozmražení dávek zmraženého spermatu se provádí ve vodní lázni teploty 37-40°C, doba potřebná k rozmražení dávky se liší podle formy a objemu dávky a pohybuje se od 10 do 100s. Bezprostředně po rozmražení se sperma používá k oplození jiker.

. Kryokonzervace a genofond kapra

5.1 Genofond kaprů chovaných v České republice

Genofond kaprů chovaných v České republice patří svoji kvalitou a ozmanitostí ke špičce v evropském i světovém měřítku. Plemena u nás hovaného kapra můžeme rozdělit na **původní česká plemena**, (ze šupinatých apř. Třeboňský šupináč, Jihočeský kapr šupinatý, Mariánskolázeňský kapr upinatý, z lysců např: Jihočeský lysec, Telčský lysec), **plemena nportovaná ze zahraničí** (ze šupinatých např. Tatajský kapr šupinatý, laďarský kapr šupinatý M4, Amurský sazan, Ropšínský kapr šupinatý, lysců především maďarští lysci M2 a 15, DOR-70), **hybridní plemena** apř. z lysců Severský lysec M72, Syntetický lysec HSM,) a užitkové říženec (např. šupinatý ROPxTAT, ASxTAT, lysci HSMxM72, M2xM72..)

V současné době je v systému plemenářské práce u kapra využívána ryokonzervace spermatu v Národním programu ochrany genových zdrojů, ezi něž patří následující plemena kapra: Žďárský lysec, Žďárský šupináč, hočeský kapr šupinatý (C73), Mariánskolázeňský kapr šupinatý, Milevský sec, Jihočeský lysec (BV), Telčský lysec, Pohořelický lysec, Třeboňský upináč, Syntetický lysec C434, Syntetický lysec C435.

5.2 Kryokonzervace spermatu kapra obecného Cyprinus carpio: motilita spermií a úspěšnost líhnivosti embryí

V této práci byla zkoušena metoda kryokonzervace spermatu kapra pro otřeby uchování českých plemen kapra. Kapří sperma bylo ředěno roztokem urokura a uchováno při teplotě 4°C; následně byl přidán kryoprotektant – MSO. Kryotuby se spermatem byly potom zchlazovány z +4°C na -9°C chlostí 4°C za minutu, z -9°C do -80°C rychlostí 11°C za minutu, teplota -0°C byla držena 6 minut a následovalo přenesení kryotub do tekutého usíku. Sperma bylo rozmražováno na vodní lázni teploty 35°C; u ozmraženého spermatu byla sledována motilita a rychlost pohybu spermií pomocí analýzy obrazu z videosnímků záznamu mikroskopického obrazu ohybu spermií) a úroveň oplozenosti a líhnivosti plůdku po použití

ozmraženého spermatu. Motilita a rychlost pohybu spermií 15 s po aktivaci yla u rozmrazeného spermatu statisticky průkazně nižší než u čerstvého permatu. ANOVA prokázala signifikantní vliv kryokonzervace na dosaženou plozenost (68±11% pro čerstvé sperma oproti 56±10%), na celkovou hnivost a procento malformací vliv neprokázala. Motilita a rychlost pohybu permií korelovaly s líhnivostí po použití čerstvého a rozmrazeného spermatu =0,51 a r=0,54).

Podrobně je práce popsána v publikaci:

Otomar Linhart, Marek Rodina, and Jacky Cosson (2000): Cryopreservation of Sperm in Common Carp Cyprinus carpio: Sperm Motility and Hatching Success of Embryos, Cryobiology 41, 241-250

a prezentována formou posterového sdělení v rámci mezinárodní onference Genetic days v Brně (2002).

5.3. Optimalizace postupů řízené reprodukce kapra

Tato část shrnuje pokusy o optimalizaci jednotlivých postupů během ízené reprodukce. Jde především o: dávku spermií (v přepočtu na 1 jikru), využití vhodného aktivačního média (aktivačního roztoku), poměr jeho bjemu a hmotnosti jiker. Z technologického hlediska je velmi důležité okonalé odlepkování jiker, kde je v případě použití enzymatické metody ůležitý druh enzymu, jeho koncentrace, čas aplikace enzymu po aktivaci iker a trvání expozice v roztoku enzymu.

Z provedených experimentů vyplynulo, že vhodná dávka spermií přepočtu na 1 jikru byla 24000 (zaokrouhleno) a vyšší, u aktivačního oztoku bylo hlavním faktorem (určujícím úroveň dosažené oplozenosti) pH 9 nikoliv předpokládaná osmotická úroveň. Použití enzymu chymotrypsinu se eosvědčilo; při použití alkalázy DX bylo dosaženo líhnivosti nelišící se od ontroly (odlepkování mlékem) a celková doba od oplození po vysazení jiker lo inkubační láhve byla zkrácena na 21 min.

Podrobně je práce popsána v publikaci:

Linhart O., Rodina M., Gela D., Kocour M. and M. Rodriguez, 2003. Improvement of common carp artificial reproduction using enzyme for elimination of eggs stickiness. Aquat.Liv.Res., 16, 450-456

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Cryopreservation of Sperm in Common Carp *Cyprinus carpio:* Sperm Motility and Hatching Success of Embryos

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In this study, fish sperm cryopreservation methods were elaborated upon for *ex situ* conservation of nine strains of Bohemian common carp. Common carp sperm were diluted in Kurokura medium and chilled to 4°C and dimethyl sulfoxide was added. Cryotubes of sperm with media were then cooled from +4 to −9°C at a rate of 4°C min^{-1} and then from −9 to −80°C at a rate of 11°C min^{-1}, held for 6 min at −80°C, and finally transferred into liquid N_2. The spermatozoa were thawed in a water bath at 35°C for 110 s and checked for fertilization yield, hatching yield of embryos, and larval malformations. Fresh and frozen/thawed sperm were evaluated for the percentage and for the velocity of motile sperm from video frames using image analysis. The percentage and velocity of sperm motility at 15 s after activation of frozen/thawed sperm was significantly lower than that of fresh sperm (nine males). ANOVA showed a significant influence of fresh vs frozen/thawed sperm on fertilization rate ($P < 0.0001$), but differences in hatching rate and in larval malformation (0–6.8%) were not significant, and different males had a significant influence on fertilization and hatching rate ($P < 0.003$ and $P < 0.007$, respectively). Multiple range analysis (LSD) showed significant differences between fresh and frozen/thawed sperm regarding fertilization rate (68 ± 11 and 56 ± 10%, respectively) and insignificant differences between fresh and frozen/thawed sperm on the hatching rate (50 ± 18 and 52 ± 9%, respectively). The percentage and velocity of fresh sperm motility were correlated, respectively, with the fertilization yield of frozen/thawed sperm at the levels $r = 0.51$ and $r = 0.54$.

Key Words: fish; common carp *Cyprinus carpio;* sperm; motility; velocity; hatching; cryopreservation.

INTRODUCTION

The common carp, *Cyprinus carpio* L., is an important fish in global aquaculture, as its annual yield is over 1 million tons, which is exceeded only by silver carp (1.9 million tons, FAO, 1995, 11). Common carp is by far the most important freshwater fish in Europe, with an annual aquaculture yield of about 220 kton. Contemporary aquaculture in the Czech Republic consists of 42,000 ha (420 km^2) of ponds with a total production of fish amounting to 18,000 tons per year, of which the common carp is around 15,000 tons per year (10). In the Czech Republic (formerly territory of Bohemia), the common carp was domesticated in different sites, resulting in the formation of different strains, e.g., Mariánské Lázně, Žďár, Milevsko, Pohorelice (12), characterized by their body and head shape (29) and their protein content (31). Different strains constitute the basis of the breeding work that is focused on gene resource conservation, selective breeding, and performance testing and genomic manipulations. The Czech Republic gene resources conservation program for fish includes nine local breeds of common carp, which are still considered pure, with banks of cryopreserved sperm (12, 21).

Cryopreservation methods for common carp sperm have been reported (6, 16, 20, 22, 28, 33). Most of the experimental work in this field has been focused on trials to find optimal saline solutions for activation or for conservation, cryoprotective agents, thawing solutions, and freezing and thawing rates for *cyprinid* sperm (1, 2, 5, 24–27).

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In this study, cryopreservation methods were elaborated for *ex situ* conservation of nine strains of Bohemian common carp. A sperm freezing procedure was developed that resulted in high motility after thawing as well as high values of velocity and percentage of sperm motility, fertilization, and hatching yield.

MATERIALS AND METHODS

Males

Male broodstock (5 to 7 years old) were individually marked and were injected intradorsaly 24 h before collection of sperm with carp pituitary acetone powder (CPP) at a dosage of 1 mg kg^{-1} body wt (bw) at a temperature of 20°C.

Cryopreservation

Common carp sperm were collected from nine males in separate plastic containers for cell culture with sperm:air volume ratios of 1:10 to 1:30 and were stored under aerobic conditions at 4°C for 2–4 h. Sperm quality was checked for the percentage of sperm motility from video records. Only sperm samples showing more than 40% motility were used for cryopreservation. Sperm were diluted 1:5 (sperm: extender) in a Kurokura-1 (16) extender (128.4 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$, 2.4 mM $NaHCO_3$) and equilibrated for 40 min at 4°C. Ten percent pure dimethyl sulfoxide (Me_2SO) was added and every milliliter of mixture was transferred to a 2-ml cryotube, and then the cryotubes were directly transferred to a preprogrammed PLANER Kryo 10 series III at +4°C and cooled from +4 to −9°C at a rate of 4°C. min^{-1} and then from −11 to −80°C at a rate of 11°C min^{-1}, held for 6 min at −80°C, and finally transferred into liquid N_2. The spermatozoa were thawed in a water bath at 35°C for 110 s.

Sperm Motility

Sperm motility from nine males was evaluated in order to determine the percentage of motility and the velocity after activation. Motility was measured using dark field microscopy. Swimming ability of spermatozoa was evaluated with fresh sperm that were prediluted 1:100 with Kurokura medium and then 1 μl of diluted sperm was directly mixed with a 29-μl drop of swimming medium (SM = 45 mM NaCl + 5 mM KCl + 20 mM Tris–HCl, pH 8), placed on a glass slide prepositioned on the microscope stage; it was examined under 200× magnification immediately after being mixed. The final dilution of fresh or of thawed sperm was from 1:1000 to 1:3000. Motile spermatozoa were video recorded within 10 s after activation for the measurement of velocity and percentage of actively swimming spermatozoa. A minimal value of 10 $\mu m.s^{-1}$ was used as the threshold velocity, below which motility was considered zero. The movements of spermatozoa were tape-recorded (Sony S-VHS) using a CCD video camera (Sony) set on a dark field microscope (Olympus BX 50), including a record of the time elapsed since the initiation of movement (see Fig. 1).

The successive positions of sperm heads were measured from successive video frames using a video-recorder (Sony SVHS, SVO-9500 MDP) and analyzed 15 s after activation (Figs. 4 and 5) and at 15-s intervals thereafter (Figs. 2 and 3), using three successive frames by Micro Image Analysis (version 3.0.1. for Windows by Olympus, with a special application from Olympus C&S, Czech Republic). The immotile sperm (velocity = 0) were not taken into account in the velocity curve of Figs. 3 and 5, i.e., only motile sperm were plotted. This is important considering (Fig. 2) that at 60 s only ⅓ of sperm swim in frozen/thawed samples (compared to native), but the speed is the same (Fig. 3).

Changes in motility (Fig. 2) and velocity (Fig. 3) of fresh and thawed sperm are described by regression curves with regression values (R), where X = time after sperm activation and Y = percentage of sperm motility or velocity. Analysis of variance (ANOVA, Statgraphics version 5) followed by multiple comparison tests LSD was used for the results shown in Figs. 2–5. Probability values lower than 0.05 were considered significant. The mean values, with standard

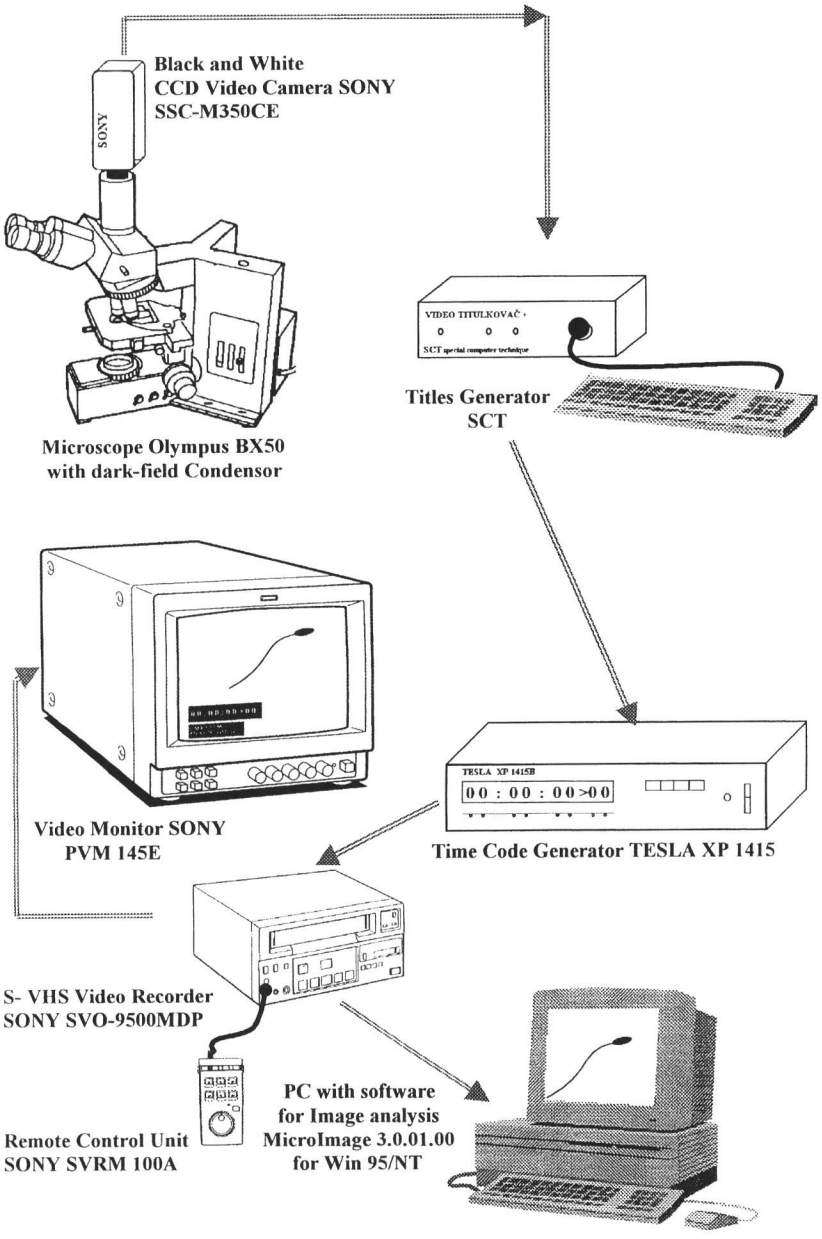

FIG. 1. Scheme of the equipment for evaluation of sperm motility.

deviation calculated from six replicates, are shown in Figs. 4 and 5.

Fertilization and Hatching

The sperm were stored for 1 week in liquid N_2. Eggs from three females after CPP treatment were used. Sperm of the same males that were used for cryopreservation were collected 24 h after CPP treatment. Frozen sperm were stored for 1 week at −196°C and then thawed in a water bath at 35°C for 110 s. A sample of sperm from each of 10 males was checked in

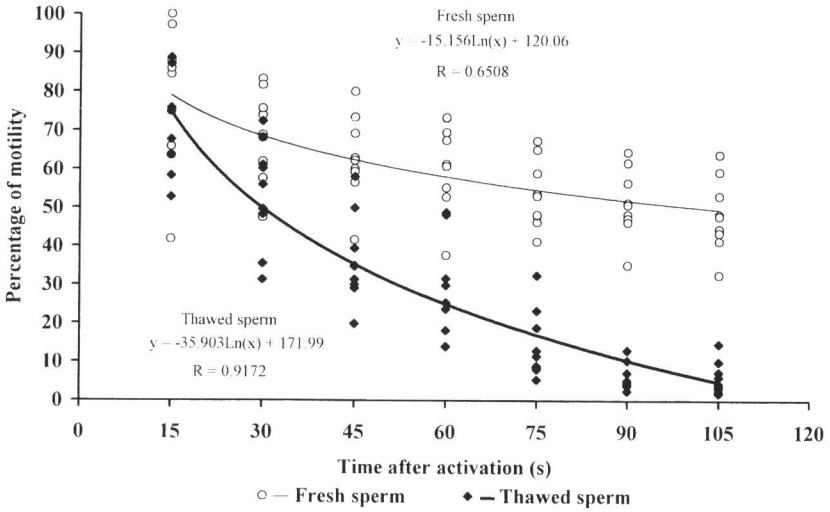

FIG. 2. Evolution of the percentage of motility of fresh and frozen/thawed sperm. Regression curves: fresh, $R = 0.65$; frozen/thawed, $R = 0.92$; X = time after sperm activation, and Y = percentage of sperm motility.

triplicate for fertilization yield, hatching yield, and larval malformations.

One gram of eggs (800 eggs) was mixed with either 200 μl of thawed sperm or 35 μl of fresh sperm (corresponding to identical numbers of spermatozoa, 1.8–2.4 $\times$ 10^5 spermatozoa per egg) and then immediately activated by the addition of 5 ml of hatchery water and incubated in incubators. Each incubator had a surface of 300 cm^2, with an individual water supply and thermoregulation at 21°C (minimum oxygen concentration of 9 $mg.ml^{-1}$). The fertilization yield was evaluated at gastrulation; viability was measured 1.5 days later and at hatching.

The means with standard deviation (SD) were evaluated from triplicates. Analysis of variance after arcsine transformation (ANOVA, Statgraphics version 5) followed by multiple comparison tests LSD was used for the results shown in Figs. 7 and 8. Probability values lower than 0.05 were considered significant.

The correlation coefficient between hatching and motility parameters was evaluated from data with fresh and thawed sperm. The means were evaluated from nine individual males (Figs. 4, 5, 7, and 8). The correlation coefficient (r) between groups of two parameters was assessed using Microsoft Excel 97.

RESULTS

Sperm Motility

Both fresh and frozen/thawed sperm showed similar types of straight trajectories after dilution, but the frozen/thawed spermatozoa had reduced velocity and percentage of motile spermatozoa 60 s after activation (Figs. 2 and 3). The percentage of motility of fresh and frozen/thawed sperm (nine males) was 78 $\pm$ 18 and 69 $\pm$ 14% 15 s after activation, respectively (Fig. 4), but with a high variability between males. The percentage of motility 15 s after activation of frozen/thawed sperm (nine males) was significantly lower ($P < 0.038$) than with the corresponding fresh sperm (Fig. 4). Sperm from different males also had a significant influence ($P < 0.0001$) on the percentage of motility of fresh and of frozen/thawed sperm 15 s after activation. A percentage of motility higher than 60% was observed at 120 s after activation in fresh sperm but at 20 s in frozen/thawed sperm. The velocity of fresh and frozen/thawed sperm of the nine males was 168 $\pm$ 61 and 94 $\pm$ 28 $\mu m.s^{-1}$, respectively, 15 s after activation. The velocity of sperm 15 s after activation of frozen/thawed sperm (nine males) was signifi-

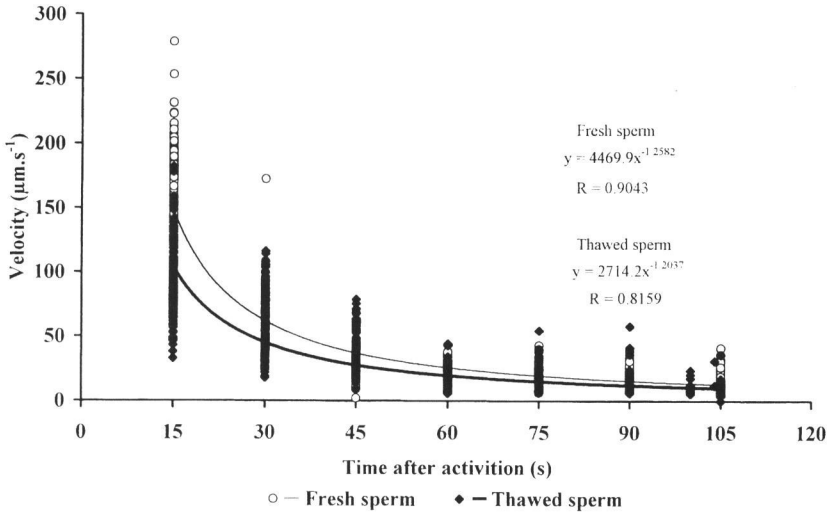

FIG. 3. Evolution of velocity. Regression curves: fresh, $R = 0.90$; frozen/thawed, $R = 0.92$, X = time after sperm activation, and Y = velocity of sperm.

cantly lower than that of fresh sperm (Fig. 5). Velocity measured from 33 to 120 s after activation was similar for fresh and for frozen/thawed sperm (50 to 20 $\mu m.s^{-1}$, respectively). The velocity of fresh sperm 15 s after activation was highly variable between males but similar for corresponding frozen/thawed sperm. Immediately after the initiation of motility, the axonemal waves were well developed on the whole length of the flagellum in both native and frozen/thawed sperm, with the same wave propagation velocity and wave amplitude (Fig. 6). ANOVA showed a significant influence of fresh vs frozen/thawed sperm from different males on

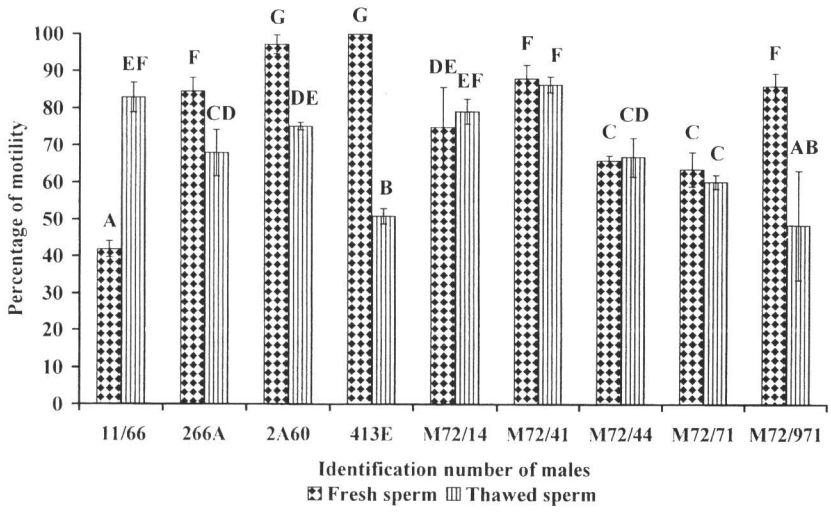

FIG. 4. Percentage of motility of fresh and frozen/thawed sperm 15 s after activation in activation solution (45 mM NaCl, 5 mM KCl, 20 mM Tris, pH 8). Groups with a common superscript do not differ significantly ($P < 0.05$).

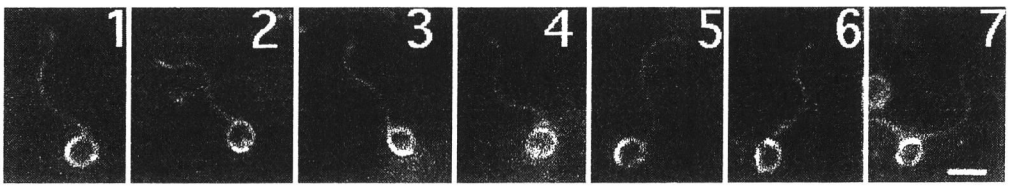

FIG. 5. Motility of frozen/thawed sperm. After 2 years of cryopreservation, sperm were thawed and diluted in the activation solution for motility observation. Individual spermatozoa were video-recorded at 10, 12, 14, 18, 20, 25, and 30 s post activation in frames 1–7 inclusive, using stroboscopic illumination (60 Hz) and a high-magnification objective lens (40X Olympus oil immersion) combined with an oil immersion condenser. Scale bar = 10 μm.

the sperm velocity 15 s after activation ($P < 0.0001$).

Fertilization and Hatching

Fresh or frozen/thawed sperm significantly influenced ($P < 0.0001$) fertilization but not the percentage of hatching or larval malformation (0–6.8%), but different males significantly influenced fertilization and hatching yield ($P < 0.003$ and $P < 0.007$, respectively). Multiple range analysis (LSD) showed significant differences between fresh and frozen/thawed sperm with respect to fertilization yield (68 ± 11 and 56 ± 10%, Fig. 7) but there were insignificant differences between fresh and frozen/thawed sperm regarding hatching yield (50 ± 18 and 52 ± 9%, respectively, Fig. 8). A total of 1.8 to $2.4.10^5$ fresh and frozen/thawed spermatozoa per egg were used for the fertilization experiment.

Correlation Coefficient between Fertilization and Motility Parameters

A correlation of $r = 0.53$ was observed between the percentage of fresh sperm motility and the percentage of fertilization yield from fresh sperm. A similar correlation ($r = 0.51$) was found between the percentage of fresh sperm motility and the percentage of fertilization yield from frozen/thawed spermatozoa. An independent correlation ($r = -0.31$) was observed between the percentage

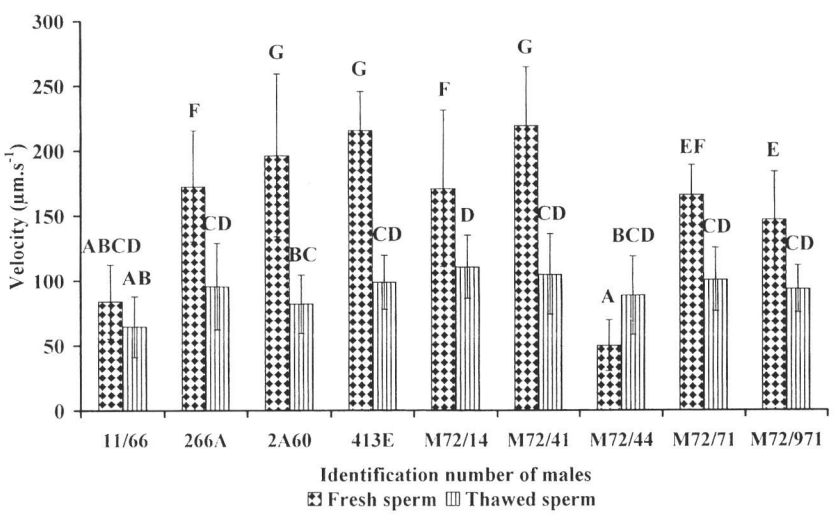

FIG. 6. Velocity of fresh and frozen/thawed sperm 15 s after activation in activation solution (45 mM NaCl, 5 mM KCl, 20 mM Tris, pH 8). Groups with a common superscript do not differ significantly ($P < 0.05$).

tion. This allows the maintenance of genetic variability in broodstock, with reduced hazard of transmission of diseases or infections (25), and preserves genetic resources, as in the present study. For common carp, sperm cryopreservation has been investigated in many studies, first successfully used by Moczarski (28) with a low fertilization yield (10–20%). Higher yields of fertilization or of hatching (30 to 70%) were obtained later (16, 24, 25, 26, 27) and also in the present results. Improved methodology of cryopreservation and adaptation in each situation have resulted in the preparation of cryopreserved carp sperm banks in the former USSR and Russia (15, Cvetkova, personal communication), in Israel (25), in Hungary (27), and in the Czech Republic (present paper). Fertilization and hatching yields were substantially improved by the use of the "seeding" technique (20), the programmed cooling rate using a Kryo-Planer (16, 24, 26), or the oxygenation of sperm before freezing (27).

Our methodology is close to that of Magyary *et al.* (26) but with several important differences. First, in our experiments, sperm were collected in plastic containers (plastic container, 200 ml for cell culture) and stored on ice at +4°C. Sperm in containers were usually stored under better conditions for respiration, as the layer of sperm in the container was only 1–3 mm. The methodology of oxygenation used by Magyary *et al.* (27) is difficult to apply under practical conditions. In contrast, a thin layer of sperm permits good oxygen exchange without an increase of glycolysis; glycolysis can decrease sperm metabolism and also decrease sperm motility and fertility (3, 23). Similar to Kurokura *et al.* (16), Lubzens *et al.* (24), and Magyary *et al.* (26), we used "Kurokura-1" extender with a dilution ratio of 1:5 between the sperm and protectant and 10% Me_2SO was added just before freezing, as is used for the sperm of other animals (19, 32). Compared to Magyary *et al.* (26), our cooling rate to −9°C was faster with the Planer Kryo III. The fertilization and hatching experiments were performed 1 week after sperm were frozen and stored at −196°C. Sperm used as fresh/control sperm was collected from the same individual males. This may explain why we obtain significantly better fertilization and hatching yields with frozen/thawed sperm compared to fresh sperm in males 72/71 (Figs. 7 and 8).

It is generally assumed that motility and fertility are well-correlated (8). Our fertilization and hatching yield results (Figs. 7 and 8) with frozen/thawed sperm are less variable than with fresh sperm. This may explain why we obtain a good correlation between percentage and velocity of sperm on the one hand and fertilization yield on the other. The percentage of motility and the velocity of fresh sperm were well and positively correlated with the fertilization yield of frozen/thawed sperm. A similar result was obtained in turbot (9) but only concerning the percentage of sperm motility. In that study, it was shown that it is the percentage of motile sperm, not the velocity, that limits the ability of sperm to fertilize. Also, Lahnsteiner (17) observed in a study of 50 different frozen-thawed sperm samples of rainbow trout a high correlation between sperm motility and fertilization yield. In contrast, Lubzens *et al.* (25) and Koosmann (14) observed no correlation between sperm motility and fertilization yield in Koi carp common carp, respectively. Koosmann (14), with Leibo and Bradley (19), also found no fertilization yield with motile sperm.

The success of fish cryopreservation is usually assessed by the yield of fertilization, with some reference to motility parameters such as percentage of motility, velocity, flagellum beat frequency (7), or other parameters obtained by computer-assisted sperm analysis. Such data have been reported for the common carp (4, 30) and other fish species (6, 13, 18); these constitute useful parameters for assessing the general viability of sperm cells. Our results dealing with the percentage of sperm motility and the velocity of fresh and frozen/thawed sperm can be applied to carp sperm and may serve as good indicative parameters for the success of fertilization yield or hatching yield with freeze/thawed sperm.

CONCLUSION

Cryopreservation of sperm appears to be a useful and reliable technique for conservation of gene resources in the common carp.

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Improvement of common carp artificial reproduction using enzyme for elimination of egg stickiness

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bstract

This study summarizes optimization of techniques for common carp artificial propagation including improvements of activation solution AS), the process of insemination, and elimination of egg stickiness. The optimum gamete ratio for good fertilization and hatching rate ranged om 8490 to 23 672 spermatozoa per egg, when dechlorinated tap water was used. Optimal ratio between eggs (weight in g) and AS (in ml) as defined as 1:1 to 1:2. Different concentrations of AS such as NaCl from 0 to 34 mM (0–68 mOsmol kg^{-1}) did not change fertilization and atching rates. An AS adopted for carp spermatozoa (45 mM NaCl, 5 mM KCl, 30 mM Tris–HCl, pH 8) was compared with other saline AS; nly the 51 mM (102 mOsmol kg^{-1}) NaCl solution decreased fertilization and hatching rate. The AS containing 20 mM Tris–HCl at H 9 increased fertilization and hatching rates compared to dechlorinated tap water of pH 7 or to AS of pH 6 and 7. Adhesiveness from the eggs as successfully removed by incubation in Alcalase DX (PLN 04715) using two successive steps with different enzyme concentrations. The rst step with an enzyme concentration of 2 ml l^{-1} was applied from 8 to 20 min after fertilization. Later in a second step, the best time for pplication of alcalase enzyme at a concentration of 20 ml l^{-1} was for 45 and 60 s at 20 min after fertilization leading to fertilization and atching rates of 80–87%. The α-Chymotrypsin (EC 3.4.21.1. Merck) was also found effective for elimination of stickiness. Results with -Chymotrypsin enzyme indicate that the response to success in elimination of stickiness is highly variable mainly due to differences in the nvironment, quality of water and carp strains.

ésumé

Amélioration de la reproduction artificielle chez la carpe en utilisant des enzymes pour l'élimination de la viscosité des œufs. ette étude résume l'optimisation des techniques pour la fécondation artificielle chez la carpe, y compris les améliorations des solutions 'activation, du processus d'insémination, et d'élimination de la viscosité des œufs. Pour obtenir de bons taux de fertilisation et d'éclosion, le apport optimal se situe entre 8490 et 23 672 spermatozoïdes par œuf, avec de l'eau du robinet déchlorée. Le rapport optimal entre les œufs poids en grammes) et la solution d'activation (en millilitres) est définie comme étant de 1:1 à 1:2. Différentes concentrations de solutions 'activation, telles que NaCl de 0 à 34 mM (0 à 68 mOsmol kg^{-1}) ne modifient pas les taux de fertilisation et d'éclosion. Une solution 'activation adoptée pour les spermatozoïdes de carpe (45 mM NaCl, 5 mM KCl, 30 mM Tris–HCl, pH 8) est comparée à d'autres solutions alines d'activation ; seule, la solution NaCl de 51 mM (102 mOsmol kg^{-1}) diminue le taux de fertilisation et d'éclosion. La solution 'activation contenant 20 mM Tris–HCl à pH 9 augmente les taux de fertilisation et d'éclosion, comparée à l'eau du robinet déchlorée de pH ou à une solution d'activation de pH 6 et 7. La viscosité des œufs est éliminée avec succès, par incubation dans l'alcalase DX (PLN 04715) vec deux étapes successives et différentes concentrations d'enzyme. La première étape, avec une concentration d'enzyme de 2 ml l^{-1} a été ppliquée de 8 à 20 min après fertilisation. Dans une seconde étape, le meilleur temps d'application de l'alcalase, 20 ml l^{-1}, a été de 45 et 60 s 20 min après fertilisation, conduisant à des taux de fertilisation et d'éclosion de 80–87 %. La α-chymotrypsine (EC 3.4.21.1. Merck) a été ussi efficace pour l'élimination de la viscosité des œufs mais avec des chances de succès beaucoup plus variables, dues aux différences nvironnementales, à la qualité de l'eau et aux différentes souches de carpe.

eywords: Reproduction; Insemination; Aquaculture; Enzyme; Common carp; *Cyprinus carpio*

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troduction

ntrolled reproduction of common carp started in the s, after establishing practical application of technolo- for artificial propagation (Woynarovich, 1962; Woy- ich and Woynarovich, 1980; Rothbard, 1981; Horvath 1984). Various studies have reviewed the physiology of perm (Billard et al., 1986, 1995), eggs and fertilization art et al., 1995), endocrine control of gametogenesis nduction of spawning (Yaron, 1995) and artificial in- ation Saad and Billard (1987), Billard (1988, 1990) illard et al. (1995).

ocedures of artificial insemination and incubation were sed by Woynarovich (1962). The method was based treating gametes in various solutions. Eggs and sperm mixed in an activating solution of 4 g NaCl and 3 g urea of water. Adhesiveness was removed by continuous g for 1.5 h in the same solution. A tannic acid treatment for 20 s. This technique was later improved (Woy- ich and Woynarovich, 1980) by the use of a second on of 4 g NaCl + 20 g urea per l, which reduced the ent time. Soin (1976) published other modifications ling the use of Milk for desticking. Khan et al. (1986) ed that full cream milk powder (20–25 g l^{-1}) was the efficient in removing the adhesiveness of eggs and it he best results in terms of fertilization and hatching compared to the methods of Woynarovich. The main eries in central Europe have applied these modifications tly to produce 100–200 millions of sac fry. Two to minutes after fertilization, the eggs are placed to a jar with milk, and the content is kept mixing by air ing for 60 min. Then running water is introduced to he milk during 10 min (Billard et al., 1995).

e present work was undertaken to re-examine various dures for artificial insemination in common carp, *Cyp- carpio*. The objectives of this study were to enhance zation and hatching rates through optimization of acti- solution (AS), process of insemination, activation of es and elimination of eggs stickiness.

terials and methods

roodstock handling and gametes collection

e reproduction and culture of common carp was carried the Department of Fish Genetics and Breeding, H, University of South Bohemia at Vodnany in the Republic in 2001–2002. Five- to 7-year-old male and e broodstock were maintained in separate ponds until n the experiments. Broodfish suitable for stripping elected in May and stocked separately in the hatchery ^{3} tanks with water flow rate of 0.2 l s^{-1}, temperature of °C and 6–7 mg l^{-1} O_2.

each experiment, 12 males were injected with carp ry (CP) at 1 mg kg^{-1}, 24 h before stripping at 21 °C. was collected individually from each male, kept in

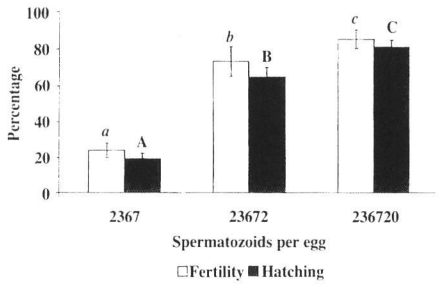


Fig. 1. The effect of increasing number of spermatozoa (2367–236 720 spermatozoa) per egg on fertilization and hatching rates with dechlorinated tap water. Groups with a common superscript do not differ significantly ($P < 0.05$).

thin layer in cell culture vessels under aerobic condition at 0 °C and proportionally pooled from five males prior to fertilization.

Eight females were used for each experiment and injected with CP at the dose of 0.4 mg kg^{-1} and then at 2.1 mg kg^{-1}, 24 and 12 h before stripping, respectively. The females were checked every 3 h after the second injection for ovulation, and stripped into separate dishes; the best egg batches were selected. Ova were stored under aerobic conditions at 17–19 °C for 2–4 h prior to artificial insemination (Rothbard et al., 1996). The eggs of at least four females were proportionally pooled just prior to conducting experiments.

Prior to the fertilization experiments with sperm pooled sperm, sperm concentration was counted with Thoma cell hemocytometer under Olympus microscope BX 41 (400×) and mean number was expressed per 20 squares of Thoma cell. Three batches of approximately 0.3 g (around 200 eggs) of unfertilized eggs were weighed to the nearest 0.0001 g, and then fixed in 4% formaldehyde for later counting and determination of mean egg weight. Number of eggs was then calculated in each experiment from the weight of eggs and

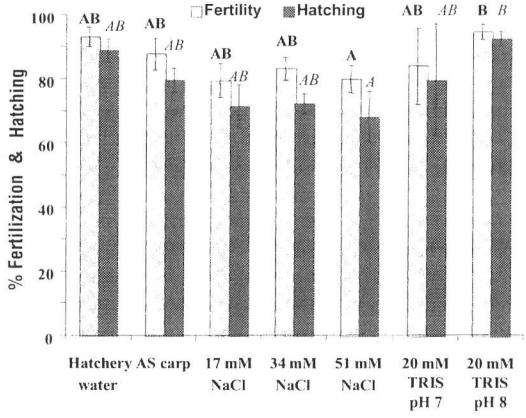


Fig. 2. Fertilization and hatching rates after artificial insemination in dechlorinated tap water; in solutions with increasing NaCl concentration; in buffered water pH 7 and 8 of 20 mM Tris–HCl; and in AS for carp (45 mM NaCl, 5 mM KCl and 30 mM Tris–HCl, pH 8; Saad and Billard, 1987). Sperm was used on the level of 13 000 spermatozoa per egg. Groups with a common superscript do not differ significantly ($P < 0.05$).

expressed as number of eggs per gram of eggs. Before injection and gamete collection, the males and females were anesthetized in a solution of 2-phenoxyethanol (1:1000).

2.2. Determination of optimal sperm/egg ratio (Experiment 1)

The quantity of sperm per egg for fertilization was 2367, 23 672 and 236 720 spermatozoa per egg, what represented 10, 100 and 1000 µl of sperm, respectively. Five grams of eggs with 586 eggs per 1 g were placed into a dish of 50 ml and accurate volume of sperm (10, 100 and 1000 µl of sperm) with estimated number of spermatozoa was dropped by micropipette. Before activation of eggs with water, seminal plasma was added at volumes of 990, 900 and 0 µl into experiments with 10, 100 and 1000 µl of sperm, respectively. Seminal plasma was made free of spermatozoa by means of centrifugation at 2500 × *g* for 5 min at room temperature. Then the dish was placed on shaking table with constant rotation 200 min^{-1}, 10 mm deflection and 5 ml of dechlorinated tap water at 22 °C was added. Two minutes later, approximately 200–300 fertilized eggs were placed with replication (four times) into a special incubator cage of 200 ml supplied with UV-sterilized recirculated dechlorinate tap water at 22 °C, 9 mg l^{-1} O_2.

2.3. Effects of different activation solutions (AS) (Experiment 2)

Five grams of eggs with 770 eggs per 1 g were placed into a dish of 50 ml and accurate volume of sperm with 13 000 spermatozoa per egg was dropped by micropipette. Then the dish was placed on shaking table with constant rotation 200 min^{-1}, 10 mm deflection and 5 ml of different AS at 22 °C were tested. Solutions differing with NaCl concentration (0, 17, 34 and 51 mM), solutions with 20 mM Tris–HCl of pH 7 and/or 8, then dechlorinated tap water as control and carp AS (45 mM NaCl, 5 mM KCl and 30 mM Tris–HCl, pH 8, Saad and Billard, 1987) were also used. Two minutes later, approximately 200–300 fertilized eggs were placed with replication (four times) into a special incubator cage of 200 ml supplied with UV-sterilized recirculated dechlorinate tap water at 22 °C, 9 mg l^{-1} O_2.

2.4. Determination of pH in activation solution (Experiment 3)

Five grams of eggs with 885 eggs per 1 g were placed into a dish of 50 ml and two levels of sperm quantity per egg were dropped by micropipette. The importance of AS pH on fertilization was more clearly defined under conditions of low numbers of sperm (4500) per egg together with the first control. On the other hand, the capacity of eggs to be fertilized was maximized with high number of spermatozoa (2 250 000 per egg) in repeated controls 2 and 3 (Fig. 3). Then the dish was placed on a shaking table; the rotation rate was 200 min^{-1} with a 10 mm deflection. Solutions of 5 ml of

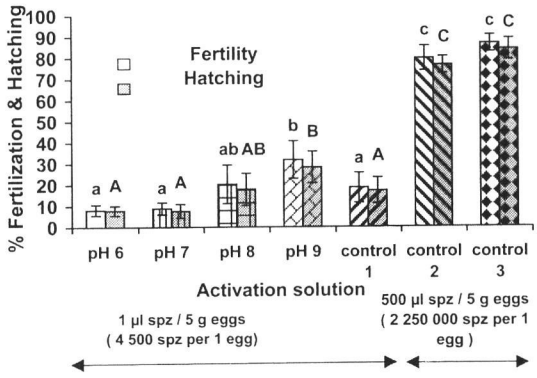


Fig. 3. Fertilization and hatching rates after artificial insemination with different pH of AS containing 20 mM Tris–HCl, dechlorinated tap water with concentration 4500 spermatozoa per egg. Sperm was used on two concentration levels, 4500 and 2 250 000 spermatozoa per egg. Groups with a common superscript do not differ significantly ($P < 0.05$).

20 mM Tris–HCl at 22 °C, were tested at different pH of 6, 7, 8 and 9 and with three controls. Controls were activated with dechlorinated tap water. Two minutes later, approximately 200–300 fertilized eggs were placed with replication (four times) into a special incubator cage of 200 ml supplied with UV-sterilized recirculated dechlorinate tap water at 22 °C, 9 mg l^{-1} O_2.

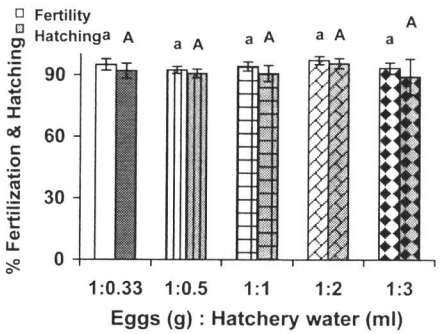
2.5. Effect of different ratio of AS volume and egg quantity (Experiment 4)

Five grams of eggs with 885 eggs per 1 g were placed into a dish of 50 ml and accurate volume of sperm with 8490 spermatozoa per egg was dropped by micropipette. Then the dish was placed on shaking table with constant rotation 200 min^{-1}, 10 mm deflection and accurate volume of 1.66 ml (1:0.33), 2.5 ml (1:0.5), 5 ml (1:1), 10 ml (1:2) and 15 ml (1:3) of dechlorinated tap water 22 °C were added. Two minutes later, approximately 200–300 fertilized eggs were placed with replication (four times) into a special incubator cage of 200 ml supplied with UV-sterilized recirculated dechlorinate tap water at 22 °C, 9 mg l^{-1} O_2.

2.6. Application of enzymes α-Chymotrypsin (EC 3.4.21.1. MERCK) and Alcalase DX (PLN 04715) for elimination of egg stickiness (Experiment 5)

Initially, seven proteolytic and polysaccharide enzymes were tested for ability to effectively eliminate egg stickiness. The α-Chymotrypsin (EC 3.4.21.1. MERCK) and Alcalase DX (PLN 04715) were selected as effective ones and also as less expensive enzymes, which could be used in practical condition.

Twenty grams of eggs with 567 eggs g^{-1} were placed into a dish of 250 ml and accurate volume of sperm with 140 130 spermatozoa per egg was dropped by micropipette. Then the dish was placed on shaking table with constant rotation 200 min^{-1}, 10 mm deflection and 20 ml of dechlorinated tap water at 22 °C was added.



Fertilization and hatching rates using different ratio of volume of water to weight of eggs with the concentration 8490 spermatozoa Groups with a common superscript do not differ significantly 5).

2, 4 and 6 min after activation, redundant water was ded and additional 20 ml hatchery water was added. nilliliters of α-Chymotrypsin (EC 3.4.21.1. MERCK) centration of 0.024 ml l^{-1} was added 8 min after ion. Fifteen minutes after activation, redundant solu- as discarded and additional 50 ml of the above motrypsin in concentration of 0.024 ml l^{-1} was added Finally, 16 (CH1), 18 (CH2), 20 (CH3) and 22 min after activation, 50 ml of α-Chymotrypsin in concen- of 0.24 ml l^{-1} was added with similar exposure for

en using Alcalase DX (PLN 04715) enzyme, the pro- was similar to the previous treatment until 8 min after on. Fifty milliliters of Alcalase DX (PLN 04715) in tration of 2 ml l^{-1} was added 8 min after activation. minutes after activation, the diluted enzyme was ed and additional 50 ml of Alcalase DX in concentra- 2 ml l^{-1} was added again. Finally, 20 min after on, 50 ml of Alcalase DX in concentration of 20 ml l^{-1} ded for exposure of 45 (A1), 60 (A2), 75 (A3) and 90 s espectively (Fig. 5).

The first control (C 1) was fertilized and at 2, 4 and 6 min after gamete activation, redundant water was discarded and additional 20 ml dechlorinated tap water was added. Water from eggs was discarded and additional 20 ml hatchery water was added. Eggs were incubated in cages since 8 min after gamete activation. The second control (C 2) was similar until 8 min after gamete activation. Eight minutes after activation, milk solution with concentration of 40 g l^{-1} dry powder was added for 60 min and since 70 min after gamete activation; the eggs were incubated in cages and jars.

The eggs after treatments were properly rinsed four times with 500 ml of hatchery water. After elimination of enzyme, 200–300 eggs were placed with replication (four times) into an incubator cage of 200 ml supplied with UV-sterilized recirculated dechlorinate tap water at 22 °C, 9 mg l^{-1} O_2. The rest of eggs were filled in the Zuger incubation jar of 2 l volume supplied with UV-sterilized recirculated dechlorinate tap water at 22 °C, 9 mg l^{-1} O_2 for observation of the success of desticking.

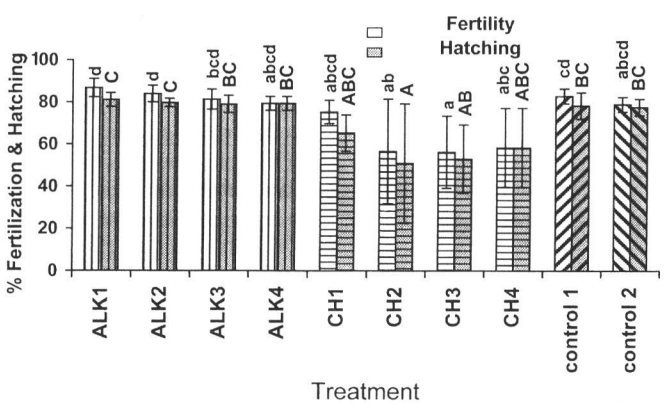
2.7. Calculation of fertilization and hatching

The eggs were counted in each cage and during incubation, dead eggs were counted and removed, then hatched fry were counted, usually up to 4.5 days of incubation at 22 °C. The percentage of fertilization rate (F_r) was then calculated for each cage from the total number of eggs placed in the cage (E_t) minus dead eggs (E_d) collected up to 24 h after fertilization as follows:

$$F_r = 100\,[(E_t - E_d)/E_t]$$

The hatching rate (H_r) percentage was calculated for each cage from the total number of eggs placed in the cage (E_t) and divided with number of hatched larvae (H_l) as follows:

$$H_r = 100\,(H_l/E_t)$$



ne different time exposition of 0.024 ml l^{-1} α-Chymotrypsin enzyme (EC 3.4.21.1. MERCK) and 20 ml l^{-1} Alcalase DX enzyme (PLN 04715) for on of egg stickiness with the concentration of 140 130 spermatozoa per egg. During the second step, α-Chymotrypsin was used for 1 (CH1), 3 (CH2), nd 7 min (CH4) exposures. The Alcalase DX enzyme was added at 20 min after activation with exposure for 45 (ALK 1), 60 (ALK 2), 75 (ALK 3) and K 4), respectively. Groups with a common superscript do not differ significantly ($P < 0.05$).

.8. Data analysis

Means of the data acquired were evaluated from four eplicates. Statistical significance was assessed using mul-iple analysis of variance (ANOVA, Statgraphics version 5), ollowed by multiple comparison Tukey HSD range test. Probability values <0.05 were considered significant.

. Results

.1. Determination of optimal sperm/egg ratio Experiment 1)

The fertilization and hatching rates (82% and 80%, re-pectively) were significantly higher for 236 720 spermato-oa per egg, than for other ratios (Fig. 1). The fertilization nd hatching rates were 70% and 64%, respectively, with the evel of 23 672 spermatozoa per egg and to 23% and 20%, espectively, with the level of 2367 spermatozoa per egg. ANOVA showed significant effects of the number of sperma-ozoa per egg ($P < 0.0001$) on the fertilization and hatching ate.

.2. Effect of different activation solutions (Experiment 2)

The highest fertilization and hatching rate (96% and 93%, espectively) was found for AS of 20 mM Tris–HCl, pH 8 with concentration 13 000 spermatozoa per egg but was nly significantly different from one solution. Fertilization nd hatching rates were significantly lower in 51 mM NaCl AS, where fertilization and hatching rates were 67% and 63% (Fig. 2).

.3. Determination of pH in activation solution Experiment 3)

The highest fertilization and hatching rates (30% and 28%, respectively) with concentration 4500 spermatozoa per egg, was for AS pH of 9. Lower levels of 15–16% fertiliza-ion and hatching rates were found with control 1, when low numbers of spermatozoa were used (Fig. 3). Fertilization and natching rates were only on the level 9–7% with AS of pH 6 and 7 and also using low number of spermatozoa. The quality and fertilizability of eggs were very good that was demonstrated by control 2 and 3 with 80–90% of fertilization and hatching rate, when 2 250 000 spermatozoa per egg were used (Fig. 3).

.4. Effect of different ratio of volume of activation solution and egg quantity (Experiment 4)

No relationship was observed between the ratios of dechlorinated tap water volume: egg quantity represented by their weight. Results varied between 90% and 96% in the range of ratios 1:0.33 to 1:3 with concentration of 8490 sper-matozoa per egg (Fig. 4). ANOVA showed insignificant effect of the ratio of AS:egg quantity ($P < 0.1$–0.4) on fertilization and hatching rates.

3.5. Application of two enzymes α-Chymotrypsin (EC 3.4.21.1. MERCK) and Alcalase DX (PLN 04715) for elimination of egg stickiness (Experiment 5)

The duration of 45–60 s treatment with alcalase enzyme was applied 20 min after gamete activation yielding fertilization and hatching rates of 80–87% with concentration of 140 131 spermatozoa per egg. However, the use of α-Chymotrypsin (EC 3.4.21.1. MERC) was not effective for elimination of stickiness and there was a significant decrease in fertilization and hatching rates compared to controls 1 and 2 (Fig. 5).

4. Discussion

4.1. Conditions of sperm storage

Sperm of common carp can be stored in vitro at 2–5 °C for 2 days (Belova, 1981; Jähnichen, 1981; Hulata and Rothbard, 1979) without decrease in fertilization. However, environmental conditions of sperm storage must be correctly adjusted, because ATP is consumed during sperm storage leading to a slight decrease after 24 h (Saad et al., 1988). If availability of O_2 and substrates for sperm is limited, it can be provided artificially in vitro, by exposition to an O_2 atmosphere in ratio 1:10 and storage at 0 °C as sperm was stored prior fertilization.

4.2. Artificial insemination

Composition of diluents for sperm activation and fertilization was developed by Woynarovich and Woynarovich (1980) and later modified by Saad and Billard (1987). Woynarovich and Woynarovich (1980) used AS containing 4 g (68 mM) of NaCl and 3 g of urea per l. Later, Saad and Billard (1987) in a more detailed study developed AS containing 45 mM NaCl, 5 mM KCl, and 30 mM Tris–HCl pH 8. Saad and Billard (1987) included KCl as important component favorable for motility of sperm (Morisawa et al., 1983; Redondo-Muller et al., 1991), but carp eggs were found not to tolerate more than 5 mM KCl (Saad and Billard, 1987). Saad and Billard (1987) formulated a media with optimal osmotic pressure where all ova were fertilized in a range of 100–150 mOsmol kg^{-1} when the number of spermatozoa per egg was high (20 000–25 000). Also, fertilization was not affected by a pH of 7–9. According to our results, it can be concluded that the minimum number of spermatozoa for good fertilization and hatching rate ranges 8500–25 000 spermatozoa per egg, according to quality of eggs (Figs. 1 and 4). Various AS with NaCl 0–34 mM (0–68 mOsmol kg^{-1}) did not change fertilization or hatching rates. Also our results did not verify those of Saad and Billard (1987) using an AS for carp (45 mM NaCl, 5 mM KCl, and 30 mM

ICl pH 8) did not change the results. Only 51 mM nOsmol kg^{-1}) NaCl significantly decreased fertiliza- nd hatching rates. The two experiments showed in- g fertilization and hatching rates when using higher stly pH 8 (Fig. 2) and secondly pH 9 (Fig. 3) instead of rinated tap water with pH 7. An overview of our results d that dechlorinated tap water or low concentration of solutions at pH 8 or 9 and 10 000–20 000 spermatozoa g are the best conditions for AS. Then the question is about the differences between our results and those of nd Billard (1987). They can be explained by the y of methodology during experiments. Saad and Bil- 987) used Petri dishes for basic experimental condi- where the ratio between weight of eggs and volume of s 1:30–40 and later they adapted these results to the al approach. However, under practical conditions the etween weight of eggs and volume of AS ranges in 2. Results obtained in these conditions could be influ- by the high mass of eggs and by the content of seminal Also, the practical approach to carp culture in Europe lly employs milk for elimination of stickiness and it is nown that during this procedure, a low ratio of milk e to volume of eggs is needed. If the quantity of milk is esults of desticking are usually not successful. The y of our study was opposite to that of Saad and Billard and we tried firstly to adapt our experimental condi- o the practical ones. The ratio between the weight of d volume of AS was 1:1 in all cases with constant time tation level during the procedure of mixing gametes lutions. Ratio 1:1 used under practical conditions as in our experiment was confirmed to be feasible with s for artificial insemination procedure.

tificial insemination and elimination of stickiness practical conditions

ed on our results, our recommended procedure for al insemination is as follows.

Fertilization

minimum volume of short-term stored sperm under conditions used for insemination was 1 ml of sperm g of short-term stored ova (3–4 h in stable temperature °C; Rothbard et al., 1996). That volume of sperm nted 236 720 spermatozoa per egg. Also, 0.1 ml of per 1 kg of eggs was found sufficient for good fertili- and hatching rates (Figs. 1, 2 and 4) when 8490– spermatozoa guaranteed successful fertilization and g rates. The mixture of eggs and sperm was directly ed with 1 or 2 l of activating solution made of 20 mM Cl, pH 9 or of dechlorinated tap water or clean hatch- ter at an optimum temperature 22 °C. It was mixed 2 min and later 1 l of dechlorinated tap water was After 2 min, the redundant solution was poured out other 1 l of dechlorinated tap water (or clean hatchery was added. This procedure was repeated again at 4 and and desticking process with milk or enzyme was started at 8 min. During that time the eggs were hydrated and swollen rapidly. Egg sticking was prevented during that period by constant mixing.

4.3.2. Desticking

The procedure for egg desticking was developed many years ago by Soin (1976) and later improved by Khan et al. (1986). In Czech Republic, the fish farming practice employs powder milk containing in 100 g of powder 27.2% of fat, 26.6 g of albumin, 37.2 g milk sugars, 5.8 g of ions, 0.2 g of lecithin and 3 g of water for preparation solution with concentration 40 g of powder milk per l of dechlorinated tap water or clean hatchery water. The milk solution is added slowly with intensive mixing in volume of 1 l kg^{-1} of freshly stripped eggs. Later usually at 15 min after activation when the eggs have swelled, additional milk is slowly added. If too much milk is added stickiness of eggs is not eliminated, therefore, it is necessary to add only small quantities of milk solution. The swelling process lasts in general for 60 min. After that time, hatchery water is slowly added within 10 min to replace milk solution and finally, the eggs are transferred to Zuger jars. Carp eggs will swell three to four times in milk than their original volume but using the method of Woynarovich and Woynarovich (1980) with urea, the swelling factor was increased six to nine times (Horvath et al., 1984).

4.3.3. Enzyme treatment

The Alcalase DX (PLN 04715) was successful in removing adhesiveness. Optimum ratio between eggs and alcalase (2 ml of enzyme per 1 l of dechlorinated tap water) was 1:2.5 (g eggs:ml enzymes) with mixing from 8 to 15 min after activation and after 15 min additional 2.5 part of enzyme solution (2 ml of enzyme per 1 l of dechlorinated tap water) was added. Twenty minutes after activation, the enzyme solution was poured out from the eggs and more concentrated alcalase solution (25 ml l^{-1} of dechlorinated tap water) was added in the rate 1:2.5 for 60 s exposure only. At 21 min after activation, the eggs were rapidly rinsed with water and transferred to Zuger jars for incubation. Fertilization and hatching rates were similar between enzyme treatment and milk treatment. The results of the study were repeated in the Czech Republic commercial condition with similar results.

Traditional technique for destickiness of eggs in common carp can be successfully shortened from 70 to 21 min when using enzyme treatment instead of milk or urea. A proteolytic enzyme (alcalase, Merck EC 3.4.21.14) has also been successfully used for elimination of stickiness in European catfish, *Silurus glanis* eggs (Linhart et al., 2002) and tench, *Tinca tinca*(Linhart et al., 2000, 2003), and it is now used routinely in hatcheries of the Czech Republic and France. Enzyme treatment in both species increased hatching success and decreased the time of egg handling from 1 h using milk/clay in tench or only clay in European catfish treatment, to about 2 min.

5. Conclusion

The present work optimized procedures for artificial insemination of common carp, *C. carpio* with increase in fertilization and hatching rates, optimization of AS, process of insemination, activation of gametes and elimination of eggs stickiness with a practical proposal for artificial reproduction in fish hatcheries.

Acknowledgements

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Genofond, specifika a kryokozervace spermatu lína

6.1 Genofond a plemenářská práce v chovu lína

Systematická plemenářská práce v chovu lína je v porovnání s kaprem ohem mladší a je spojena s působením Ing. Kvasničky v 80. a 90. letech stol. na šlechtitelské stanici VÚRH Ve Vodňanech. Výchozím materiálem současná plemena se staly ryby z různých populací chovaných v rámci dejšího Československa a pozdější dovozy ze zahraničí. Stejně jako u pra dělíme tedy plemena na původní česká (lín vodňanský, komeziříčský, hlubocký, táborský, mariánskolázeňský), mezi něž řadíme i evné variety lína zlatého, modrého a bílého, a dále importovaná (lín necký –Konigswartha, maďarský a rumunský.)

6.2 Močový měchýř, iontové složení seminánní plazmy a moči a charakteristika pohybu spermií lina obecného (*Tinca tinca L.*)

Práce se spermatem lína je komplikovaná močí, která pří odběru sperma taminuje a zhoršuje jeho kvalitu. Proto byla při práci s línem nejprve ornost zaměřena na zdokumentování této problematiky a vlivu na yblivost spermií.

Močový měchýř u lína byl zdokumentován jako malý bílý váček o emu 0,5-2 ml těsně přiléhající k chámovodům v nejkaudálnější části tělní iny. Sperma odebírané během experimentu mělo bělavou barvu a jeho centrace závisela na stupni naředění močí. Semenná plazma obsahovala 4±1,3 mM Na, 1,9±0,6 mM K, 0,6±0,2 mM Ca a 0,5±0,1 mM Mg; nolalita byla naměřena 203±82 mOsmoll. Kg^{-1}. Moč obsahovala 30,9±8,9 Na, 4,3±2,9 mM K, 0,9±0,5mM Ca a 0,6±0,2 mM Mg; osmolalita moči a 85±58 $mOsmoll.kg^{-1}$. V moči byla dále detekována močovina. Dále byla orována spontánní aktivace spermií močí. Motilita spermií 90 -100% byla lována bezprostředně po aktivaci v destilované vodě nebo aktivačním liu (30-45 mM KCl), naměřená rychlost pohybu spermií 120-140 $\mu m.s^{-1}$. kvence pohybu bičíku se pohybovala bezprostředně po aktivaci mezi 60- Iz dopředný pohyb spermií bylo možno zaznamenat do 80 s po aktivaci

řípadě aktivace destilovanou vodou a do 180s v případě aktivace ivačním roztokem.

drobně je práce popsána v publikaci:

hart O., Rodina M., Bastl J. and Cosson J.,: Urinary bladder, ionic nposition of seminal fluid and urine with charakterizatio of sperm tility in tench (Tinca tinca L.) Journal of Appl. Ichthyology 19: 177- , 2003

6.3 Imobilizační roztoky

Pro eliminaci vlivu moči je možné použít pro odběr spermatu různé obilizační roztoky. Jako první použil u lína takový roztok Linhart a asnička (1992), přičemž použil roztok složení:171,2 mOsmol NaCl, 53,7 smol KCl, 75,1 mOsmol glycinu (Linhart, Billard and Koldras 1992).

6.3.1 Roztok Kurokura jako imobilizační médium pro spermie lína obecného *(Tinca tinca L.)*

Pro potřeby krátkodobého uchování a kryokonzervace byla testována žnost použití roztoku Kurokura (1984) a jeho modifikací jako obilizačního roztoku pro sperma lína. Sperma bylo odebíráno do injekční kačky s imobilizačním roztokem (IR) v poměru IR: sperma 2:1 (kontrola IR)a uchováno v aerobních podmínkách při teplotě 0-4°C po dobu 10h. U rmatu byla po odběru a po 10 hod uchování sledována motilita, rychlost ybu spermií, oplozenost a líhnivost plůdku po použití uchovávaného rmatu. Ze získaných výsledků vyplývá, že pro úspěšné oplození jiker ovávaným spermatem je použití IR nezbytné, přičemž z testovaných difikací IR vyšel jako nejlepší Kurokura 180 (180 mM NaCl. 2,86 mM l, 1,36 mM $CaCl_2.2H_2O$ a 2,38 mM $NaHCO_3$), kde bylo dosaženo ozenosti a líhnivosti 41%, přičemž nebylo průkazného rozdílu mezi stvým a uchovávaným spermatem. Naproti tomu v kontrole bez IR bylo aženo oplozenosti a líhnivosti v rozmezí 6-7%.

drobně je práce popsána v publikaci:

odina M., Cosson J., Gela D. and Linhart O.: Kurokura solution as mobilizing medium for spermatozoa of tench (Tinca tinca L.) quaculture International 12: 119-131, 2004

formou posteru byla předběžně prezentována na workshopu Reprodukce pra konaném v rámci mezinárodní konference Genetic days Brno 2002

6.4 Zmražování spermatu lína:

Cílem těchto pokusů bylo navrhnout metodiku zmražování spermatu na, především vhodnou kombinaci imobilizačního roztoku, kryoprotektantu, rmy a objemu mražené dávky a zmrazovacího programu. Úspěšnost byla dnocena jako motilita a rychlost pohybu rozmrazených spermií a dále lozenost a líhnivost jiker po použití rozmrazených spermií. Sperma bylo ebíráno přímo do roztoků Kurokura a Kurokura 180 a uchováváno do racování při teplotě do 4°C. Ke spermatu byl přidán kryoprotektant (DMSO směs DMSO s propandiolem v poměru 1:1) a sperma bylo rozpipetováno do yotub (1,8 ml) či (pejet 0,5 1 a 5 ml) a mraženo ve zmrazovacím automatu aner Kryo 10 serie III podle programu K4 (z +4°C do -9°C rychlostí 4°C za inutu, z -9°C do -80°C tychlosti -11°C za minutu, výdrž na teplotě -80°C 6 inut) a podle programu L1 (z teploty +4°C na -80°C rychlostí 20°C za inutu, výdrž na teplotě -80°C 6 minut). Poté byly kryotuby a pejety eneseny do tekutého dusíku. Zmražené dávky byly rozmrazovány na vodní zni teploty 40°C (doba rozmražování podle objemu dávky) a bezprostředně užity k testu fertility a kontrole motility; motilita a rychlost byly stanoveny videozáznamu mikroskopického obrazu pohybu spermií). Anova prokázala všech případech negativní vliv mražení. Nejpříznivějších výsledků nnivosti plůdku 33,8% bylo dosaženo u spermatu odebraného do roztoku urokura 180, mraženého s kryoprotektantem DMSO+propandiol (10%) v 5 l pejetách programem L1. Rychlost pohybu spermií rozmraženého spermatu pohybovala bezprostředně po aktivaci v rozmezí 31-46μm.s^{-1}, motilita 8,6-

3,9%. Úspěšné zmražování spermatu lína ve větším objemu (5ml) se jeví ako vhodná varianta pro praktické použití.

odrobně byla uvedená práce shrnuta a přijata k publikaci ve článku:
Marek Rodina, David Gela, Martin Kocour, S.M. Hadi Alavi, Martin Hulak and Otomar Linhart*: Cryopreservation of Tench *Tinca tinca* Sperm: Sperm Motility and Hatching Success of Embryos Theriogenology (přijato do tisku)

Výsledky byly dále prezentovány formou posteru v rámci IV International Workshop on Biology and culture of the Tench Tinca tinca (L.) 2004 Wierzba Poland

6.5 Optimalizace inseminace a oplozování jiker lína

Cílem této práce bylo zlepšit oplozenost a líhnivost optimalizací aktivačního roztoku, vlastní inseminace a aktivace gamet a odlepkování jiker.

Sperma bylo odebíráno do modifikovaného roztoku Kurokura (Kurokura 180) a chováno při 2°C 2,5-5 hodin do použití. Při použití dechlorované vodovodní vody k aktivaci spermií a jiker bylo průkazně nejlepší oplozenosti a líhnivosti dosaženo při dávce spermií na 1 jikru 11500 a vyšší. Optimální poměr mezi objemem aktivačního média (ml) a hmotmostí oplozovaných jiker (g) byl 1:1. Aktivační roztok na bázi NaCl v koncentracích 0-68 mM bez úpravy a stabilizace pH průkazně snížil oplozenost i líhnivost oproti dechlorované vodovodní vodě pH 7, naopak 17 mM NaCl s 10mM TRIS-HCL pH 8 a 9 průkazně zvýšil oplozenost i líhnivost oproti dechlorované vodovodní vodě pH 7. Odlepkování bylo optimalizováno na použití enzymu alkaláza aktivity 3,16 Ansonu na ml.

Kompletně je práce popsaná v publikaci:
Linhart O., Rodina M., Kocour M. and Gela D. 2006: Insemination, fertilization and gamete management in tench, *Tinca tinca* (L.) Aquaculture International 14: 61-73

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ry bladder, ionic composition of seminal fluid and urine with characterization erm motility in tench (*Tinca tinca* L.)

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aboratory of Genetics, Physiology and Reproduction of Fish, Institute of Animal Physiology and Genetics, Academy of of the Czech Republic, University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech ; [2]University of South Bohemia, Faculty of Agriculture, Ceske Budejovice, Czech Republic; [3]Centre National de Recherche que, Station marine, Villefranche sur Mer, France

y

urinary bladder attached to the seminal duct in caudal he abdominal cavity was registered for the first time in l males of tench. The urinary bladder wall was of color and the bladder contained 0.5–2 ml of urine. llected in the experiment, the tench sperm was white- Spermatozoa density is highly variable due to con- on by urine, and the latter additionally activates eous motility of the spermatozoa. Seminal fluid con- s such as Na^+ (18.4 ± 1.3 mM), K^+ (1.9 ± 0.6 mM), .6 ± 0.2 mM) and Mg^{2+} (0.5 ± 0.1 mM), leading to ty of 230 ± 82 mOsmol kg^{-1} depending on the dilu- urine. Urea was detected in urine samples uncontami- sperm with an osmolality of 85 ± 58 mOsmol kg^{-1}. so contained high concentrations of ions such as Na^+ 8.9 mM), K^+ (4.3 ± 2.9 mM), Ca^{2+} (0.9 ± 0.5 mM) $^{+}$ (0.6 ± 0.2 mM). The spontaneous sperm activation was up to 100%, but could be prevented by collection mmobilizing solution. Motility was observed for % spermatozoa just after their transfer to distilled water vimming medium (SM, 30–45 mM KCl) with a velocity 40 $\mu m\ s^{-1}$. A flagellar beat frequency of 60–70 Hz and motility lasted up to 80 s in distilled water, and up to SM at room temperature.

tion

nary bladder has a significant role in freshwater fish or recapturing ions in the absence of water reabsorp- rties and Wood, 1991). In freshwater species such as trout (*Oncorhynchus mykiss*), the kidney has a grouping of tubules containing urine (Karnaky, r there is macroscopically visible urinary bladder as , tilapia *Oreochromis mossambicus* (Linhart et al., nd European catfish *Silurus glanis* (Linhart et al.,

collection in laboratory conditions, tench sperm is lored and the spermatozoa density highly variable as a urine contamination (Kouril et al., 1981,1984). When h sperm is collected during the process of artificial ction (Linhart and Billard, 1995), the spermatozoa ontaneous movement as a result of urine contamin- hich triggers this movement by osmolality decrease to that of seminal fluid (Linhart et al., 1986; Linhart snička, 1992). Such spontaneous sperm activation by n be prevented by collection of the sperm in an immobilizing solution (IS) (Linhart and Kvasnička, 1992; Linhart et al., 1995a).

After dilution in hatchery water, the average motility time was 25–52 s for the mass progressive motion (Moczarski and Kodras, 1982; Linhart et al., 1986) and 93 s for progressive motion (Zuromska, 1981). Total motility duration was 161–210 s (Moczarski and Kodras, 1982; Linhart et al., 1986; Linhart and Kvasnička, 1992). Tench sperm cannot be stored for even short terms in the absence of IS due to extensive dilution by urine (Linhart et al., 1986). Spermatozoa motility was observed to decrease during the spawning season (Zuromska, 1981; Zuromska and Markowska, 1984). The percentage of morphologically normal-appearing spermatozoa differed considerably (0–90%) according to the sample and during the spawning season. It was not possible to demonstrate any relationship between duration of the spermatozoa motility and presence of abnormal spermatozoa (Zuromska, 1981).

In the present study the position of the urinary bladder, analysis of ionic composition of urine and seminal fluid with motility parameters, such as percentage motility, velocity and frequency of tench spermatozoa, were investigated.

Materials and methods

The experiment was performed at the experimental hatchery of the University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Department of Fish Genetics and Breeding at Vodnany, Czech Republic. During the reproductive season (June), sperm was individually collected from five mature 5-year-old tench males [400–700 g body weight (BW)]. Males were judged for maturity by abdominal compression and spermiation was detected by sperm production. Each male was treated with a single injection of 1 mg kg^{-1} BW of carp hypophysis. Sperm was collected 24 h later by aspiration with a syringe. Fish were dissected after sampling. A urinary bladder of small size was observed in the abdominal cavity of dissected males: the bladder was attached to the seminal duct very near to the anal aperture in the caudal part of the abdominal cavity (Fig. 1). For volumetric assessment, fresh urine was aspirated by Luer syringe with needle size no. 2. Eventual urine contamination with sperm was assessed by means of dark field microscopy (Linhart et al., 2002) whereby a 29 μl drop of urine was placed on a glass slide and viewed under the microscope at 200× magnification.

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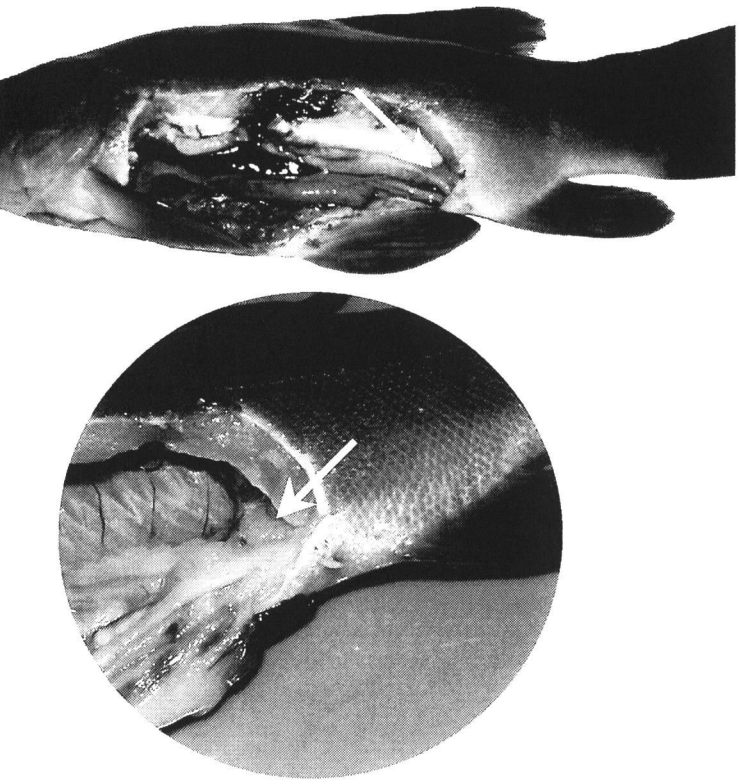


Fig. 1. Arrows show the position of a small urinary bladder very near the anal aperture in the ventral cavity of dissected tench (A) in general and (B) in detail

minal fluid separation, the sperm was centrifuged at r 5 min at room temperature and supernatant was collected. The osmolality of pure urine and of seminal measured using a Vapour Pressure Osmometer USA) and expressed as mOsmol kg^{-1}. Ionic compo- a^+, K^+, Ca^{2+} and Mg^{2+}) of urine and of seminal e measured with a flame spectrometer SpectrAA 640 Techtron, Australia).

of sperm motility parameters

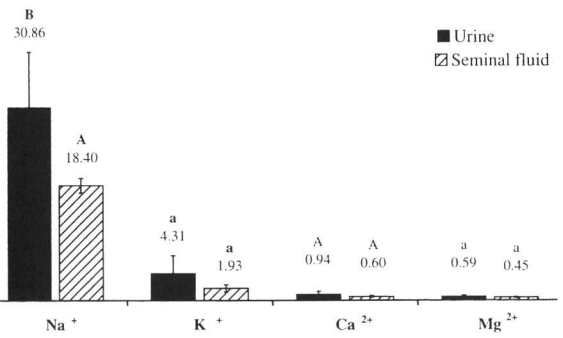
otility was evaluated for the percentage of motile zoa, velocity and beat frequency. Measurements of parameters were carried out using dark field micro-

iately after milt collection, spermatozoa were ob- spontaneous movement prior to any dilution; a drop undiluted milt was spread directly onto a glass slide ined at 200× magnification under the microscope. sting the spermatozoa swimming ability, 0.1 μl of directly mixed with a 49 μl drop of distilled water or ning medium (SM – 30–45 mM KCl), placed on a de previously set on the microscope stage and ely after mixing was examined under 200× magni- vithout a cover slip; therefore in this study the final vas 1 : 500. Motile spermatozoa were video-recorded ithin 10 s for measurement of velocity and percent- ctively swimming spermatozoa. The movements of zoa were observed through a 20× lens using dark roscopy and recorded at 50 frames per second using ideo camera (SONY SSC-M350CE) mounted on a microscope (Olympus BX50, Japan). The focal s always positioned near the glass slide surface. ozoa movement was recorded using a tape recorder (SONY S-VHS, SVO 9500 MDP, Japan), visualized on a color video monitor and using stroboscopic illumination by a Strobex (Chadwick-Helmut, 96300, USA). The stroboscopic flash illumination with adjustable frequency was set in automatic register with video frames (50 Hz). Successive positions of the recorded sperm heads were measured from video frames using a still video-recorder and analyzed each time from three frames by microimage analysis (Version 3.0.1. for Windows, Olympus, with special application from Czech Olympus) velocity and percentage of moving spermatozoa.

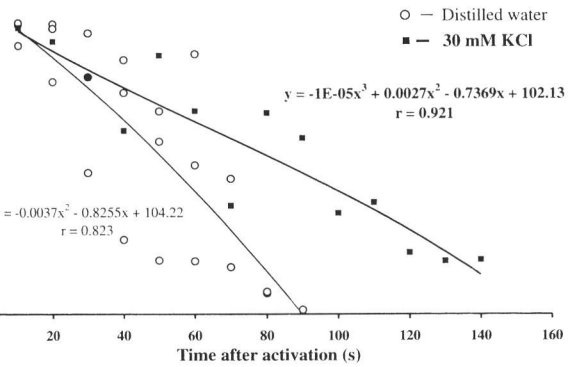
For the measurement of the beat frequency of the flagellum, 0.1 μl of milt was directly mixed with a 49 μl drop of distilled water or SM – 45 mM KCl and placed on a glass slide previously set on the microscope stage. Immediately thereafter the beating of the spermatozoa flagellum was measured using a Chadwick-Helmut stroboscopic flash (9630, USA), adjusting the flash-light frequency to stabilize the flagella waves in synchrony.

Data evaluation and analysis

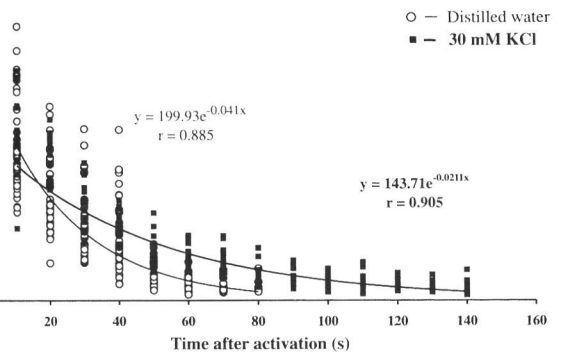
The data were acquired from the following replications: percentage of spermatozoa motility one time, frequency of sperm flagellum two times, and velocity three times per male and time of observation after sperm activation (Figs 3–5), ionic composition and osmolality five times per species (Fig. 2). Data were evaluated and statistical significance was assessed using one-analysis of variance (ANOVA, Statgraphics version 5), followed by multiple comparison Tukey HSD range test (Fig. 2). Linear regression analysis and correlation were obtained using Microsoft Excel 97 for establishment of the relationship in Figs 3–5. Probability values < 0.05 were considered significant.



onic composition of tench seminal fluid and urine. Groups of n with common superscript do not differ significantly 05)



Percentage of motility of tench spermatozoa activated in water or in SM = 30 mM KCl



Velocity of tench spermatozoa activated in distilled water or in 0 mM KCl

bladder and ionic parameters in urine and seminal fluid

inary bladder of tench was found to be of whitish color olumes of urine 1.2 ± 0.5 ml in the abdominal cavity of ed males. It was attached to the seminal duct very near al aperture (Fig. 1a) in the caudal part of the abdominal (Fig. 1b). The osmolality of urine and of seminal fluid was 85 ± 58 and 230 ± 82 mOsmol kg^{-1}, respectively; ility of the latter indicated a low dilution rate of the l fluid by urine. Urine contained high concentrations of uch as Na^+ (30.9 ± 8.9 mM), K^+ (4.3 ± 2.9 mM), 0.9 ± 0.5 mM) and Mg^{2+} (0.6 ± 0.2 mM); the seminal

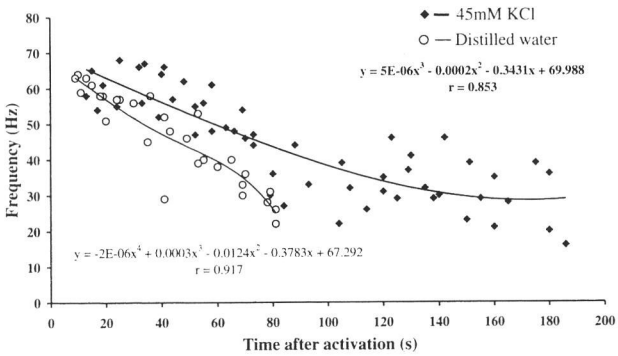


Fig. 5. Flagella beat-frequency of tench spermatozoa activated in distilled water or in SM = 45 mM KCl

fluid contained a significantly lower level of Na^+ (18.4 ± 1.3 mM) and lower, but insignificant, levels of other ions as K^+ (1.9 ± 0.6 mM), Ca^{2+} (0.6 ± 0.2 mM) and Mg^{2+} (0.5 ± 0.1 mM) (Fig. 2).

Swimming parameters at initiation of sperm motility

Direct observation of undiluted sperm by dark field microscopy showed that most spermatozoa in the seminal plasma of tench were motile for less than 60 s. Many of the sperm cells were not translated even though flagella transiently showed propagated curvatures along the flagella at very low frequencies (1 Hz or lower), so-called 'shivering'. Right after activation by transfer to distilled water, the motility of tench spermatozoa presented the following characteristics: the motile spermatozoa percentage was from 90 to 100% (Fig. 3), velocity was 140 $\mu m\ s^{-1}$ (Fig. 4), beat frequency was 65 Hz s^{-1} (Fig. 5) and the period of motility was up to 80 s. The effects were tested of a SM composed of 30 mM KCl (Figs 3 and 4) and 45 mM KCl (Fig. 5). After sperm dilution in such SM, a majority of the samples showed 95% motility with a flagellar beat frequency of 70 Hz within the first 20 s and a period of motility up to 180 s. The spermatozoa of tench showed in such a SM a lower velocity within 10 s, 120 $\mu m\ s^{-1}$, but significantly higher velocity within 20, 40, 60 and 80 s, compared with values in distilled water. After activation of tench spermatozoa with the SM, the time of motility was prolonged up to two to three times. ANOVA showed a significant influence of the SM ($P < 0.001$) on percentage of sperm motility, velocity of spermatozoa and flagellar beat frequency.

From the comparison of resistance of the tench spermatozoa swimming ability in various media, it appears mostly at activation that sperm cells are exposed to a drastic environment, i.e. low osmolality compared with that of the seminal fluid. In this respect, the activation in distilled water is certainly the most harmful, because of its lowest osmolality. After transfer for a short period (20 s) in distilled water, blebs are observed to appear along the flagellum, which prevents correct and efficient waves to propagate along the flagellum. At later periods in the progress of the motility phase (30 s), the flagella tips become curled and present a loop which shortens the efficient part of the flagellum and restricts waves to one-third or one-fourth the flagellar length. These defects are virtually not detected for flagella of spermatozoa induced to swim in a solution containing 30–45 mM KCl.

bladder of tench had approximately the same
ıt of tilapia *O. mossambicus* (Linhart et al., 1999),
r than that of European catfish (Legendre et al.,
ch, urine osmolality is the key factor for activation
zoa, as it is one to two times lower than the
seminal fluid. Negative effects of sperm contam-
urine on the sperm quality have been observed in
Cyprinus carpio (Perchec et al., 1995) and turbot
maximus (Suquet et al., 1998). However, urine
trigger spontaneous sperm movement not only in
ossambicus (Linhart et al., 1999), but also in
tfish (Linhart et al., 1987), in asp *Aspius aspius*
Benešovský, 1991), in common carp (Poupard
in paddlefish *Polyodon spathula* (Linhart et al.,
others. Osmolality of urine in other freshwater
as common carp, tilapia and European catfish
and 50 mOsmol kg^{-1}, respectively; Linhart et al.,
Poupard et al., 1998) was found to be lower in
with tench.

ty parameters (frequency, velocity) evolve very
g the motility period as seen in Figs 2–4. Present
provide a more detailed analysis of sperm
ameters compared with older methodologies of
ty evaluation (e.g. Zuromska, 1981; Moczarski
1982; Zuromska and Markowska, 1984; Linhart
Linhart and Kvasnička, 1992). During the earliest
ı spermatozoa move at velocities of 120–140 μM
Thereafter, the forward motility of the spermato-
y slows to values of 50–100 μm per second at
asionally some spermatozoa swim up to 3 min.
vior was previously described in trout *O. mykiss*
l., 1989), European catfish (Billard at al., 1997),
p (Perchec et al., 1993), *Cottus gobio* (Lahnsteiner
perch *Perca fluviatilis* (Lahnsteiner et al., 1995),
eon species (Cosson et al., 1995; Tsvetkova et al.,
ı et al., 2000), paddlefish (Linhart et al., 1995b;
, 2000; Linhart et al., 2002), sea bass *Dicentrachus*
anno et al., 1999), turbot *Scopthalmus maximus*
t al., 1995), and tilapia *O. mossambicus* (Linhart
Nevertheless, this decrease in swimming perfor-
ost species finds its roots partly in the parallel
nergy stores observed during the motility period.
f tench, however, it was noted that a small part of
pulation can swim for long periods of time, up to
arski and Kodras, 1982) and 161–188 s (Linhart
Linhart and Kvasnička, 1992). These motility
nch were probably obtained for only a small
ıe spermatozoa population which was asynchro-
ered for motility and which could probably not
ıctual period of motility for the entire sperm
Also it is worth emphasizing that in our experi-
sperm samples that show more than 90% of the
which synchronously activate movement are
experiments.

ined on tench sperm motility, swimming velocity,
capability and subsequent hatching rate (Rodina
showed that only sperm collected in the various
successfully used for artificial insemination and
p to 10 h at 0–4°C (Linhart and Billard, 1995;
Rodina et al., 2002). The IS is used to counteract the co-occurring sample contamination by urine, causing poor fitness of spermatozoa and resulting in impaired motility and reduced swimming speed, with subsequent effects on development (Rodina et al., 2002). The best tench IS was found to be Kurokura 180 (180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2 \cdot 2H_2O$ and 2.38 mM $NaHCO_3$; Rodina et al., 2002). During stripping of tench males, sperm must be sampled into a syringe containing IS, allowing to maintain the IS:sperm ratio at 2 : 1 under aerobic conditions and at 0–4°C while at the same time minimizing contact with urine. Sperm can be stored in such IS for 10 h and then used as usual for artificial insemination of eggs.

Acknowledgements

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Kurokura solution as immobilizing medium for spermatozoa of tench (*Tinca tinca* L.)

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Abstract. This study tested KUROKURA solution (Kurokura et al., 1984, Aquaculture 37, 267–274) and its modifications (by increasing NaCl content to 160, 180 and 200 mM) on immobilizing properties for sampling and short-term preservation of potential motility of tench spermatozoa. The immobilizing solution is used because, when collected, the sperm of most samples is contaminated by urine, causing spermatozoa to be of poor quality, with low motilities and velocities (almost 0), thus resulting in a worsened fertilization and hatching rate. Sperm was sampled with a syringe containing an immobilizing solution (IS), allowing an IS:sperm ratio of 2:1, under aerobic conditions at 0–4°C. This sperm solution was stored for 10 h and untreated sperm was collected prior to fertilization as a control. Spermatozoa quality was evaluated for the cell motility and velocity parameters and also for fertilization ability and hatching rate. Results obtained for tench sperm motility, velocity, fertilization and hatching rate showed that only sperm collected in the various immobilizing solutions can be successfully used for artificial insemination and preservation after 10 h at 0–4°C. The best immobilizing solution was found to be KUROKURA 180 (180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2 \cdot 2H_2O$ and 2.38 mM $NaHCO_3$), giving a fertility and hatching rate of 41%, with no change in rates after 10 h storage of sperm. Control sperm without immobilizing solution showed a fertilization and hatching rate of only 6–7%.

Key words: Aquaculture, Fish, Immobilizing solution, Motility, Spermatozoa, Tench, *Tinca tinca*

Introduction

When sampled, various concentrations of urine usually contaminate sperm of tench and cause decreased spermatozoa motility, thus resulting in bad/low fertilization rate. Without special care, tench sperm cannot be stored even for short-terms without use of immobilizing solution. Linhart and Billard (1995) and Linhart et al. (2003) previously described this phenomenon. Urine-contaminated sperm has also been observed in many other fish species such as common carp (*Cyprinus carpio* L.; Poupard et al., 1998), European

catfish (*Silurus glanis* L.; Legendre et al., 1996), asp (*Aspius aspius* L., Linhart and Benesovsky, 1991), turbot (*Psetta maxima*, Dreanno et al., 1998, 2000), Atlantic salmon (*Salmo salar*, Maisse et al., 1998), rainbow trout (*Oncorhynchus mykiss*, Glogowski et al., 2000), Mossambic tilapia (*Oreochromis mossambicus*, Linhart et al., 1999), etc. Usually, the low osmolality of urine is seen to be responsible for the degradation in the spermatozoa quality of common carp (Perchec et al., 1995), tench (Linhart et al., 2003), European catfish (Billard et al., 1997), Mossambic tilapia (Linhart et al., 1999), pejerrey (*Odontesthes bonariensis*, Strussman et al., 1994), etc. Maisse et al. (1998) proposed that the high magnesium content of urine of Atlantic salmon could be responsible for motility activation of the sperm, leading to a low ATP content and a low ability to fertilize. In order to avoid such contamination with urine and therefore to maintain motility and fertilization capacity of sperm, a catheter has been used for sperm collection in rainbow trout (Glogowski et al., 2000) and in ocean pout (*Macrozoarces americanus* L., Yao and Crim, 1995) or, alternatively, the urinary bladder is emptied prior to sperm stripping, as described for turbot by Dreanno et al. (1998) and for European catfish by Linhart et al. (1987). Other techniques for preservation of sperm quality and prevention of degradation by urine include direct addition of contaminated sperm to immobilizing solution or artificial seminal fluid, as detailed for European catfish (Linhart et al., 1987; Saad and Billard, 1995), for catfish *Pangasius bocourti* (Cacot et al., 2003), in tench (Linhart and Kvasnicka, 1992; Linhart and Billard, 1995) and in turbot (Chereguini et al., 1997).

The motility of tench spermatozoa is partly controlled by osmotic pressure (Linhart et al., 2003), similar to common carp (Redondo-Müller et al., 1991) and marine fish (Morisawa and Suzuki, 1980), but little is known about the way in which tench spermatozoa motility is controlled by concentration of different ions. After dilution in hatchery water, the average motility time of tench spermatozoa was 25–52 s for the mass progressive motion (Moczarski and Koldras, 1982; Linhart et al., 1986) and up to 93 s for progressive motion (Zuromska, 1981). The total duration of motility was 161–210 s, as reported by Moczarski and Koldras (1982) and by Linhart et al. (1986) and it decreased during the spawning season (Zuromska, 1981; Zuromska and Markowska, 1984). Recently, using image microscopy analysis for evaluation of spermatozoa motility, tench spermatozoa have been shown to present the following characteristics: velocity of 120–140 $\mu m\ s^{-1}$ with beat frequency of 63–65 $Hz\ s^{-1}$ at 10 s after activation and period of motility up to 80 s (Linhart et al., 2003).

The main aims of the present study were to examine the effects of KUROKURA solution and of its variants as immobilizing solutions for sampling and preservation of tench spermatozoa.

Material and methods

The experiment was performed at the experimental hatchery of the University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Department of Fish Genetics and Breeding at Vodnany, Czech Republic. After transfer from ponds to the hatchery, mature males and females were initially separated in tanks at 20–23°C. Artificial propagation followed methods described by Linhart and Billard (1995) and Linhart et al. (2003). During the reproductive season (June), eggs and sperm were individually collected from three and seven mature 5-year-old tench females and males (400–700 g body weight), respectively. Broodstock was assessed for maturity by means of abdominal compression and spermiation was detected by sperm production. Males were treated with single intramuscular injection of carp pituitary, 1 mg kg^{-1} body weight. Females were stimulated for ovulation 30 h prior to egg stripping by injection of GnRH analogue [D-Ala^6, GnRH ProNHEt, Kobarelin] at a dose of 5 μg kg^{-1} b.w. Before injection and gamete collection, males and females were anesthetized in a solution of 2-phenoxyethanol (1:1000).

Sperm of each male (7 males in total) was sampled with a syringe filled with various modifications of KUROKURA immobilizing solution (IS; Kurokura et al., 1984) with NaCl concentration of 128, 160, 180 and 200 mmol l^{-1} (mM) (Table 1), maintaining the volume ratio of IS:sperm as 2:1. Directly after sampling, the motility of spermatozoa was videorecorded (Linhart et al., 2002). Then the sperm was stored for 10 h on ice at 0°C under aerobic conditions (1 part of sperm or sperm with IS and 1 part of air) and spermatozoa motion was recorded again. Prior to the fertilization experiments, fresh sperm was again collected from the same 7 males and spermatozoa motility was again recorded. Sperm without any IS was used as control and also stored at 0°C under aerobic conditions. Sperm concentration was evaluated Thoma haemocytometer under Olympus BX50 microscope

Table 1. Composition of KUROKURA solutions. Unmodified solution was KUROKURA 130 (Kurokura et al., 1984). In other KUROKURA solutions concentration of NaCl was modified

Solutions	NaCl (mmol l^{-1})	Other components (mmol l^{-1})	Osmolality measured (mmol kg^{-1})
KUROKURA 130	128.34		235
KUROKURA 160	160	KCl 2.68	305
KUROKURA 180	180	$CaCl_2 \cdot 2H_2O$ 1.36	343
KUROKURA 200	200	$NaHCO_3$ 2.38	381

(200×), and average concentration of spermatozoa was calculated from 20 square Thoma cells. The osmolality of IS was measured using a Vapour Pressure Osmometer (Wescor, USA) and expressed as mOsmol (mmol) kg^{-1}.

Observation of sperm motility and velocity

Spermatozoa were evaluated for cell motility and velocity. Measurements were taken using dark field microscopy and a Sony camera setup as described by Billard et al. (2000), Cosson et al. (2000) and Linhart et al. (2002). Cell activity was examined under 200× magnification immediately after mixing 0.5 µl of sperm with 49.5 µl of swimming medium (SM = 40 mM NaCl + 0.1% BSA), on a glass slide prepositioned on the microscope stage. The final dilution was 1:300. Within 10 s after mixing, a video recording was made for 2-min to be used in the evaluation of spermatozoa swimming activity. The focal plane was always positioned in the vicinity of the glass slide surface. The movements of spermatozoa were recorded at 25 frames s^{-1} using a CCD video camera (SONY) mounted on a dark-field microscope (Olympus BX 50, Japan) illuminated with a stroboscopic lamp of Strobex (Chadvick-Helmut, 9630, USA) and visualized on a video monitor. The adjustable frequency stroboscopic flash illumination was set in 50 Hz for sperm velocity measurement.

Evaluation of the velocity and percentage of motile spermatozoa

The successive positions of the recorded sperm heads were measured from video frames using a video-recorder (SONY SVHS, SVO-9500 MDP) at each 15 s after activation from three frames (25 frames s^{-1} were recorded) analyzed by the image analyzer Olympus Micro Image v. 4.0.1. for Windows with a special macro created by Olympus C & S s.r.o., Czech Republic. The velocity and percentage of movement were measured by evaluating spermatozoa head positions on three successive frames with three different colors (blue, green and red). The analyses were repeated three times at 10-s intervals, that is, frames 1–3, then frames 11–13, and finally from frames 21–23. Thirty to forty spermatozoa were evaluated in each frame. Every actively moving spermatozoon was identified as a track in three colors, while non-moving spermatozoa were white. Percentage of motile/non-motile spermatozoa was easily calculated from the ratio between the number of red tracks versus the sum of white plus red tracks. Velocity of each spermatozoon was calculated only from motile spermatozoa as µm s^{-1} based on length traces of spermatozoa from blue to green and red heads, after calibration for magnification. Both values were automatically calculated with Excel'97.

Evaluation of hatching rate with 5000 spermatozoa per 1 egg

Sperm stored for 10 h as well as sperm freshly collected prior to fertilization was used for the experiment. One gram of eggs (1600 eggs per 1 g; Linhart and Billard, 1995) were placed in a dish of 20 ml. An accurate volume of experimental sperm with 5000 spermatozoa per 1 egg was dropped on eggs with a micropipette. The dish was then placed on a shaking table with constant 200 rpm and with 10-mm deflection. An accurate volume of 1 ml of dechlorinated tap water at 22°C was added. Two minutes later, approximately 200 eggs were placed in a special incubator cage of 200 ml supplied with UV sterile recirculated and dechlorinated tap water at 23°C, 9 mg l^{-1} O_2. For each experiment, the procedure was replicated four times. Dead eggs were counted and eliminated 24 h after fertilization and used for calculation of fertilization rate. Number of hatched and the total number of eggs that died during incubation in cages were counted 4 days after fertilization and results were used for calculation of hatching rate.

The percentage of fertilization rate (F_r) was then calculated for each cage from the total number of eggs placed in the cage (E_t) minus dead eggs (E_d) collected up to 24 h after fertilization as follows:

$$F_r = \left(\frac{E_t - E_d}{E_t}\right) \cdot 100$$

The percentage of hatching rate (H_r) was also calculated for each cage from the total number of eggs placed in the cage (E_t) and divided with number of hatched larvae (H_l) as follows:

$$H_r = \left(\frac{H_l}{E_t}\right) \cdot 100$$

Data analysis

The data acquired from the three/four replications were evaluated as means with SD and statistical significance was assessed using multiple analysis of variance (ANOVA, Statgraphics version 5), followed by multiple comparison Tukey HSD range tests. Probability values <0.05 were considered significant.

Results

The evolution of spermatozoa velocity and of the rate of motile spermatozoa during 120 s post-activation is shown in Figures 1 and 2. Fresh sperm in immobilizing KUROKURA 180 solution, containing 180 mM NaCl showed

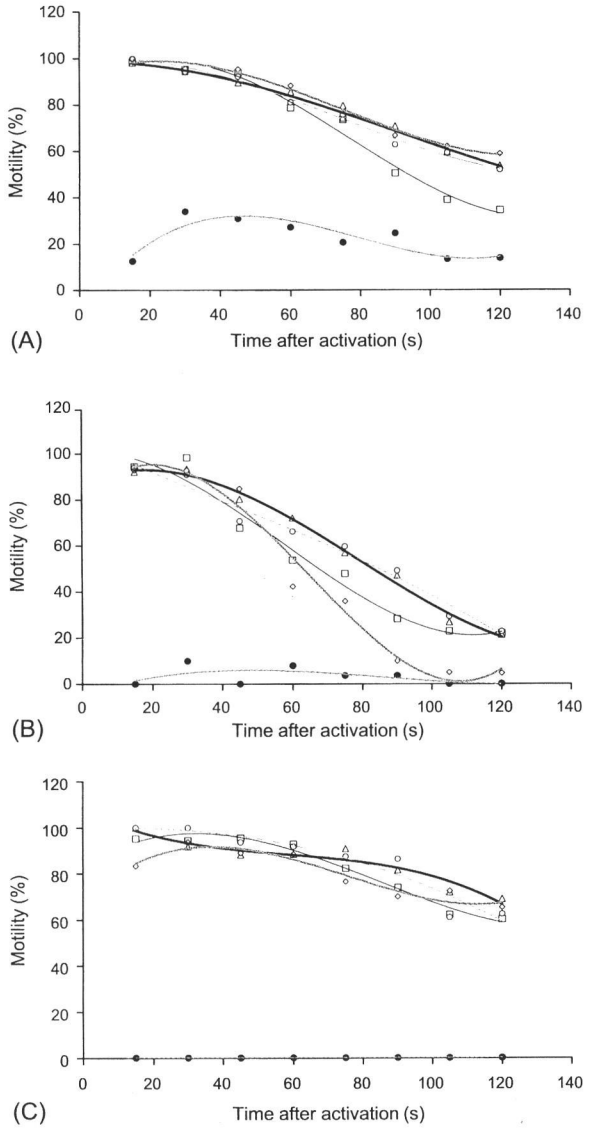

Figure 1. Motility of tench spermatozoa at percentage: (A) – initial fresh sperm; (B) – sperm after 10 h preservation in immobilizing solution; (C) – the second fresh sperm sampled prior to the fertility test. Sperm without IS was used as control. Coefficient of mean variability was 0–7.9%. (•) Control; (□) KUROKURA 130; (△) KUROKURA 160; (◊) KUROKURA 180; (○) KUROKURA 200.

high motility rates from 100% at 15 s after activation to 60–80% after 120 s. Sperm stored for 10 h in immobilizing KUROKURA 180 solution showed a decrease in spermatozoa motility to 20% at 120 s after activation. Use of

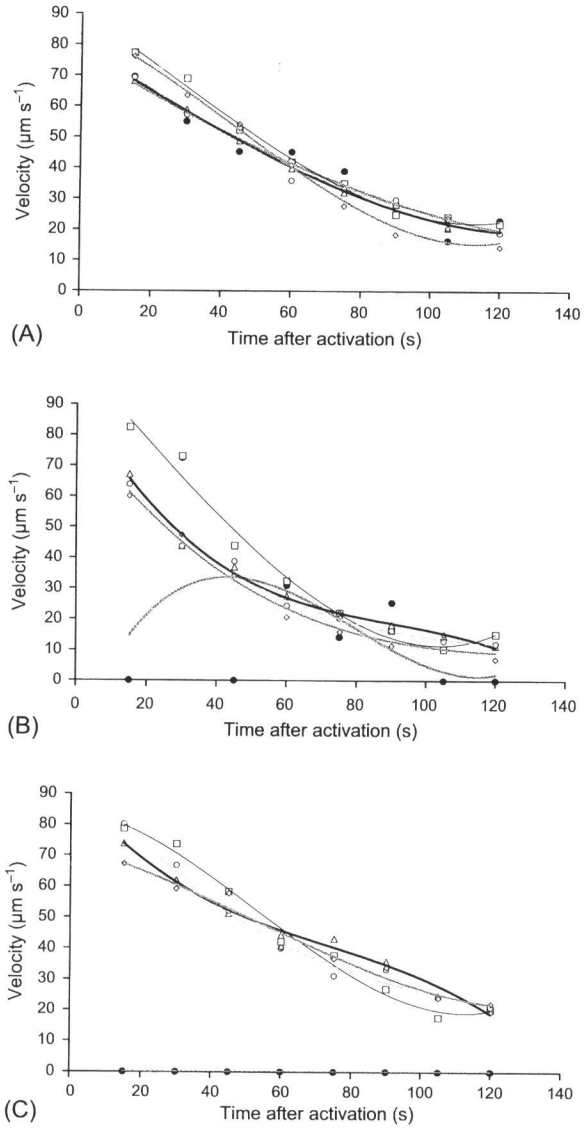

Figure 2. Velocity of tench spermatozoa at $\mu m\, s^{-1}$: (A) – the initial fresh sperm; (B) – sperm after 10 h preservation in immobilizing solution; (C) – the second fresh sperm sampled prior to the fertility test. Sperm without IS was used as control. Coefficient of mean variability was 0–75.2%. (•) Control; (□) KUROKURA 130; (△) KUROKURA 160; (◊) KUROKURA 180; (○) KUROKURA 200.

the same immobilizing solution with fresh sperm revealed velocity at a level of 70 $\mu m\, s^{-1}$ at 15 s after activation and 20 $\mu m\, s^{-1}$ at 120 s after activation. Sperm stored for 10 h in immobilizing KUROKURA 180 solution also

showed a decrease in velocity to $10\,\mu m\,s^{-1}$ at 120 s after activation. In all cases fresh sperm or sperm stored without any immobilizing solution exhibited very low motility rates (0–20%) and very low velocity. The ANOVA showed highly significant effects of the sperm preservation method (with or without IS), of preservation medium and time of records after activation on spermatozoa motility and velocity ($P < 0.0001$). Modifications of the IS composition did not lead to significantly different values of motility and velocity. The only significant difference in spermatozoa motility was found in the control sample (sperm without IS) recorded in both the fresh sperm and sperm stored for 10 h. The spermatozoa velocity was significantly different between the first fresh sperm and sperm stored for 10 h. Sperm preserved for 10 h showed significantly lower spermatozoa motility and velocity ($P < 0.0001$) than fresh sperm.

Results of fertility and hatching test are shown in Figures 3 and 4. No significant effect of sperm preservation on fertilization and hatching rate was observed but individual modifications of the IS composition were significantly

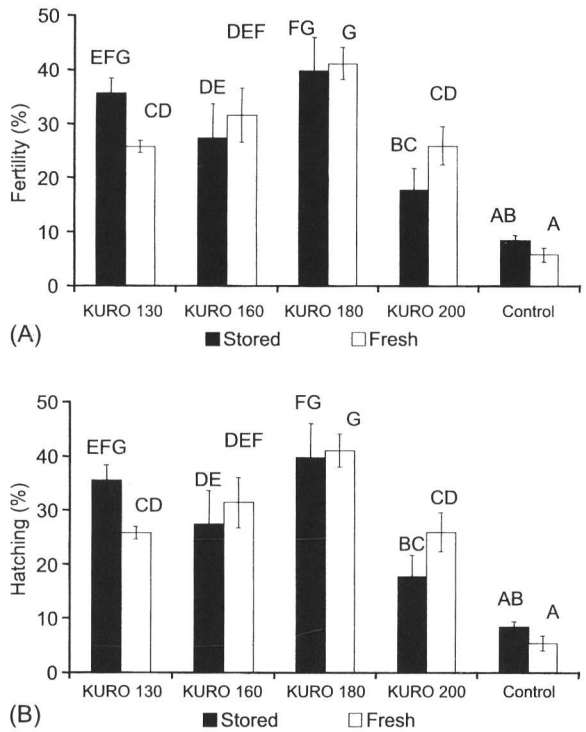

Figure 3. Fertilization rate (A) and hatching rate (B) with SD of individual KUROKURA IS (KURO), for sperm stored 10 h and for fresh sperm. Sperm without immobilizing solution was used as control. Groups with a common superscript do not differ significantly ($P < 0.05$).

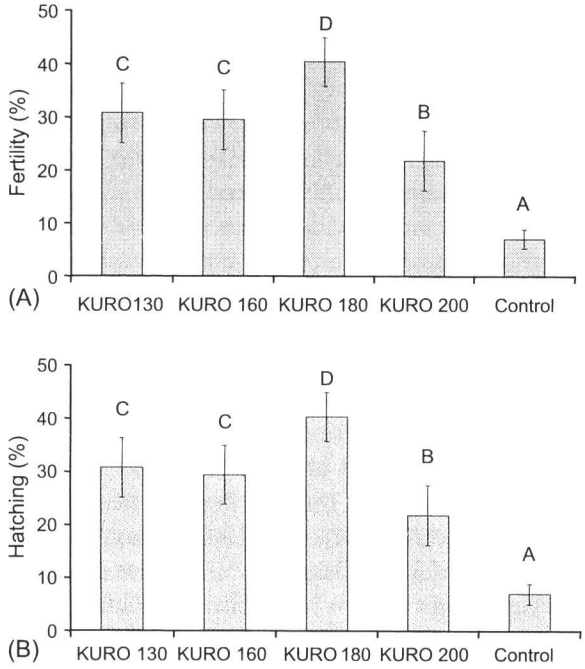

Figure 4. Fertilization rate (A) and hatching rate (B) with SD of individual KUROKURA IS (KURO) with sum of sperm stored 10 h and fresh sperm. Sperm without immobilizing solution was used as control. Groups with a common superscript do not differ significantly ($P < 0.05$).

different ($P < 0.0001$). KUROKURA 180 was observed as the best immobilizing solution leading to fertility and hatching rate of 41% without changes after 10 h storage of sperm. The sperm without immobilizing solution showed a fertilization and hatching rate of only 6–7%.

Altogether, our results on tench spermatozoa motility, velocity, fertilization and hatching rate showed that only the sperm saved in immobilizing solution can be successfully used for artificial insemination and for preservation for 10 h at 0–4°C. KUROKURA solution with concentration of 180 mM NaCl was considered to be the most positive modification with a relationship between sperm motility and velocity (during first period of movement) and fertilization/hatching rate.

Discussion

The artificial propagation of tench has been recently improved by techniques developed in the Czech Republic; currently, the fry of this species is primarily

produced in hatcheries. Nowadays, the artificial insemination of tench under the conditions of Czech traditional fish farming is a very rapid procedure; the use of the milk/clay treatment for elimination of egg stickiness takes almost 1 h while this is reduced to 5 min when using enzymatic elimination of egg stickiness, and the hatching rates increase (Linhart et al., 2000). Other methods, such as semi-artificial reproduction or natural reproduction (Horvath et al., 1984), are not used in Czech farming. The weakest point of artificial reproduction of tench is a poorly controlled hatching rate, varying from 30 to 90%, mainly caused by the low quality of sperm due to contamination by urine. In tench, the low osmolality of urine is the main key factor for spontaneous activation of spermatozoa (Linhart et al., 2003), as urine exhibits osmolality down to half of that of seminal fluid. Osmolality of contaminated tench sperm was re-equilibrated successfully during our experiment, with a storage potential of 10 h, thanks to the KUROKURA 180 immobilizing solution containing 180 mM NaCl. The total concentration of soluble compounds in the KUROKURA 180 solution was around 190 mM leading to an osmolality of 343 mOsmol kg^{-1}. Similar osmolality at a level of 340–400 mOsmol kg^{-1} was used in immobilizing solution (artificial seminal fluid/storage solution) for European catfish (Saad and Billard, 1995; Linhart et al., 1987), catfish *Pangasius bocourti* (Cacot et al., 2003), asp (Linhart and Benesovsky, 1991) and turbot (Chereguini et al., 1997). For all species, the major ions present in the immobilizing solution are Na^+ with addition of K^+, Ca^{++} and eventually Mg^{++} to allow recovery of the potential energetic content used by the motility apparatus. Fish spermatozoa cannot sustain for long periods of time the high-energy requirement of ATP necessary for their very rapid motility, and for the latter reason the motility of, for example, rainbow trout spermatozoa lasts for 30 s as a maximum. The decrease in velocity of spermatozoa as well as that of the beat frequency of flagella wave motion corresponds to the decrease of intracellular ATP concentration. In *in vitro* studies of demembranated spermatozoa, using an ATP concentration similar to that present inside cells at the initiation period, it was possible to maintain a high flagella beat frequency up to 30 min (Billard and Cosson, 1990), showing that the machinery itself is able to sustain very long periods of activity. The capacity of mitochondria to supply energy is too slow relative to the consumption by the motility mechanics of a fish spermatozoon during its motility phase: this is consistent with results showing that respiration is at the same level before and after initiation of motility in rainbow trout spermatozoa (Christen et al., 1987). Energy to be used for the needs of flagella motion is generated mostly from the ATP accumulated in the flagellum before the onset of activation. After cessation of motility, initial values of intracellular concentration of ATP can be regained after 15 min incubation in a non-swimming

solution. Respiration of mitochondria can be experimentally blocked by cyanide (Christen et al., 1983), despite the observation that the same decrease in phosphocreatin level occurred after the same period of motility as it was in absence of respiration blockage (Robitaille et al., 1987). This means that the amount of phosphocreatin needed to cover the ATP degradation never sufficiently meets the ATP requirement necessary to balance the need during the motility period.

The principle of arrest of the spermatozoa motility in rainbow trout is certainly more complicated than a simple exhaustion of the ATP stores. In fact, the intracellular ATP level is not exhausted at the arrest of the flagella motion: a beat wave frequency of 20 Hz is measured right before arrest and an intracellular ATP concentration of 0.5–1 mM still remains. Nevertheless, the wave motion of the flagellum fully stops 1–2 s later. This sudden stop occurs exactly when a high level of intracellular calcium is observed, the latter causing the sudden decrease of flagella frequency to zero while the intracellular ATP level is maintained uselessly high (Billard and Cosson, 1990). In tench spermatozoa a similar explanation of potential for energy recovery for spermatozoa motility probably applies. This would explain that the role of the IS (e.g., KUROKURA 180 in tench) is not only to stop movement of spermatozoa spontaneously activated by urine, but also to allow the recovery of the energetic stores. This prediction would explain why tench spermatozoa do not really need energetic organic components in IS (as it is the case in mammalian sperm) because the motility period of tench spermatozoa is very short.

Conclusion

With tench spermatozoa a high level of motility, velocity, fertilization and hatching rate were obtained only when sperm contaminated by urine was added to an immobilizing solution. Immobilizing KUROKURA 180 solution can be used as IS for sampling of tench sperm and for its preservation for 10 h at 0–4°C.

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ract

aim of the present study was to elaborate cryopreservation methods for *ex situ* ervation of tench. Success of cryopreservation was tested during two series of iments:

irst set of experiments studied the effects of two types of cryoprotectants (DMSO and a ination of DMSO with propanediol at ratio 1:1) at concentrations of 8 and 10 % and different equilibration times in 2 different immobilization solutions (IS) (Kurokura 180 Kurokura) before freezing (0.0, 2.0 and 4.0 h after T_0). The K4 cooling programme was to freeze 1 ml of cryoextended sperm using 1.8 ml cryotubes. Main monitored parameter natching rate after using of cryopreserved sperm.

second set of experiments studied the volume effect of 0.5, 1 and 5 ml straws and pared these with 1.8 ml cryotubes as well as the effect of the cooling programme (K4 and Following the results of the first study, a combination of DMSO and propanediol (ratio at concentration of 10% was added to extended sperm in Kurokura 180 IS. Main itored parameter was hatching rate after using of cryopreserved sperm, supplementary meters were sperm velocity and motility percentage assessed at 10 s post activation.

m was collected directly into IS and stored at 4 °C for 2.5 h. Thereafter were sperm les pooled, equlibred in IS (first set of experiments) or directly mixed with protectants (DMSO or a mixture of DMSO with propanediol at ratio 1:1) and transferred 8 ml cryotubes or straws (0.5, 1 and 5 ml) . Then the cryotubes/straws were directly sferred to pre-programmed PLANER Kryo 10 series III and cooled using two different ing programmes including a slow cooling programme (a) named K4 (from +4 °C to -9 °C rate of 4 °C min^{-1} and then from -9 °C to –80 °C at a rate of 11 °C min^{-1}) and a rapid ing programme (b) named L1 (directly from +4 °C to -80 °C at a rate of 20 °C min^{-1}). slow (K4) and rapid (L1) cooled samples were held 6 min at -80 °C. Finally, samples

ransferred into liquid N_2. The frozen spermatozoa were thawed in a water bath (40 °C) ling to the frozen volume and checked for fertilization and hatching rates. Percentage of motility and sperm velocity were measured using video recorded frames. ANOVA d a significant influence of frozen and fresh sperm in all treatments. The hatching rates 8 % were obtained when sperm was equilibrated for 0 h before freezing in IS of ura 180 and frozen with a 10 % of mixture 1:1 of DMSO and propanediol into straws of and cooled using program L1. The velocity of frozen-thawed spermatozoa ranged from 46 $\mu m.s^{-1}$ and in post-thawed sperm was not significantly different according to frozen volume, but a higher velocity was obtained when sperm was fast frozen using amme L1. A large volume of frozen sperm could reveal the best procedure for freezing, lso for simulating methods of artificial propagation for future practical use of frozen sperm at a large scale.

***words*:** Cryopreservation, hatching rate, Tench *Tinca tinca*, sperm motility

duction

n, *Tinca tinca* (L.) are small members of the family Cyprinidae inhabiting fresh water. urrent distribution of tench extends throughout Europe (except the north of Scotland, ern Scandinavia, the Crimea and the western Balkan peninsula) and Asia (up to the asus Mountains, river Yenisey in Siberia and North-western China) (38,4). Interest in oping aquaculture of tench is growing; the total production was 2402 t in 1990 and grew 4984 t in 2004 (FAO 2006). Tench are mostly produced in polyculture, either in small arge ponds.

the 1970s, some rearing trials have been conducted to develop aquaculture techniques nch at the Research Institute of Fish Culture and Hydrobiology at Vodňany. In this l, several topics in tench breeding and cultivation were investigated including selective ing and a testing programme (16), genetic resources of breeds as a part of the Czech al heritage (11), a gene resource bank of cryopreserved sperm of different breeds (18), te biology (17,25,28,35), artificial reproduction under hatchery conditions 4,29,12,7) as well pond as aquaculture management (29,13) for effects on fish survival rowth.

preservation of sperm is considered as a valuable technique for the artificial duction and genetic improvement as well as for biological conservation programmes (39, sh sperm cryopreservation has been well established for many years in more than 200 pecies (6). Nevertheless, because of clear intra- and inter-species differences observed in haracteristics (1), some key parameters (e.g. ionic composition and osmolality) should nsidered when cryopreservation methods for sperm are developed. There are no data to e optimum procedure in the case of tench sperm.

notility of tench spermatozoa is inhibited by the high osmolality present in the seminal a (230±82 mOsmol Kg^{-1}, (25)); exposure to hypo-osmotic media triggers sperm motility

5). But spontaneous movement of tench sperm was reported due to contamination by
at osmolalities down to half of that in seminal fluid (85±58 mOsmol Kg^{-1}, (25,29)).
ıer studies revealed that the spontaneous sperm activation could be prevented using an
obilizing solution containing ionic components with osmolality higher than 230 mOsmol
(35). Contamination of sperm by urine also creates a problem for storage of sperm even
short time (19,25). Recent results revealed higher percentages of sperm motility, sperm
city and fertilizing ability of tench sperm collected in immobilizing solution compared to
ı without immobilizing solution, whether or not the sperm was fresh or stored for a short
(35). On the other hand, there were small and non-significant differences between fresh
short time stored sperm after collection in different types of immobilizing solution.
zarski and Koldras (1982) reported results of cryopreservation trials on tench sperm
cted from an induced spermiation (2 mg of carp pituitary homogenate per kg body
ht) after diluting into a phosphate buffer (ratio: 1:1 or 1:2) containing 15% ethylene
ol. Five minutes post equilibration, to balance the osmotic pressures by allowing the
sion of a protective substance inside the spermatozoa, samples were frozen at -80 °C and
transferred to liquid nitrogen. Six hours post storage, the frozen sperm samples were
ed in 30-40°C water baths and motility rate was evaluated. A very low sperm motility of
n-thawed samples was observed, but no test was carried out to study the fertilizing
y of frozen-thawed sperm and success in hatching of embryos.

aim of the present study was to optimize cryopreservation methods for *ex situ*
ervation of tench sperm by manipulation of the immobilizing solution (IS), storage time
erm in the IS, composition of the cryoprotectant, the cryoprotectant: sperm ratio (volume
eezing sperm) and the freezing programme. Cryopreservation success was evaluated by
ı motility parameters (velocity and percentage of motile sperm) and hatching rate after
ınination of tench eggs with frozen/thawed sperm.

erials and methods

experiments were carried out on tench broodstock at the University of South Bohemia, arch Institute of Fish Culture and Hydrobiology in Vodnany, Czech Republic.

dstock handling: Sperm and egg collections

to seven-year-old broodstock (400-700 g body weight, Hungrian strain) were caught breeding ponds in April, brought into the hatchery, kept in two natural ponds (0.01 ha), with pelleted feed for carp and monitored for sexual development by means of abdominal pression. Fish were separated by sex.. Those fish exhibiting gonadal maturity were duced to spawning induction. Spawnable broodstock were selected in June and held in ndoor flow-through tanks (4000 l), supplied with irrigation water (temperature 18-22 nd O_2 6-7 $mg.l^{-1}$) at a flow rate of 0.2 $l.s^{-1}$.

miation was induced by a single intramuscular injection of 1 $mg.kg^{-1}$ body weight of carp tary extract dissolved in physiological solution. Ovulation was also induced by muscular injection of 5 $\mu g.kg^{-1}$ b.w. of GnRH analogue [D-Ala^6, GnRH ProNHEt, relin]. Before each injection and gamete collection, the fish were anaesthetized in a ion containing 2-phenoxyethanol (1:1000).

y hours post injection; ovulated eggs stripped from 5 females were pooled and stored at a erature of 17-18 °C until fertilization experiments (approximately 1 hour). Prior to the ization experiments, three batches of approximately 0.2 g (around 300 eggs) of pooled rtilized eggs were weighed to the nearest 0.0001 g and fixed in 4 % formaldehyde for counting and determination of mean egg weight. On the basis of this, the number of eggs sample was expressed as the weight of the sample (g) multiplied by 1600.

m was collected individually from the genital papilla from each of 40 males into 5 ml ges containing 2 ml of different immobilization solutions (IS); Kurokura 180 composed 80 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$ and 2.4 mM $NaHCO_3$ (35) and Kurokura

osed of 128.4 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$, 2.4 mM $NaHCO_3$ (15). To nt spontaneous initiation of motility, not more than 1.0 ml of sperm was taken into one ge to keep dilution rate 2:1 (IS: sperm) (29,35). The quality of stripping samples was ated after inducing sperm motility in freshwater. Thirty males out of forty showed natozoa motility higher than 80 %. These samples selected for further experiments were l under aerobic conditions on ice at 4 °C for 2.5 hours (time from collection to pooling) hen samples were pooled at zero time (T_0). Sperm concentration was determined by ing cells in physiological solution at a final dilution of 1: 1000 by means of a Burker cell ocytometer under an Olympus microscope BX 41 (400x). The mean number of natozoa counted in 16 squares of the Burker cell was finally expressed as number of natozoa per unit volume.

preservation and Thawing

erent concentrations of pure cryoprotectant (%, v/v) or mutual combinations (1,2-anediol P-1009, Sigma; Me_2SO-DMSO – Sigma kat. no D-8779) were added to extended m and every 0.5-5 ml of mixture was transferred into 1.8 ml cryotubes (Nunce, 375418) raws (0.5ml - IMV AA101; 1 ml – Koh-i-noor 30.6; and 5 ml IMV -AA303). Then the tubes/straws were directly transferred to a pre-programmed PLANER Kryo 10 series III cooled using two different cooling programmes including (a) a long cooling programme ed K4 (from +4 °C to -9 °C at a rate of 4 °C min^{-1} and then from -9 °C to –80 °C at a rate 1 °C min^{-1}) and (b) a rapid cooling programme named L1 (directly from +4 °C to -80 °C at e of 20 °C min^{-1}) (Figure 1). Both long (K4) and rapid (L1) cooled samples were held 6 at -80 °C. Finally, samples were transferred into liquid N_2. To study sperm parameters Experimental Design), the sperm was thawed in a water bath at 40 °C for 105 s (2 ml tubes), 8 s (0.5 ml straw), 20 s (1ml straw) and 35 s (5 ml straw). A part of the pooled m in IS kept on ice (0- 4°C) for 19 h was used as a control (unfrozen sperm).

rimental Design

pools of sperm and eggs were used for all experiments. For each treatment, 6 cryotubes raws containing the same volume of sperm were cryopreserved. The sperm parameters ding sperm velocity, percentage of sperm motility, fertilization as well as hatching rates evaluated after 19 h in case of frozen sperm. Two series of experiments were established llows:

ts of IS, storage time and cryoprotectant

first studied the effects of two different IS, two types of cryoprotectants (DMSO and a bination of DMSO with propanediol at ratio 1:1) at concentrations of 8 and 10 % and different equilibration times in IS before freezing (0.0, 2.0 and 4.0 h after T_0). Both IS okura 180 and Kurokura) were used at ratio 2:1 (IS: sperm). The K4 cooling programme used to freeze 1 ml of cryoextended sperm using 1.8 ml cryotubes. Four nonfrozen sperm rols were used: controls 1 (collected to Kurokura and Kurokura 180, used for freezing, d 19 h on ice at 0-4 °C) and controls 2 (fresh sperm collected to Kurokura and Kurokura before fertilizing the eggs).

m volume and programme used for cryopreservation

second set of experiments studied the volume effect of 0.5, 1 and 5 ml straws and pared these with 1.8 ml cryotubes as well as the effect of the cooling programme. owing the results of the first study, a combination of DMSO and propanediol (ratio 1:1) at centration of 10% was added to extended sperm in Kurokura 180 IS. No attempt was e to equilibrate sperm in this step (equilibratio time: 0.0h) To freeze the extended sperm, mixture was gently mixed and subsequently 1 ml was frozen in 1.8 ml cryotubes and 0.5, and 5.0 ml frozen in 0.5, 1 and 5 ml straws, respectively. Cryotubes and straws were ctly transferred to pre-programmed PLANER Kryo 10 and cooled under programmes K4 L1.

tilization and Hatching Trial

grams of eggs (1600 eggs per 1 g) were placed into a 20 ml dish; an accurate volume of m (thawed or non frozen stored sperm as control) with 100,000 spermatozoa per egg was pped on them from a micropipette (100,000 spermatozoa per egg was selected upon prior eriments). The dish was then placed on a shaking table set to 200 rpm and 10 mm ection. Two ml of hatchery water at 22 °C was added as a sperm activation medium. Two utes later, approximately 200 eggs were placed on a petri dish into incubators of 200 ml plied with UV-sterilized recirculated tap water at 23 °C and 9 $mg.l^{-1}$ O_2. Each experiment repeated four times. The unfertilized eggs were counted and removed from each bator a day after fertilization. To determine hatching success, the number of hatched ryos (larvae) was counted in each incubator approximately 3 days after fertilization.

percentage fertilization rate (F_r) was calculated as follows:

$$F_r = [(E_t - E_d)/E_t]x100$$

which (E_t) and (E_d) are total number of eggs placed in a incubator and the number of ertilized eggs removed 24 h after fertilization. The hatching rate (H_r) was calculated for n incubator from the number of hatched larvae (H_l) divided by the total number of eggs ced in the incubator (E_t) as follows:

$$H_r = (H_l/E_t)x100$$

servation and measurement of sperm motility and velocity

dark field microscope and a video camera were used to measure sperm velocity and centage of motility as described by Linhart et al. (27). Percentage motility and velocity re examined at 200x magnification immediately after mixing 0.5 µl of sperm with 49.5 µl distilled water (final dilution ratio of 1:100) as the swimming medium containing 0.1% A on a glass slide prepositioned on the microscope stage to prevent sperm heads from

ng to the glass slide. Sperm motility was recorded on video tape from 0 to 2 min post ation. The focal plane was always positioned near the glass slide surface. Video records made with a S-VHS (SONY, SVO-9500 MDP) video recorder at 25 frames.s^{-1} using a video camera (SONY, SSC-DC50AP) mounted on a dark-field microscope (Olympus 0) with a Strobex stroboscopic lamp (Chadvick-Helmut, 9630, USA) at a frequency of z, and visualized on a video monitor.

n velocity and motility percentage were assessed at 10 s post activation using the ssive positions of the video - recorded sperm heads by means of Olympus Micro Image are (Version 4.0.1. for Windows with a special macro by Olympus C&S). In this od, five successive frames in which frames 1, 2 to 4 and 5 were red, green and blue, ctively, provided the opportunity to measure velocity and percentage motility. Motile natozoa were visible in three colours (red, green and blue), while non-moving natozoa were white. The analyses were repeated 3 times from 3 records at 10s after ation. Twenty spermatozoa were grabbed from each frame. Percentage of motile natozoa was given by the proportion of red cells to all cells (red and white cells). city of spermatozoa was calculated as $\mu m.s^{-1}$ based on the length of traces of sperm heads e motile spermatozoa after calibration of magnification.

analysis

ie first experiment (fig.2) mean and SD values were calculated from 3 replicates for ization and hatching and in the second experiment (figs 3-5) from 4 replicates for ization, hatching, velocity and percentage of sperm motility. Statistical significance was ssed using multiple analysis of variance (ANOVA, Statgraphics version 5), followed by ey HSD multiple range test comparisons. Probability values < 0.05 were considered as ificant.

ılts

all experiments, ANOVA showed no significant differences ($P > 0.05$) between lization and hatching rate. Therefore, only results of hatching rate are presented below.

cts of IS, storage time and cryoprotectant

trol sperm (control 1) stored 19 h in IS Kurokura and Kurokura 180 showed good quality hatching rates of 75.8±5.6 % and 84.6±3.9 % (Fig. 2); the eggs were also of good ity, because control 2 (fresh sperm collected in Kurokura and Kurokura 180 before lizing the eggs) showed hatching rates of 78.7±6.1% and 75.9±6.4%. There were erences depending on treatment. Hatching rates of frozen/thawed sperm were significantly er than those of controls ($P<0.05$).

rozen/thawed sperm, hatching rates were highest (39.2±5.4) when non-eqilibrated sperm Kurokura 180 IS was extended by combination of DMSO and propanediol and lowest 3±5.4) when non-eqilibrated sperm was extended by just 10% DMSO (fig. 2). vertheless, in general, hatching rates decreased as time of eqilibration increased, either in erent IS or different cryoprotectants. Also hatching rates were higher when sperm was ected in Kurokura 180 compared to Kurokura, and when sperm was extended in a mixture DMSO and propanediol compared to DMSO. On the other hand, the first experiment ified that the best IS, cryoprotectant and storage time of sperm in IS before freezing were okura 180, a mixture of cryoprotectant DMSO and propanediol at the level 8 or 10 %, and - eqilibrated sperm, respectively.

rm volume and programme used for cryopreservation

ntrol sperm stored in IS Kurokura 180 had good quality with a hatching rate of 70.7±4.1 % g. 3), sperm motility of 95.3±0.6 % (Fig. 4) and sperm velocity of 84.8±15.3µm.s^{-1} (Fig. after 19 hours storage. All results with thawed sperm were significantly lower than the ntrol (Figs 3, 4 and 5).

highest (33.8±7.1%) and lowest (9.3±5.4%) hatching rates were observed when sperm frozen in 5 ml and 0.5ml straws under cooling programmes L1 and K4, respectively (fig. Nevertheless, there were no significant differences in hatching rates among different sizes traws either by rapid or slow cooling programme (P>0.05). In general, hatching rates were ıer when sperm was frozen in 5ml straws and 1.8ml tubes either in K4 or L1. There were statistical differences between rapid L1 and slow K4 freezing programmes.

percentage motility in frozen/thawed sperm ranged from 8.6±0.7% to 13.9±4.4% in ml straws and 1 ml tubes respectively, when frozen under L1. There were no significant ferences when different volumes of sperm were frozen or different freezing programmes d (Fig 4). Minimum (30.5±14.5 $\mu m.s^{-1}$) and maximum (49.5±17.2 $\mu m.s^{-1}$) sperm velocities frozen/thawed sperm were observed in 1 ml tubes and 1 ml straws when frozen under K4 l L1, respectively. There were small significant differences among treatments (Fig.5). wever, in general, sperm velocity was higher when sperm was frozen rapidly (programme).

scussion

gh variability of sperm quality after freezing-thawing has been reported in different fish ecies. However, successful fish sperm cryopreservation can be achieved by optimizing the ezing regime, the cryoprotectant medium, the volume and storage time of extended sperm d the thawing regime.

have good quality sperm after short-term storage and cryopreservation, the sperm must be good quality at the outset. It is very important to avoid contamination of sperm with blood, eces, urine and water during sperm collection. In tench, the common contamination of erm with urine is a reason for spontaneous movement of sperm in seminal fluid (25), where sh sperm usually is inactive (20,8). But the present negative effect of urine could be

come by collecting sperm in an IS (19). Further studies have been carried out to optimize composition of IS (35). The role of the IS in tench is not only to stop spontaneous ement of spermatozoa, but also to allow the recovery of energy stores as in common carp *rinus carpio* L.) (5). If availability of O_2 and organic energy substrates for sperm is ed, it can be provided artificially *in vitro*, by exposure to an aerobic atmosphere (5) in 1:10 and storage at 0 °C (26). Nevertheless, environmental conditions of sperm storage be correctly adjusted, because energy components such as ATP are consumed during m storage (36). Therefore, in the present study the sperm - urine mixture was collected ctly into IS and stored at 0°C in aerobic conditions. Both the IS used successfully were out organic components. This shows that tench spermatozoa may not need organic energy ponents in IS (35).

en fertilization assays with cryopreserved sperm are conducted to detect the optimal zing protocol, it is necessary to use a low or medium number of spermatozoa per egg. A ber of 11,500 to 46,000 fresh spermatozoa per egg were required for artificial mination in tench (29). In the present experiments after thawing there were 1000-20000 ile spermatozoa in the different replicates (calculated from a range of 1-20 % of motile thawed spermatozoa). Logically, sperm motility and hatching rate should be correlated, use of the limited number of motile spermatozoa used per egg. But high variability ng replicates and relatively low levels of sperm motility unfortunately masked any elation between sperm motility and hatching rate under the different experimental ditions. But in some of the samples in 5 ml straws and 1 ml tubes motility was up to 20 % *c.* 20000 motile spermatozoa per egg), which perhaps positively influenced hatching rate. millions of spermatozoa per egg are used, low viability and low quality of post-thawed m could be masked (27). Moreover, using more than 100,000 spermatozoa is not practical artificial propagation, as male tench generally have a low volume of sperm and so sperm

stage should be avoided. The dilution ratios of sperm and egg with water are other rameters which must be adjusted. Optimum sperm: water: egg ratios have been reported as 200: 100 for artificial propagation of tench (29). The present idea was to use a "practical" antity of sperm, which could reveal the best procedure for freezing sperm, but also to nulate methods of artificial propagation for future practical use of frozen tench sperm on a ge scale.

e freezing procedure applied was modified from cryopreservation studies on European fish (*Silurus glanis*) (27) and common carp (23). Newly used and simple L1 cooling ogramme, which was only "single-rate" and faster than K4, is suitable for freezing of tench erm especially for a large frozen volume. Also this programme is expected to provide a gher velocity of frozen/thawed sperm.

though DMSO is widely used for cryopreservation of fish sperm, as any other yoprotectant it has negative sides. It is not stable during storage and it is known to cause gle strand breaks of DNA. The increase of its concentration above 10% might activate erm. It was found that a mixture of DMSO and propanediol resulted in a significantly better tching rate. Such cryoprotectant composition has not been used before in tench sperm yopreservation. Despite the vast range of cryoprotectants tested in fish, the most common es for protection of common carp sperm during freezing remain: DMSO, glycerol, thanol and dimethyl acetamide (DMA) (15,30,31,3,32,33,23,2,14,40). According to our ults, 8 or 10 % of DMSO with propanediol at ratio 1:1 can be used in tench as well as in ropean catfish. Recent studies (40) in roach (*Rutilus rutilus*), bream (*Abramis brama*), ver bream (*Blicca bjoerkna*) and barbel (*Barbus barbus*) showed improved sperm motility d fertility in the post-thawed sperm when methanol was applied.

e freezing volume is generally an important factor for the freezing procedure in yopreservation of fish sperm. All of the volumes tested (1 ml tubes and 0.5, 1 and 5 ml

ws) were suitable for cryopreservation of tench sperm. 5 ml straws and 1 ml tubes were best, especially the 5 ml straw which provides for storage of a large volume of sperm at a er volume of nitrogen with a shorter operating time for freezing and for low cost of age. Christensen and Tiersch (10) found that sperm motility in 0.25 ml straws was better n in 0.5 ml straws in channel catfish. For prospective tench sperm banking, it is more venient to freeze a larger volume of sperm in one straw. This procedure enables the total age capacity of sperm in one container to be increased as the empty space among tubes is minated. Manipulation with larger straws is more practical, too.

opreservation of sperm appears to be a useful and reliable technique for conservation of e resources in the tench. The optimum procedure according the present study is shown in 6.

knowledgement

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ends

re 1: Cooling programme K4 and L1 used during tench sperm cryopreservation

re 2: The effect on the level of hatching of two immobilizing solutions (IS), Kurokura and Kurokura, immobilizing sperm equilibrated for 0, 2 or 4 h before freezing with 8 or cryoprotectant concentrations. Groups of different storage time before freezing, different ps of cryoprotectant and different immobilizing solutions with the same superscript do differ significantly ($P<0.05$). Four nonfrozen sperm controls were used: controls 1 ected into Kurokura and Kurokura 180, used for freezing, stored 19 h on ice at 0-4 °C) controls 2 (fresh sperm collected into Kurokura and Kurokura 180 before fertilizing the).

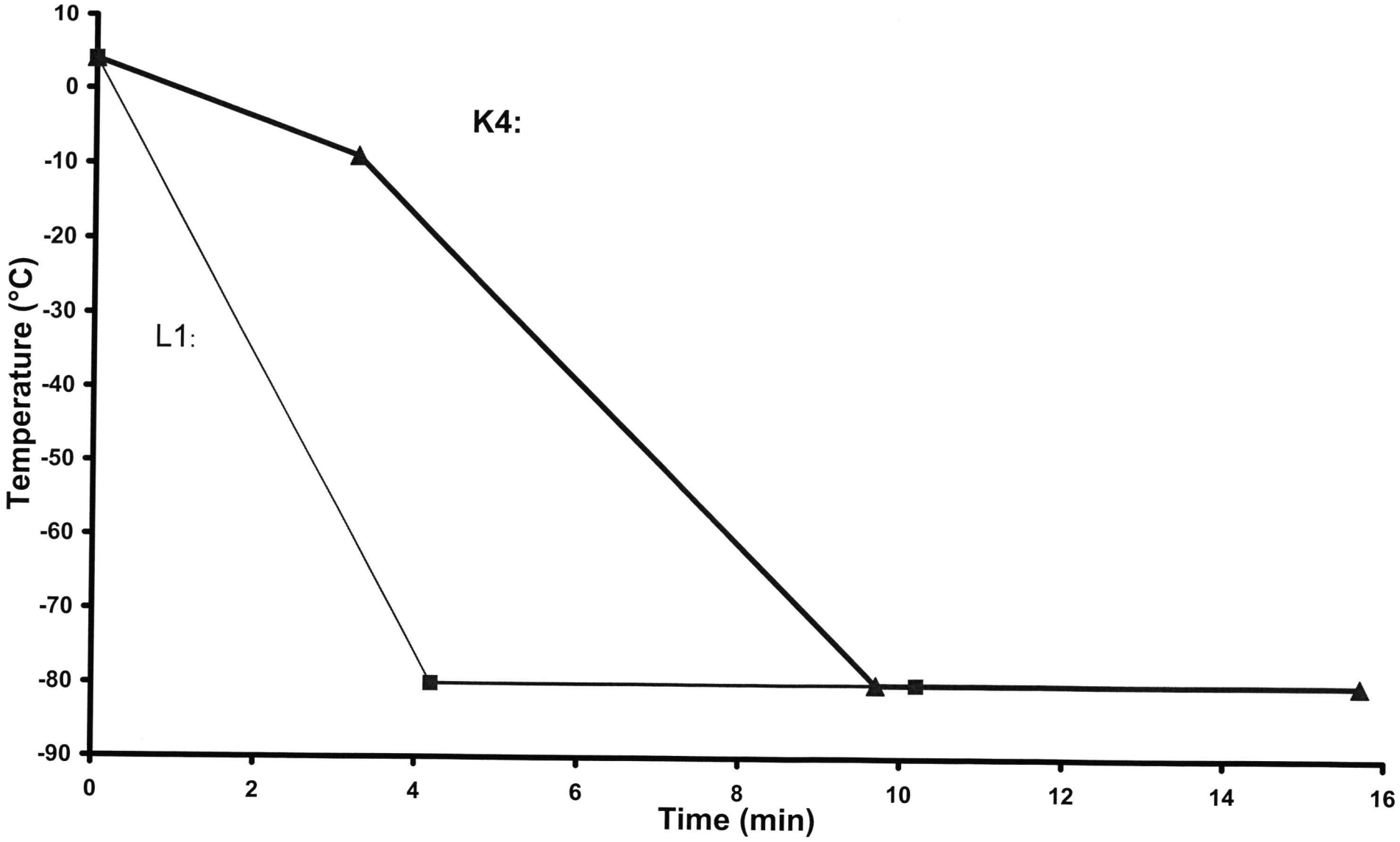
re 3: Effect on hatching rate of frozen/thawed sperm: 1 ml extended sperm frozen in 1.8 ryotubes and 0.5, 1 and 5 ml sperm in 0.5, 1 and 5 ml straws frozen under two different grammes. Groups with the same superscript do not differ significantly ($P<0.05$).

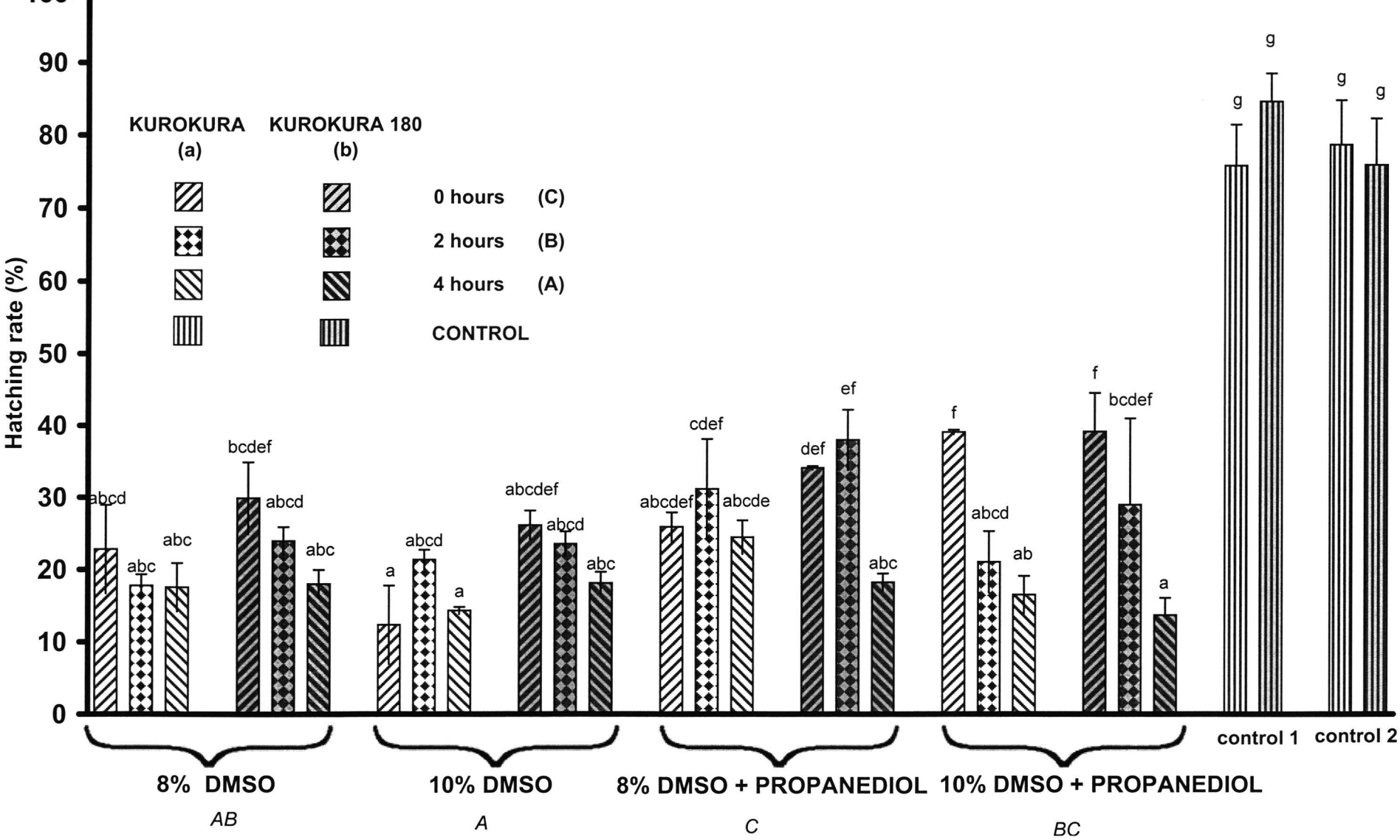
re 4: Effect on percentage motility 10s after activation of frozen/thawed sperm: 1 ml nded sperm frozen in 1.8 ml cryotubes and 0.5, 1 and 5 ml sperm in 0.5, 1 and 5 ml ws frozen under two different programmes. Groups with the same superscript do not differ ificantly ($P<0.05$).

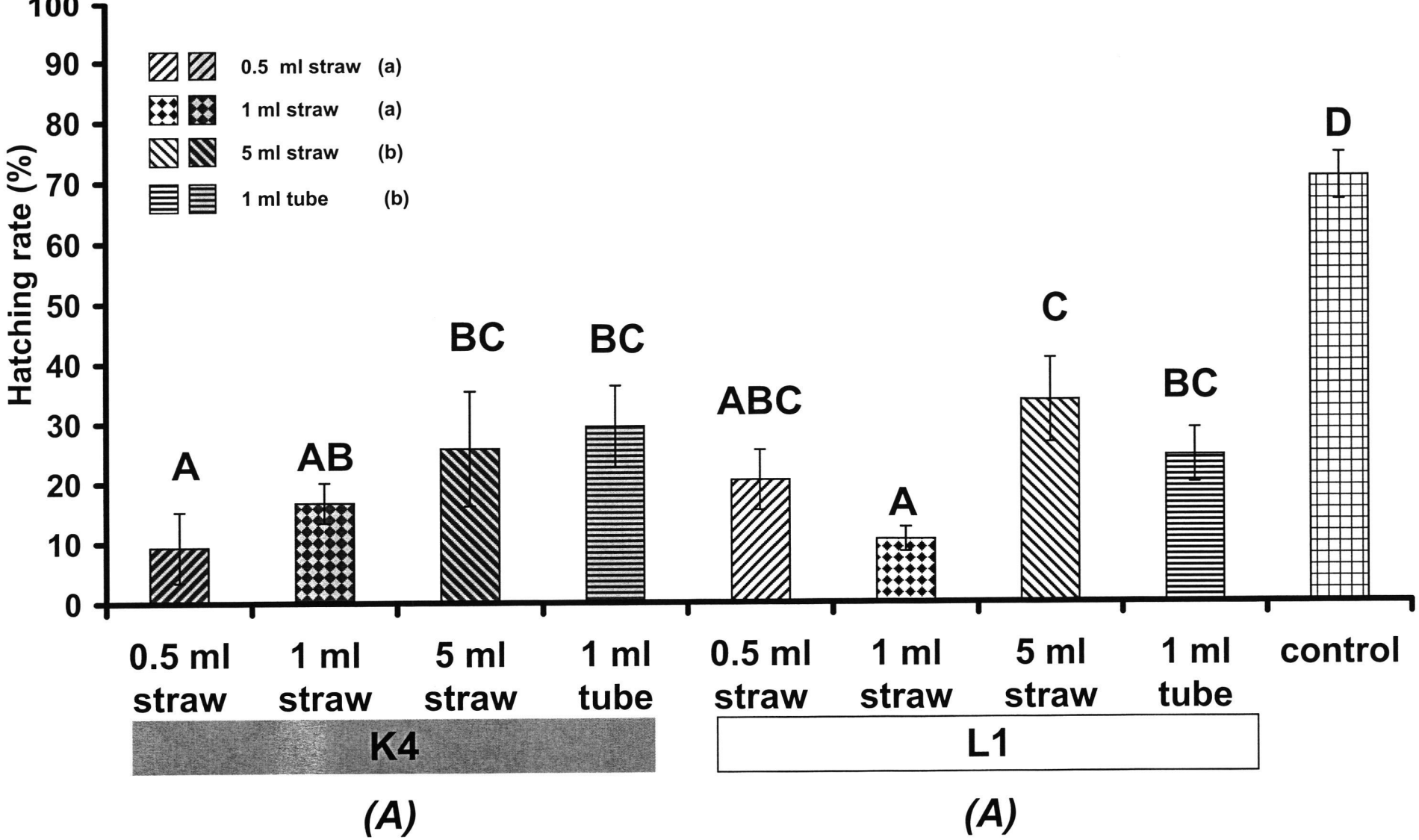
re 5: Effect on sperm velocity 10s after activation of frozen/thawed sperm: 1 ml extended m frozen in 1.8 ml cryotubes and 0.5, 1 and 5 ml sperm in 0.5, 1 and 5 ml straws frozen er two different programmes. Groups with the same superscript do not differ significantly 0.05).

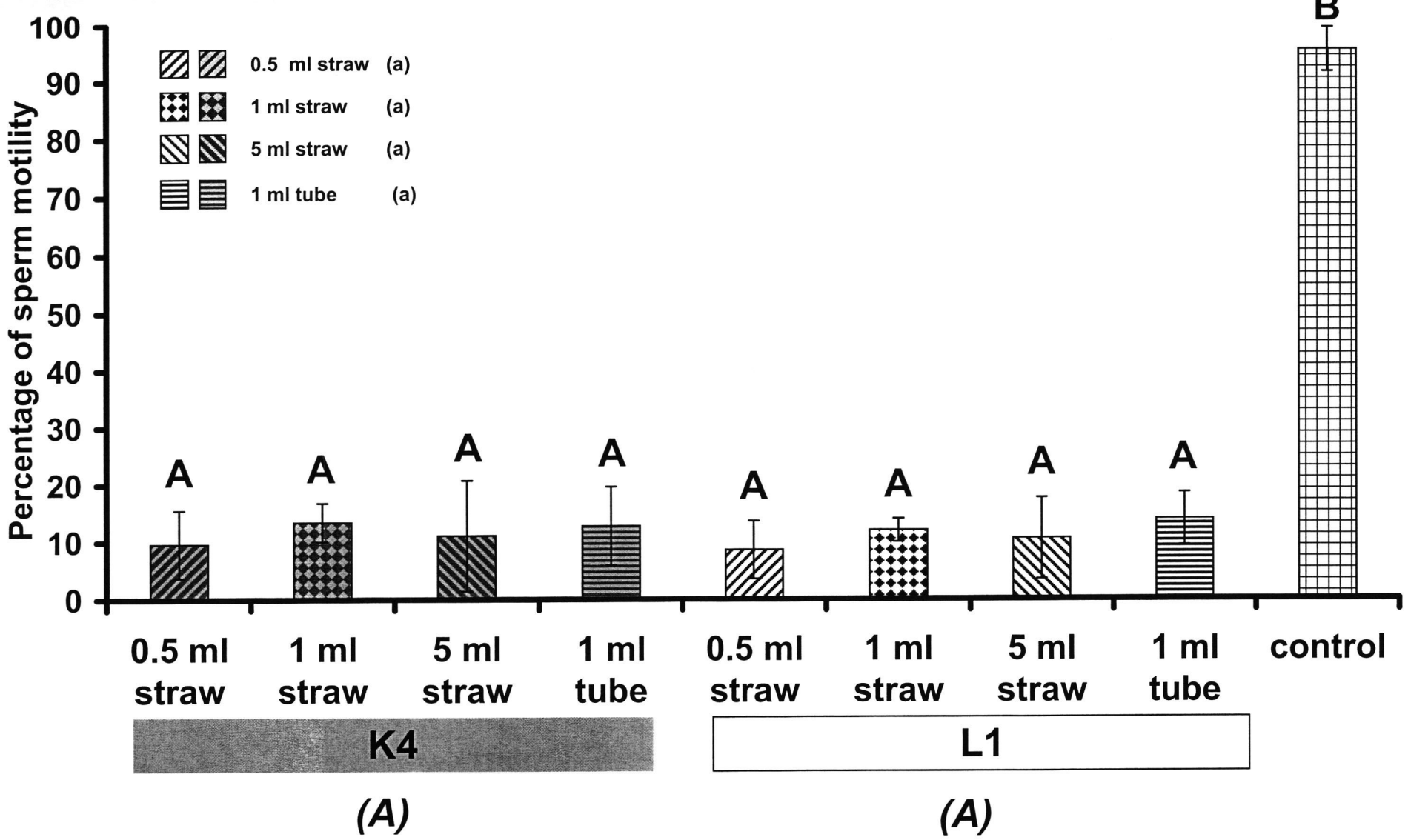
ıre 6: Procedure of sperm cryopreservation in tench after stripping of hormonally treated

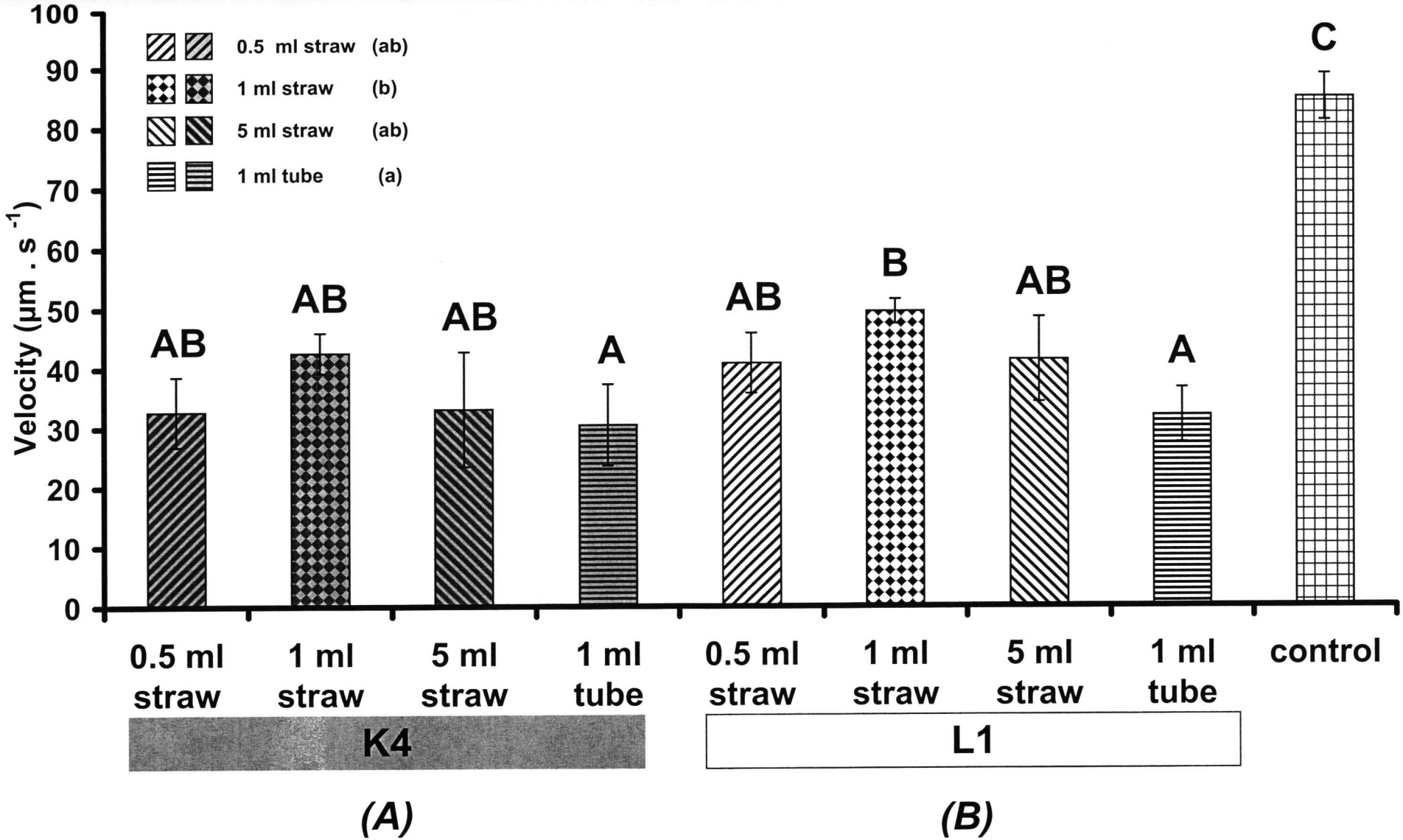
ɔdstock.

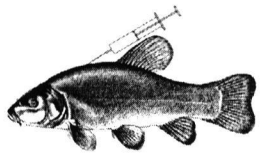

K4:
L1:
Temperature (°C)
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
0
2
4
6
8
10
12
14
16
Time (min)


Hatching rate (%)
0
10
20
30
40
50
60
70
80
90
KUROKURA (a)
KUROKURA 180 (b)
0 hours (C)
2 hours (B)
4 hours (A)
CONTROL
abcd
abc
abc
bcdef
abcd
abc
a
abcd
a
abcdef
abcd
abc
abcdef
cdef
abcde
def
ef
abc
f
abcd
ab
f
bcdef
a
g
g
g
g
control 1
control 2
8% DMSO
AB
10% DMSO
A
8% DMSO + PROPANEDIOL
C
10% DMSO + PROPANEDIOL
BC

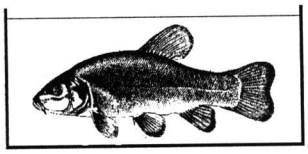

Hatching rate (%)
100
90
80
70
60
50
40
30
20
10
0
0.5 ml straw (a)
1 ml straw (a)
5 ml straw (b)
1 ml tube (b)
A
AB
BC
BC
ABC
A
C
BC
D
0.5 ml straw
1 ml straw
5 ml straw
1 ml tube
0.5 ml straw
1 ml straw
5 ml straw
1 ml tube
control
K4
L1
(A)
(A)


Percentage of sperm motility
100
90
80
70
60
50
40
30
20
10
0
0.5 ml straw (a)
1 ml straw (a)
5 ml straw (a)
1 ml tube (a)
A
A
A
A
A
A
A
A
B
0.5 ml straw
1 ml straw
5 ml straw
1 ml tube
0.5 ml straw
1 ml straw
5 ml straw
1 ml tube
control
K4
L1
(A)
(A)

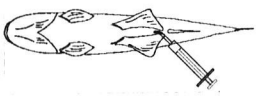

0.5 ml straw (ab)
1 ml straw (b)
5 ml straw (ab)
1 ml tube (a)
Velocity (µm . s -1)
100
90
80
70
60
50
40
30
20
10
0
AB
AB
AB
A
AB
B
AB
A
C
0.5 ml straw
1 ml straw
5 ml straw
1 ml tube
0.5 ml straw
1 ml straw
5 ml straw
1 ml tube
control
K4
L1
(A)
(B)



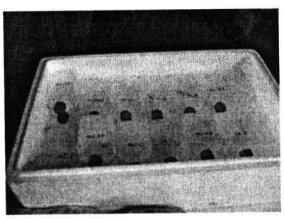
Hormonal stimulation: carp pituitary powder suspension, dosage 1mg per 1 kg of male b.w.

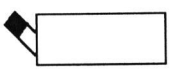


Anestezia



Collection of sperm in the imobilization solution containing NaCl 180mM, KCl mM, $CaCl_2.2H_2O$ mM, $NaHCO_3$ mM, dilution rate 2:1 (IS:sperm)

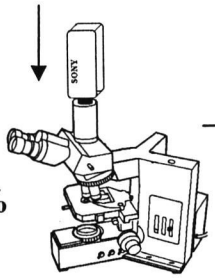


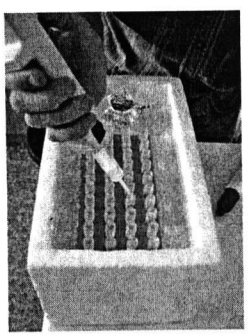


Storage of sperm in IS at 0-4°C.

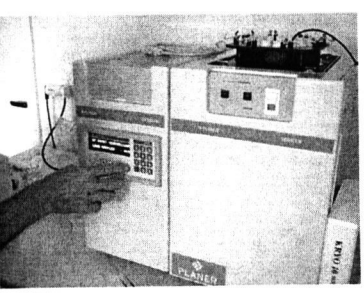
tion of sperm

with at least 80%
y)

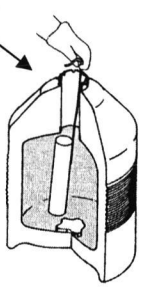




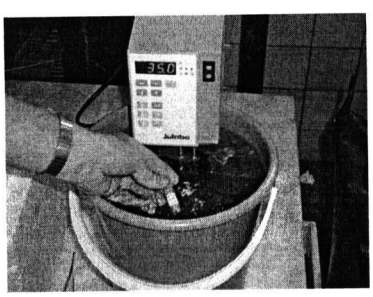
Addition of cryoprotectant: DMSO + PROPANEDIOL (1:1) 10% of total volume



Freezing using the PLANER KRYO 10 III



Transfer to the liquid nitrogen (LN2)



Thawing of frozen sperm at 40 °C

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Insemination, fertilization and gamete management in tench, *Tinca tinca* (L.)

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Key words: Aquaculture, Fertilization, Hatching, Reproduction, *Tinca tinca*

Abstract. Various procedures for artificial insemination in tench, *Tinca tinca* (L.) were re-examined with evaluation of fecundity of males and females among different tench strains. The objectives of this study were to enhance fertilization and hatching rates through optimization of the activation solution, the insemination process, the activation of gametes, and the elimination of eggs stickiness. Sperm for all experiments was collected directly into immobilization solution of modified Kurokura solution containing 180 mM of NaCl and stored at 2 °C for 2.5–5 h prior to the experiment. When dechlorinated tap water was used for activation a gamete ratio of 1150 spermatozoa per egg showed the best significant fertilisation and hatching rates. Optimal ratio between eggs (weight in g) and activation solution (in cm^3) was 1:1. Different concentrations of activation solutions such as NaCl from 0 to 68 mM (0–136 mOsmol kg^{-1}) without buffer statistically decreased fertilization and hatching rates. The activation solution containing 17 mM of NaCl, 10 mM Tris–HCl, pH 8 and 9 significantly increased fertilization and hatching rates compared to dechlorinated tap water of pH 7 or activation solution containing 17 mM of NaCl, 10 mM Tris–HCl, pH 6 and 7. Adhesiveness of the eggs was successfully removed by incubation in Alcalase and activity: 3.16 Anson units per cm^3.

Introduction

Controlled reproduction of tench started in the 1970s, after establishing technologies for artificial reproduction (Brylinski and Pyka 1976; Kouril and Chabera 1976; Horvath 1977; Horvath et al. 1984; Kouril et al. 1986). Approaches either through basic or applied research were highlighted in a review of tench gamete biology and artificial reproduction by Linhart and Billard (1995).

In tench, as in other cyprinids, temperature is the major environmental factor for the development of gametogenesis and spawning (Pimpicka 1989). A water temperature of 6–9 °C during the winter period, up to mid-February, ensures favorable conditions for the stages of ovogonial proliferation and beginning of vacuolization. Vitellogenesis is slowly stimulated from February to the end of April by increasing the temperature up to 17 °C. The spawning season begins in May when temperature increases from 17 to 22 °C (Horo-

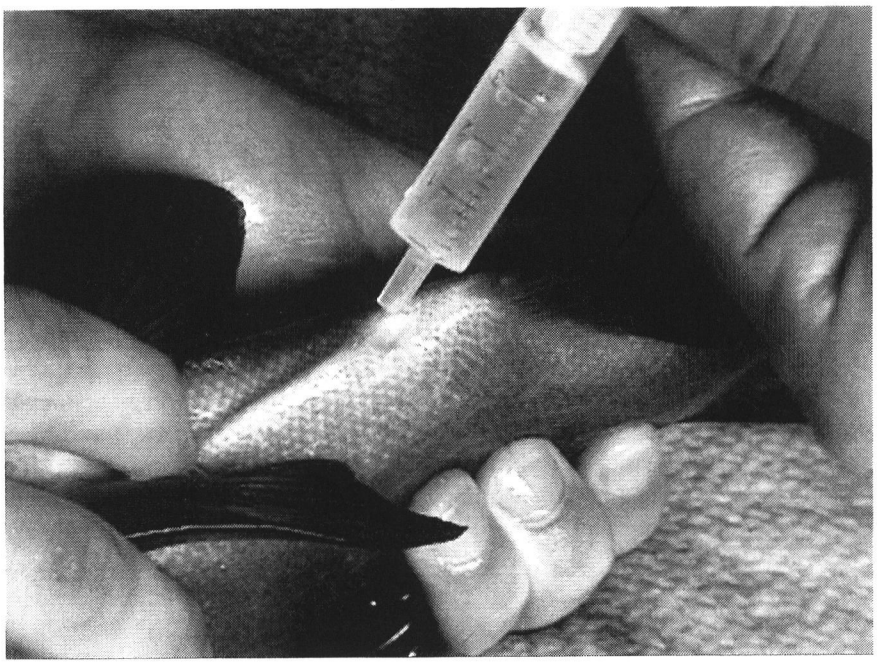

Figure 1. Demonstration of sperm collection from the genital papilla of tench into 5 cm^3 syringe with 2 cm^3 of immobilization solution and 1 cm^3 of sperm.

spermatozoa per cm^3 was dropped on them from a micropipette. The dish was then placed on a shaking table (Figure 2) with constant agitation at 200 rpm and with 10 mm deflection. One cm^3 of dechlorinated tap water or experimental activation solution at 22 °C was added. Two minutes later, approximatley 250–360 eggs were placed into a incubator cage of 200 cm^3 supplied with UV sterilized recirculated tap water at 22 °C, 9 mg dm^{-3} O_2. For each experiment the procedure was replicated four times. The eggs were exactly counted in each cage, and later, during incubation of eggs the dead eggs and hatched larvae were counted, usually up to 4.5 days. The percentage of fertilization rate (F_r) was then calculated for each cage from the total number of eggs placed in the cage (E_t) minus dead eggs (E_d) collected up to 24 h after fertilization as follows:

$$F_r = [(E_t - E_d)/E_t] \cdot 100$$

The percentage of hatching rate (H_r) was also calculated for each cage from the number of hatched larvae (H_l) divided with the total number of eggs placed in the cage (E_t) as follows:

$$H_r = (H_l/E_t) \cdot 100$$

Figure 2. Dishes for fertilization experiments on a shaking table.

Determination of optimal sperm/egg ratio (Experiment 1)

The quantity of sperm per egg for fertilization was 115, 1150, 11,500 and 46,100 spermatozoa per egg, which represents 0.1, 1, 10 and 40 mm^{-3} of sperm, respectively. One gram of eggs was placed into a dish and accurate volume of sperm (0.1, 1, 10 or 40 mm^{-3} of sperm) with estimated number of spermatozoa (115, 1150, 11,500 or 46,100 spermatozoa per egg) was dropped using a micropipette. Before activation of eggs with water, additional IS was added at volumes of 39.9, 39, 30 or 0 mm^{-3} into experiments with 0.1, 1, 10 or 40 mm^{-3} of sperm, respectively. Then the dish was placed on a shaking table and 1 cm^3 of dechlorinated tap water (22 °C) was added.

Effects of different NaCl concentrations in activation solutions (Experiment 2)

One gram of eggs was placed into a dish and an accurate volume of sperm with a low number (500) of spermatozoa per egg was dropped with a micropipette. Then the dish was placed on a shaking table and different activation solutions at volume of 1 cm^3 were tested. The solutions used had differing NaCl concentrations: 0, 17, 34 and 51 mM (in distilled water). Dechlorinated tap water was used as a control. After 2 min, the fertilized eggs were placed in incubator cages with four replicates.

Determination of optimal pH in activation solution (Experiment 3)

One gram of eggs was placed into a dish and 500 spermatozoa per egg were added by micropipette. The importance of the pH, of the activation solution, on fertilization and hatching rate was greater with very low number of sperm (500) per egg used for experiment. Then the dish was placed on shaking table and 1 cm^3 of 17 mM NaCl with 10 mM Tris–HCl at pH 6, 7, 8 and 9 of 22 °C was added. The control was activated with dechlorinated tap water. Two min later, the fertilized eggs were placed in special incubator cages with four replications.

Data analysis

Means of the data acquired were evaluated from 4 replicates. Statistical significance was assessed using multiple analysis of variance (ANOVA, Statgraphics version 5), followed by multiple comparison Tukey HSD range test. Probability values < 0.05 were considered significant.

Results

Determination of optimal sperm/egg ratio (Experiment 1)

The fertilization and hatching rates (80.6% and 80%, respectively) were significantly the highest for 11,500 spermatozoa per egg, than for other ratios (Figure 3). The fertilization and hatching rates were 42.7% and 42.2%, respectively, with the ratio of 1150 spermatozoa per egg and 17.8% and 17.4%, respectively, with the ration of 115 spermatozoa per egg. The fertilization and hatching also insignificantly decreased to 75.5% and 74.5% at the ration of 46,100 spermatozoa per egg. ANOVA showed significant effect of number of spermatozoa per egg ($p < 0.0001$) on the fertilization and hatching success.

Effects of different NaCl concentrations in activation solutions (Experiment 2)

Dechlorinated tap water (pH 7) and a concentration of 500 spermatozoa per egg gave a significantly highest fertilization rate (expressed as percentage of hatching) of 54.6%. Fertilization and hatching rates were significantly lower in all NaCl solutions from 17 to 51 mM NaCl activation solution, where fertilization and hatching rates were 0.8–9.2% and 0.8–7.2% (Figure 4). ANOVA showed the significant effect of the NaCl concentration in the activation solution on fertilization and hatching success ($p < 0.0001$).

Determination of the optimal pH of the activation solution (Experiment 3)

The highest fertilization and hatching rates (71.2 and 71.7%, and 71.2 and 71.3%, respectively) with concentration of 500 spermatozoa per egg, was found for activation solutions with pH 8 and 9 (Figure 5). Lower but insignificant levels, of 54.6 and 58.7% hatching rates, were found with dechlorinated tap water (control) and the activation solution at pH 7. Fertilization and hatching rates were only on the level 43.2 and 42.1% with activation solution of pH 6 and also using lower ratio of spermatozoa to egg (Figure 5). The experiment also demonstrated good quality and fertilization capacity of eggs. ANOVA showed significant effect of the pH of activation solution ($p < 0.0001$) on fertilization and hatching success.

Discussion

Conditions of sperm storage

In tench, the low osmolality of urine is the main key factor for spontaneous activation of spermatozoa (Linhart et al. 2003b), as urine exhibits osmolality down to half of that of seminal fluid. Osmolality of contaminated tench sperm was re-equilibrated successfully by Rodina et al. (2004) with storage potential for 10 h owing to the "KUROKURA 180" IS containing 180 mM NaCl. The

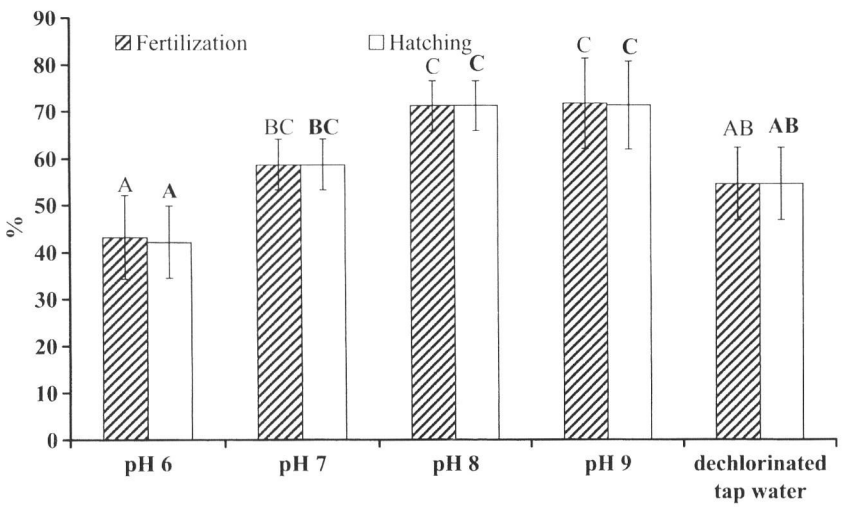

Figure 5. Fertilization and hatching success after artificial insemination with different pH of activation solution containing 17 mM NaCl + 10 mM Tris–HCl with the same level of 500 spermatozoa egg^{-1}. Mean values of four replicates are shown, vertical lines are SD. Groups with a common superscript do not differ significantly ($p < 0.05$).

total concentration of soluble compounds in the IS solution was around 190 mM leading to osmolality 343 mOsmol kg^{-1}. The role of the IS in tench is not only to stop movement of spermatozoa spontaneously activated by contaminating urine, but also to allow the recovery of the energetic stores. This prediction would explain why tench spermatozoa do not really need energetic organic components in IS, because the motility period of tench spermatozoa is very short (Rodina et al. 2004). However, environmental conditions of sperm storage must be correctly adjusted, because an energetic component as ATP is consumed during sperm storage (Saad et al. 1988). If availability of O_2 and substrates for sperm is limited, it can be provided artificially *in vitro*, by exposure to aerobic atmosphere (Billard et al. 1995) in ratio 1:10 and storage at 0 °C (Linhart et al. 2003c).

Artificial insemination

Composition of diluents for sperm activation and fertilization was developed by Kouril et al. (1976), Horvath et al. (1984) and Linhart and Kvasnicka (1992). Kouril et al. (1976) and Linhart and Kvasnicka (1992) used activation solution containing 2 g (34 mM) of NaCl with or without milk solution. Horvath et al. (1984) recommended traditional Woynarovich and Woynarovich (1980) method for common carp, using 4 g (68 mM) of NaCl and 3 g of urea per dm^3. Later, Geldhauser (1992) in a detailed study observed that activation solution from Woynarovich and Woynarovich (1980) decreased fertilization success in tench. According to our results, it can be concluded that the minimum number of spermatozoa for optimal fertilization and hatching rate ranges 500 to 10,000 spermatozoa per egg, according to quality of eggs (Figures 3 and 5). Various activation solutions with NaCl 0–68 mM (0–136 mOsmol kg^{-1}) in distilled water dramatically decrease fertilization or hatching rates (Figure 4). NaCl with buffer solution significantly decreased fertilization and hatching rates. The experiment showed increasing fertilization and hatching rates when using higher pH such as 8 and 9 (Figure 5) instead of pH 6. An overview of our results showed that dechlorinated tap water or low concentration of buffer solutions at pH 8 or 9 and 11,000 spermatozoa per egg are the best conditions for activation solution. Then the question is raised about the differences between our results and those of Kouril et al. (1976) and Linhart and Kvasnicka (1992). They can be explained by the strategy of methodology during experiments. Linhart and Kvasnicka (1992) used Petri dishes for basic experimental conditions where the ratio between weight of eggs and volume of activation solution was 1:30–40 and later they adapted these results to the practical field conditions. However, under practical conditions the rate between weight of eggs and volume of activation solution is 1:1. Results obtained in these conditions could be influenced by the high mass of eggs and by the content of seminal fluid. The strategy of our study was opposite to that of Linhart and Kvasnicka (1992). We tried to adapt our experimental

conditions to the practical ones. The ratio between the weight of eggs and volume of activation solution was 1:1 in all cases with constant time and rotation level during the procedure of mixing gametes and solutions. The ratio 1:1 used under practical conditions as well as in our experiment was confirmed to be feasible with success for artificial insemination procedure. Also in the experiment, optimal low sperm : egg ratio was used for discrimination of fertilization effect (Billard and Cosson 1992), when the high fertilization/hatching variability was used as potential predictor of gametes or environmental quality, etc.

Artificial insemination and elimination of stickiness under practical conditions

Based on our results, our recommended procedure for artificial insemination is as follows:

Fertilization (Figure 6). The minimum volume of short-term stored sperm under aerobic conditions used for insemination was 1 cm^3 of sperm collected in IS 1:2 (volume ratio) per 100 g of short-term stored ova. Ova contaminated by urine during stripping should be discarded (Linhart and Billard 1995). The *in vitro* storage of ova should not exceed a few hours at a stable temperature of 17–18 °C and it was advised to carry out the insemination and activation steps as soon as possible after ovulation. Also, 0.1 cm^3 of diluted sperm with IS per 100 g of eggs was found sufficient for good fertilization and hatching rates as documented in Figures 3 and 5, when 500–10,000 spermatozoa guaranteed successful fertilization and hatching rates. The mixture of eggs and sperm was directly activated with 100 cm^3 of activating solution made of 17 mM NaCl + 10 mM Tris–HCl, pH 9 or of dechlorinated tap water or clean hatchery water at an optimum temperature 22 °C. After 2.5 min, the excess solution was poured out and desticking process with enzyme was started at 3 min. During that time the eggs were hydrated and swelled rapidly. Egg sticking was prevented during that period by constant mixing.

Desticking (Figure 5). The procedure for egg desticking with milk and clay suspension was developed by Kouril et al. (1976), later Linhart et al. (2000) used enzyme. In the Czech Republic, fish farming practice employs enzyme diluted in dechlorinated tap water or clean hatchery water. For the elimination of egg stickiness alcalase enzyme is used 3 min. after fertilization. Optimum rates between eggs and diluted enzymes (2 cm^3 of Alcalase enzyme, *Bacillus licheniformis*; CALBIOCHEM cat. no. 12674120, diluted in 998 cm^3 of hatchery water or AS; unpublished results) is 1:1 (g eggs: cm^3 of diluted enzymes) with stirring for 2 min. After 2 min of exposure in enzyme solution, the eggs are rapidly rinsed with hatchery water and transferred to Weis jars. The duration of egg incubation after enzyme treatment was about 4–5 h shorter, than the classical method using milk solution and talc suspension. This prolongation for the classical method may be explained by hardening of the egg envelopes. Hatching is expected to start 3 days (57 D°) after

gametes and elimination of egg stickines. A practical scheme for artificial propagation at fish farm hatcheries was proposed.

Acknowledgements

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yokonzervace spermatu a genofond sumce velkého

7.1 Současný stav chovu a genofond sumce

Přestože sumec nepředstavuje v Čechách z hlediska produkce ležitější rybu, je nedílnou součástí sortimentu produkce, která je závislá zené reprodukci – umělém výtěru. Produkce sumce byla až do 80 let ého století závislá převážně na poloumělém výtěru, protože pokusy o výtěr ztroskotávaly na nízké oplozenosti v důsledku nízké aktivity atu. Řešením bylo až zavedení používání imobilizačních a aktivačních ků pro spermie, což vedlo k rapidnímu nárůstu produkce plůdku sumce v epublice. Vzhledem k velké reprodukční kapacitě generačních ryb sumce ěšnosti umělého výtěru v kombinaci s poměrně snadným a úspěšným nem plůdku v řízených pomínkách může poptávku po plůdku sumce kojit jediná rybí líheň z jediného výtěru několika generačních ryb! Tato čnost je jedním z důvodů, proč se věnovat kryokonzervaci spermatu e – pro uchování rozmanitosti genofondu.

Genofond sumce v ČR představují jednak populace původem z povodí y, které jsou brány jako tzv. sumec vodňanský, jednak populace dem z povodí řeky Moravy, které jsou brány jako sumec hodonínský. v řadě je albinotická (alampická) varieta sumce nazývaná sumec otický.

7.2 Zmražování spermatu sumce: motilita spermií, životnost spermií a líhnivost plůdku

Cílem této práce bylo pokusit se vyvinout metodiku zmražování spermatu e použitelnou pro potřeby exsitu uchování genofondu. Úspěšnost byla zována motilitou a rychlostí pohybu spermií po rozmražení, procentem ch spermií stanovovaných metodou diferenciálního fluorescenčního ení spermií po rozmražení a úrovní líhnivosti plůdku po použití razeného spermatu. Nejlepších výsledků líhnivosti (82-86%) bylo ženo u spermatu uchovaného 5hodin po odběru v imobilizačním roztoku a eno s 8-10% DMSO nebo směsi DMSO a propandiolu. Tyto výsledky ly statisticky průkazné rozdílné od kontroly! Podíl živých spermií

ekoreloval s líhnivostí plůdku ani s motilitou, ale negativně koreloval rychlostí pohybu spermií. Motilita rozmrazeného spermatu se pohybovala od do 62% oproti 96% u kontroly, korelovala s líhnivostí (r=0,76), ale ekorelovala ani s podílem živých spermií, ani s rychlostí pohybu spermií. růměrná rychlost pohybu spermií se pohybovala v rozmezí 33-106 $\mu m.s^{-1}$, řičemž nejvyšší průměrné hodnoty byly naměřeny při použití 10%metanolu ako kryoprotektiva. Objem mražené dávky neměl na dosaženou líhnivost tatisticky průkazný vliv, přesto, nejlepší výsledky byly zjištěny u objemu 2 l.

Kompletně je práce popsaná v publikaci:

Linhart O., Rodina M., Flajšhans M., Gela D., Kocour M. 2005: ryopreservation of European catfish Silurus glanis sperm: Sperm otility, viability and hatching succes of embryo, Cryobiology 51: 250-61

7.3. Optimalizace postupů řízené reprodukce sumce

U sumce bylo předmětem testování a optimalizace: složení (osmotická oncentrace) imobilizačního roztoku pro spermie a aktivačního roztoku, pH ktivačního roztoku, poměr objemu aktivačního roztoku a hmotnosti jiker a ávka spermatu (spermií) v přepočtu na 1jikru.

Výsledky pokusů ukázaly, že nejvhodnější koncentrace NaCl imobilizačním roztoku byla 170mM, přičemž líhnivost nebyla průkazně dlišná od metodikou doporučované koncentrace 200 mM. Osmotická úroveň ktivačního roztoku daná koncentrací NaCl vzrůstající z 0 na 51mM neměla a dosaženou líhnivost plůdku žádný vliv, průkazný pokles líhnivost byl aznamenán až u koncentrace NaCl 68 mM. Nejvhodnější pH aktivačního oztoku se překvapivě ukázalo pH 6 a 7, při jehož použití byla dosažená hnivost průkazně vyšší, než doposud doporučované pH 8. Jako průkazně ejvhodnější poměr objemu aktivačního roztoku a objemu jiker vyšel poměr :2. Dávka spermií 800 na jikru a vyšší zajistí spolehlivě líhnivost plůdku rozmezí 80-90%, přičemž dávky o řády vyšší již nevedou ke statisticky okazatelnému zvýšení dosažené oplozenosti a líhnivosti.

Kompletně je práce popsaná v publikaci:

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Cryopreservation of European catfish *Silurus glanis* sperm: Sperm motility, viability, and hatching success of embryos ☆

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act

e aim of this study was to elaborate cryopreservation methods for ex situ conservation of European catfish. The ss of sperm cryopreservation was evaluated by post-thaw sperm motility and velocity, percentage of live sperma- and fertility (hatching rates) using frozen/thawed sperm. The best hatching rates of 82–86% were obtained with stored for 5 h before freezing in immobilizing solution and frozen with Me_2SO in concentrations of 8, 10, and or with a mixture of 5% Me_2SO and 5% propandiole. These results did not significantly differ from the fresh sperm ol sample. The percentage of live spermatozoa in frozen/thawed sperm did not correlate with hatching rate or ty of spermatozoa, but was negatively correlated with velocity of spermatozoa ($r = -0.47$, $P = 0.05$). The percent- otility in frozen/thawed sperm ranged from 8 to 62%, when sperm was stored in immobilizing solution 5 h before ng. The average value in the fresh sperm (control) was 96%. The frozen/thawed sperm motility rate significantly ated with the hatching rate ($r = 0.76$, $P = 0.0002$), but not with the percentage of live spermatozoa ($r = 0.16$, 52) or the sperm velocity ($r = 0.07$, $P = 0.79$). The velocity of frozen/thawed spermatozoa ranged from 37 to /s, whereby methanol concentrations of 7.5 and 10% resulted in highest velocities. Freezing sperm volumes of l did not affect the quality of frozen/thawed sperm.

rds: Fish; European catfish *Silurus glanis*; Sperm; Aquaculture; Cryopreservation; Sperm motility; Velocity; Fertility; Cryo- Immobilizing medium; Reproduction

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The European catfish (*Silurus glanis* L., also called wels or sheat fish) is a commercially important freshwater fish species in Europe. Its total aquaculture production in 10 European countries

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increased from 602 ton in 1993 to about ton now [23]. The domesticated stocks of pean catfish showed less genetic diversity the wild ones, possibly due to passive selec- under hatchery conditions [15]. Therefore, ervation programmes for isolated European lations of *S. glanis* should be undertaken. In Zech Republic, live gene resources of Bohemi- opulations of European catfish are maintained bank of cryopreserved sperm [10,19].

ryopreservation of sperm could be a valuable ique for the artificial reproduction and genetic ovement of this species. The first successful pt to cryopreserve European catfish sperm chieved by Marian and Krasznai [26] who ob- d 40–95% fertilization capacity from frozen ular sperm. The testicular sperm was used for ing, because the artificially stripped sperm found polluted with urine, which activated destroyed the fertilization capacity of sperma- within a few minutes [20]. This problem was ssfully solved by development of immobilizing ion (IS) [21,25] and cryopreservation tech- es were refined in later studies, which reported ne optimal cryoprotectant to be used for IS ded sperm and freezing rates [21]. The cryo- rvation procedures have been successfully oped for five other catfish species, African cat- *Clarias gariepinus* B. [14,31,34,37–41]; channel sh, *Ictalurus punctatus* [5,36]; blue catfish, *urus furcatus* [17]; *Pseudoplatystoma corruscans* nd striped catfish, *Pangasius hypophthalmus* but only using sperm extracted from testes.

ne aim of the present study was to elaborate preservation methods for ex situ conservation uropean catfish. The success of the sperm ing procedure was evaluated by sperm veloci- ercentage of sperm motility, percentage of live matozoa, fertilization, and hatching rates in n/thawed sperm.

erials and methods

ials

ne experiments were performed at the experi- al hatchery of the University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Department of Fish Genetics and Breeding at Vodnany, Czech Republic. The broodfish were cultivated and prepared for reproduction according to Linhart et al. [23,25]. Individual broodfish suitable for stripping were selected in May–July and kept isolated in 4000 litre tanks divided into 3–4 compartments with a water flow rate of 0.2 L/s at 22–23 °C. The spermiation and ovulation were stimulated by carp pituitary (CP) injected intramuscularly at doses of 5 mg per kg of body weight. Sperm was collected 48 h after CP stimulation. Ovulated oocytes were stripped from individual females. Females were stripped at 500–510 degree-hours post-stimulation (degree-hours = sum of consecutive average temperatures registered each hour; about 23 h). Eggs from three females were then pooled, short-term stored at a temperature of 18–20 °C, and directly used for experiments. Prior to the fertilization experiments, three batches of approximately 1 g (around 150 eggs) of pooled unfertilized eggs were weighed to the nearest 0.0001 g and fixed in 4% formaldehyde for later counting and determination of mean egg weight. On this basis, the number of eggs in a sample was expressed as the weight of the sample (*g*) multiplied by 145. Before each injection and gamete collection, the males and females were anaesthetized in a solution of 2-phenoxyethanol (1:1000).

Sperm collection and dilution

Sperm was collected individually (8 males) from the genital papilla into 250 ml plastic containers for cell culture filled with 10 ml of immobilizing solution (IS = 200 mM NaCl, 30 mM Tris–HCl, pH 7 [18]. Maximally, 8 ml of sperm was taken into one container to keep dilution rate at $\leqslant$1:0.8 (IS/sperm) to prevent spontaneous initiation of motility [25]. After collection, the containers were stored under aerobic conditions on ice for 1.5 h. The quality of the extended sperm was checked for the percentage of sperm motility from video records. Only four males showed spermatozoa motility higher than 80%; their sperm was pooled and used for cryopreservation. Pooled sperm concentration was counted in a Thoma cell haemocytometer under an Olympus

oscope BX 41 (400×) and the value was ex-
sed as the mean number of spermatozoa
ted in 20 squares of the Thoma cell.

preservation and thawing

ifferent concentrations of pure cryoprotectant
/v) or mutual combinations (methanol—Fluka
65543; 1,2-propandiole P-1009, Sigma;
SO—Sigma Kat. No. D-8779) were added to
ded sperm and every 1 ml of mixture was
ferred into a 1.8 ml cryotube (Nunce, 375418)
immediately and directly transferred to a pre-
rammed device PLANER Kryo 10 series III
4 °C and cooled from +4 to −9 °C at a rate of
/min, then from −9 to −80 °C at a rate of
C/min, held for 6 min at −80 °C, and finally
ferred into liquid nitrogen (LN_2) and stored
until next morning (see Fig. 2). The sperm
hawed in a water bath at 40 °C for 105 s. A part
e pooled sperm in IS was kept on ice (0–4 °C)
next morning and used as an unfrozen control.

rimental design

ne pool of sperm and one egg pool was used for
periments. For each treatment, six cryotubes
cryopreserved and the following parameters
evaluated after 24 h storage for each: velocity
erm, percentage of sperm motility, percentage
e sperm, percentage of fertility, and percentage
tching. The same parameters were measured in
ontrol sample of unfrozen fresh sperm.
the first series of experiments, the effect of
cryoprotectants, in different concentration
ombinations, and two different storage times
5 h) of sperm cryopreservation were studied.
ryoprotectants were Me_2SO at final concen-
ns of 8, 10, and 12%; methanol at final con-
ations of 5, 7.5, and 10%, and a mixture of
O with propandiole (ratio 1:1) at final con-
ations of 8, 10, and 12%. One milliliter of
xtended sperm was frozen in cryotubes of
l.
the second series of experiments, three equil-
on periods were tested. Ten percentage of
O was added to the extended sperm stored
h. The solution was gently homogenized
and equilibrated for 0, 5, and 20 min on ice (0–4 °C). One milliliter of the solution was transferred into cryotubes of 1.8 ml and frozen.

In the third series of experiments, three different volumes of cryotubes were tested. Ten percentage of Me_2SO was added to extended sperm stored for 5 h. The mixture was gently mixed, and volumes of 1 and 1.8 were frozen in 1.8 ml cryotubes and a volume of 4 ml in 4 ml cryotubes.

Fertilization and hatching trial

Five grams of eggs (145 eggs per 1 g) was placed into a 50 ml dish; an accurate volume of sperm (thawed or nonfrozen as control) with 104,000 spermatozoa per one egg was dropped on them from a micropipette. The dish was then placed on a shaking table set to 200 rpm and 10 mm deflection. Five milliliters of activation solution (AS = 17 mM NaCl, 5 mM Tris–HCl, pH 8) at 22 °C was added. After 3 min, around 100 eggs were placed into a special incubator cage of 200 ml supplied with UV-sterilized recirculated tap water at 23 °C, 9 mg/L O_2. For each experiment, the procedure was repeated four times. The dead eggs were counted and removed from each cage a day after fertilization and after hatching (approximately 2.5 days after fertilization); the number of larvae was counted in each cage as well.

The percentage fertilization rate (F_r) was calculated for each cage from the total number of eggs placed in the cage (E_t) and from the number of dead eggs (E_d) collected 24 h after fertilization as follows:

$$F_r = [(E_t - E_d)/E_t] \times 100.$$

The percentage hatching rate (H_r) was calculated for each cage from the number of hatched larvae (H_l) and from the total number of eggs placed in the cage (E_t) as follows:

$$H_r = (H_l/E_t) \times 100.$$

Observation and measurement of sperm motility and velocity

Dark-field microscope and a Sony camera were used as described by Billard et al. [3], Cosson

et al. [6], and Linhart et al. [24] (see scheme in Fig. 1). Percentage motility and velocity was examined at 200× magnification immediately after mixing 1 μl of sperm with 49 μl of swimming medium supplemented with 0.1% BSA on a glass slide prepositioned on the microscope stage. The final dilution was 1:50. BSA was added to prevent sperm heads from sticking to the glass slide. Sperm motility was recorded on video tape from 0 to 2 min after activation. The focal plane was always positioned near the glass slide surface. Video records were made with a S-VHS (SONY, SVO-9500 MDP) video recorder at 50 half frames per second using a CCD video camera (SONY, SSC-DC50AP) mounted on a dark-field microscope (Olympus BX 50) and visualized on a video monitor illuminated with a Strobex stroboscopic lamp (Chadvick-Helmut, 9630, USA) with a frequency of 50 Hz.

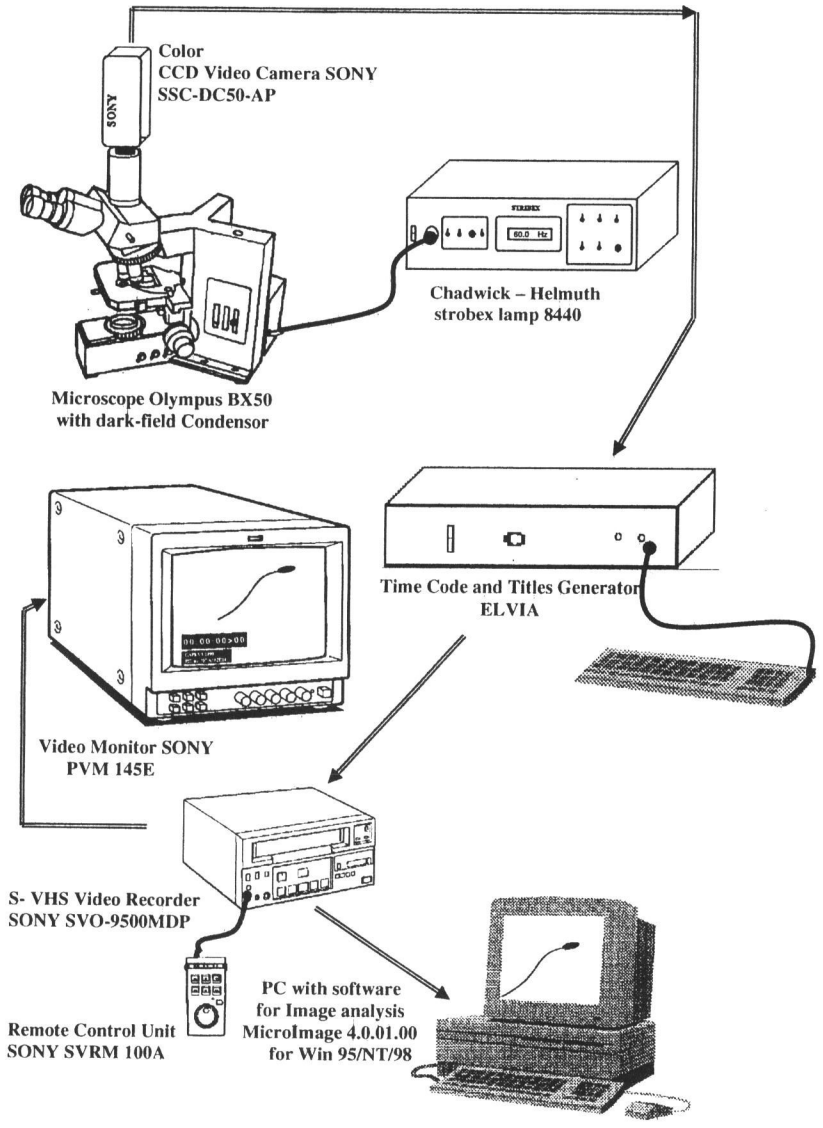

Fig. 1. Scheme of the equipment for evaluation of sperm motility.

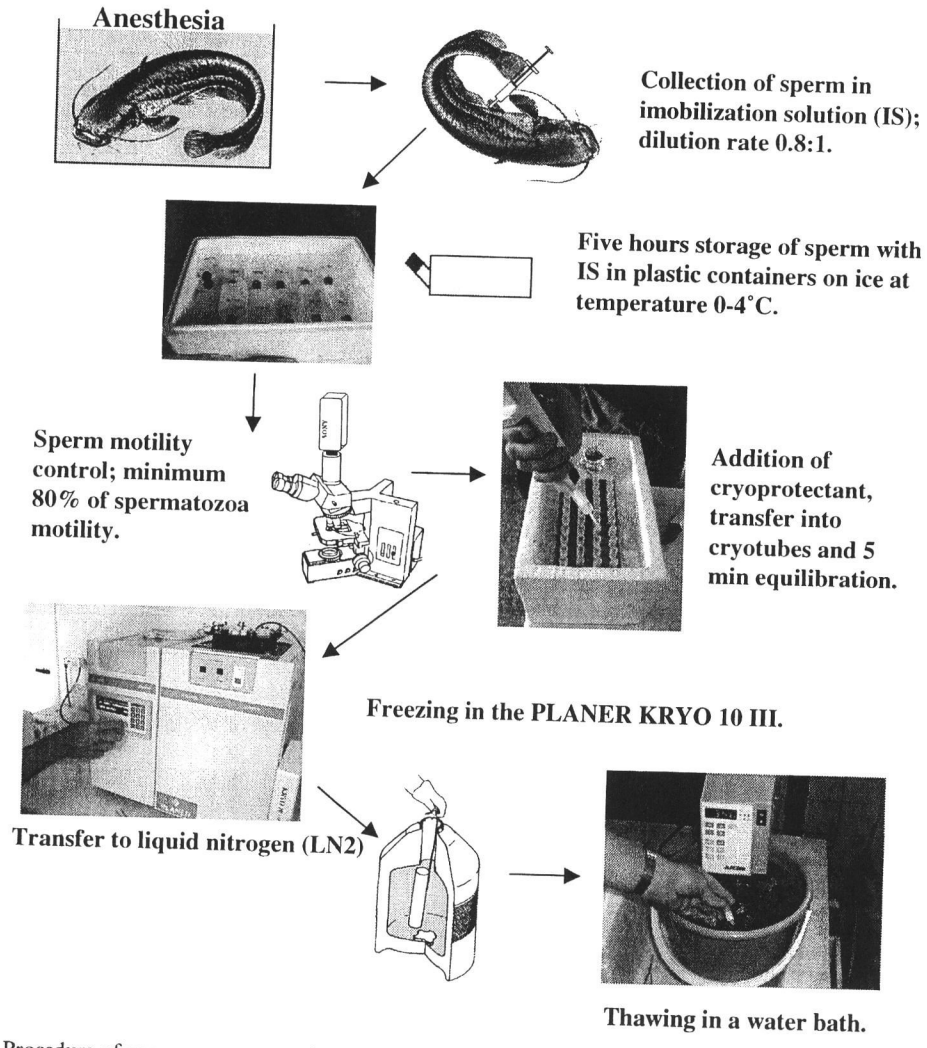

Fig. 2. Procedure of sperm cryopreservation in European catfish after stripping of hormonally treated broodstock.

Velocity and motility were assessed at 20 s after activation: the successive positions of the video-recorded sperm heads were analysed from video frames by means of Olympus Micro Image software (Version 4.0.1. for Windows with a special macro by Olympus C&S). The velocity and percentage motility were measured by evaluating spermatozoa head positions on five successive frames with three different colours (frame 1—blue, frames 2–4—green, and frame 5—red). The analyses were repeated three times from three records at 20 s after activation, i.e., frames 1–5. Thirty to forty spermatozoa were evaluated from each frame.

Motile spermatozoa were visible in three colours, while non-moving spermatozoa were white. Percentage of motile spermatozoa was easily calculated from white versus red coloured cells (green was not used). Velocity of spermatozoa was calculated as μm/s based on the length traces of sperm heads (blue–green–green–green–red) of the motile spermatozoa after calibration of magnification.

Sperm viability test

Absolute numbers and frequencies of live and dead spermatozoa were determined according to

Table 1
Effect of nine different cryoprotectants and two different storage times on the fertility (percentage of hatching) of frozen/thawed sperm

Cryoprotectants	Sperm storage, 1 h (%)				Sperm storage, 5 h (%)			
	Mean	SD	Sign.	Sign.	Mean	SD	Sign.	Sign.
Me_2SO 8%	71.6	±4.8	bcde	A	85.8	±6.5	cd	B
Me_2SO 10%	58.1	±3.7	bc	A	83.3	±5.1	cd	B
Me_2SO 12%	59.6	±10.3	bc	A	76.4	±3.4	cd	B
Me_2SO 4% + propandiole 4%	78.0	±2.3	de	A	84.1	±5.0	cd	A
Me_2SO 5% + propandiole 5%	70.7	±5.6	bcd	A	82.4	±3.2	cd	B
Me_2SO 6% + propandiole 6%	73.5	±4.4	cde	A	73.4	±6.2	bc	A
MeOH 5%	38.0	±7.3	a	A	62.2	±3.7	ab	B
MeOH 7.5%	58.8	±14.2	bc	A	52.9	±8.7	a	A
MeOH 10%	56.0	±7.0	b	A	51.9	±4.6	a	A
Control of fresh sperm	87.9	±2.6	e	A	87.9	±2.6	d	A

Mean values of four replicates are shown with SD. Groups with a common superscript (small type for columns and high type for rows) for each parameter do not differ significantly ($P < 0.05$).

Table 2
Effect of nine different cryoprotectants and two different storage times on percentage of live spermatozoa in frozen/thawed sperm

Cryoprotectants	Sperm storage, 1 h (%)				Sperm storage, 5 h (%)			
	Mean	SD	Sign.	Sign.	Mean	SD	Sign.	Sign.
Me_2SO 8%	30.5	±5.2	bcd	B	7.7	±4.7	a	A
Me_2SO 10%	19.1	±11.9	b	A	16.8	±9.3	ab	A
Me_2SO 12%	22.8	±10.0	bc	A	19.2	±7.9	ab	A
Me_2SO 4% + propandiole 4%	49.4	±5.4	e	A	52.4	±8.6	c	A
Me_2SO 5% + propandiole 5%	35.1	±6.6	cde	A	38.4	±24.2	bc	A
Me_2SO 6% + propandiole 6%	43.7	±4.6	de	A	59.0	±5.1	c	B
MeOH 5%	30.6	±13.5	bcd	A	45.2	±10.4	c	A
MeOH 7.5%	34.4	±3.5	bcde	A	52.4	±6.0	c	B
MeOH 10%	3.8	±2.6	a	A	42.7	±12.0	c	B
Control of fresh sperm	91.6	±7.2	f	A	91.6	±7.2	d	A

Mean values of 10 replicates are shown with SD. Groups with a common superscript (small type for columns and high type for rows) for each parameter do not differ significantly ($P < 0.05$).

Rajshans et al. [11]. A Sperm Viability Kit (Molecular Probes, USA) for live/dead spermatozoa was used for dual DNA staining. A membrane-permeant SYBR 14 stained live spermatozoa green, while membrane-impermeant propidium iodide (PI) stained dead spermatozoa red. Both the dyes (10 μl) were added to 1 ml sperm samples, vortexed, and a 40 μl drop was incubated for 10 min on a microscopic slide under an Olympus BX 60 microscope in dark. Epifluorescence microscopy employed a 100 W Ushio mercury lamp, an Olympus MWB filter cube (wide band, blue excitation 450–480 nm, and emission above 515 nm), and an Olympus objective UMPlanFl 20×. Ten fluorescent images per sample were recorded with a 3CCD Sony DXC-9100P colour camera and saved using Olympus Micro Image v. 4.0 software. Time elapsed between recording the 1st and the 10th images was not allowed to exceed 1.2 min. Images were then examined for the presence of green and red fluorescent signals. A "Ranges Statistics" function of the Olympus Micro Image v. 4.0 software was employed to count the absolute numbers of red and green objects and to compute their frequencies as percentages of green (live) or red (dead) objects.

Data analysis

Mean values of the investigated parameters and SD were calculated from 4, 3, and 10 repli-

Table 3
Effect of nine different cryoprotectants and two different storage times on percentage of sperm motility of frozen/thawed sperm

Cryoprotectants	Sperm storage, 1 h (%)				Sperm storage, 5 h (%)			
	Mean	SD	Sign.	Sign.	Mean	SD	Sign.	Sign.
Me_2SO 8%	35.0	±2.3	ab	A	50.0	±0	bc	B
Me_2SO 10%	20.9	±11.4	ab	A	61.6	±0.3	c	B
Me_2SO 12%	44.6	±1.2	b	A	49.3	±3.4	bc	A
Me_2SO 4% + propandiole 4%	37.9	±12.0	b	A	57.0	±5.2	c	A
Me_2SO 5% + propandiole 5%	28.6	±5.0	ab	A	50.6	±13.3	bc	A
Me_2SO 6% + propandiole 6%	41.7	±19.6	b	A	58.7	±0.5	c	A
MeOH 5%	0	±0	a	A	8.3	±0	a	B
MeOH 7.5%	14.4	±4.6	ab	A	19.8	±6.3	a	A
MeOH 10%	12.1	±8.2	ab	A	24.3	±14.1	ab	A
Control of fresh sperm	96.0	±0.8	c	A	96.0	±0.8	d	A

Measurements were made 20 s after activation. Mean values of three replicates are shown with SD. Groups with a common superscript (small type for columns and high type for rows) for each parameter do not differ significantly ($P < 0.05$).

Table 4
Effect of nine different cryoprotectants and two different storage times on sperm velocity (μm/s) of frozen/thawed sperm

Cryoprotectants	Sperm storage, 1 h (%)				Sperm storage, 5 h (%)			
	Mean	SD	Sign.	Sign.	Mean	SD	Sign.	Sign.
Me_2SO 8%	51.9	±21.8	Abc	A	44.9	±17.4	abc	A
Me_2SO 10%	33.0	±12.3	Ab	A	42.3	±15.5	ab	B
Me_2SO 12%	34.1	±13.7	Abc	A	37.3	±17.6	a	A
Me_2SO 4% + propandiole 4%	41.6	±24.9	Abc	A	61.2	±13.2	c	B
Me_2SO 5% + propandiole 5%	49.0	±22.3	Abc	A	57.9	±17.2	bc	A
Me_2SO 6% + propandiole 6%	35.0	±13.5	Abc	A	44.5	±11.8	abc	B
MeOH 5%	0	±0	A	A	45.5	±20.2	abc	A
MeOH 7.5%	55.7	±25.8	Abc	A	82.6	±18.4	d	B
MeOH 10%	105.9	±32.4	d	B	84.7	±27.7	d	A
Control of fresh sperm	84.8	±17.7	d	A	84.8	±17.7	d	A

Measurements were made 20 s after activation. Mean values of three replicates are shown with SD. Groups with a common superscript (small type for columns and high type for rows) for each parameter do not differ significantly ($P < 0.05$).

Table 5
Effect of three equilibration periods on the quality of frozen/thawed sperm

Equilibration time (min)	Hatching rate (%)			Live spermatozoa (%)			Motility of sperm 20 s after activation (%)			Velocity of spermatozoa 20 s after activation (μm/s)		
	Mean	SD	Sign.	Mean	SD	Sign.	Mean	SD	Sign.	Mean	SD	Sign.
0	77.6	±7.3	ab	44.7	±4.1	ab	34.4	±10.6	a	44.0	±16.4	a
5	84.0	±3.4	bc	32.3	±12.0	a	49.4	±11.4	a	41.5	±16.2	a
20	73.8	±0.7	a	57.6	±3.0	b	62.5	±5.9	ab	41.4	±17.4	a
Control of fresh sperm	87.9	±2.6	c	91.6	±7.2	c	96.0	±0.8	b	84.8	±17.7	b

Extender: IS +sperm and 10% Me_2SO. Sperm motility and velocity were measured 20 s after activation. Mean values of 4 (fertilization and hatching), 10 (live sperm), and 3 (motility and velocity of sperm) replicates are shown with SD. Groups with a common superscript for each parameter do not differ significantly ($P < 0.05$).

Table 6
Effect of cryopreservation of different sperm volumes on quality of frozen/thawed sperm

Frozen volume (ml)	Hatching rate (%)			Live spermatozoa (%)			Motility of sperm 20 s after activation (%)			Velocity of spermatozoa 20 s after activation (μm/s)		
	Mean	SD	Sign.	Mean	SD	Sign.	Mean	SD	Sign.	Mean	SD	Sign.
1	80.2	±8.1	ab	3.3	±1.7	a	46.7	±2.9	a	46.4	±17.0	A
2	80.7	±5.5	ab	14.7	±5.2	ab	36.5	±9.6	a	39.6	±14.2	A
4	70.9	±6.4	a	19.6	±10.0	b	51.1	±4.2	a	47.1	±14.8	A
Control of fresh sperm	87.9	±2.6	b	91.6	±7.2	c	96.0	±0.8	b	84.8	±17.7	B

Extender: IS +sperm and 10% Me_2SO. Sperm motility and velocity were measured 20 s after activation. Mean values of 4 (fertilization and hatching), 10 (live sperm), and 3 (motility and velocity of sperm) replicates are shown with SD. Groups with a common superscript for each parameter do not differ significantly ($P < 0.05$).

cates for fertilization and hatching, velocity, and percentage of sperm motility, and live/dead spermatozoa, respectively. Statistical significance was assessed using multiple analysis of variance (ANOVA, Statgraphics version 5), followed by Tukey HSD multiple range test comparisons. Correlation was calculated from the nine mean values of the following parameters: velocity of post-thawed sperm, percentage of post-thawed sperm motility, percentage of live post-thawed sperm, percentage of fertility, and percentage of hatching. Probability values <0.05 were considered as significant.

Results

In all experiments, analysis of variance (ANOVA, Statgraphics version 5) showed no significant differences ($P < 0.05$) between fertilization and hatching rate. Therefore, only results of hatching rate are subsequently presented.

Effects of cryoprotectant and storage time

Control sperm had good quality (Tables 1–4). In experiments, the sperm quality changed depending on treatment. In many treatments, better results were obtained with sperm stored for 5 h instead of only 1 h.

The best hatching rates of 82–86% were obtained when sperm was stored for 5 h before freezing in IS and frozen either with Me_2SO in concentrations of 8, 10, or 12% or a mixture of 5% Me_2SO and 5% propandiole. These results were not significantly different from that of the control sample (Table 1).

The percentage of live spermatozoa in the post-thawed sperm of European catfish ranged from 8 to 59%. The highest percentages of live spermatozoa were found in a mixture of Me_2SO (6%) and propandiole (6%) and in methanol (7.5 and 10%; Table 2). The percentage of live spermatozoa in post-thawed sperm was not correlated with the hatching rate ($r = 0.24$, $P = 0.33$) nor with the motility rate of spermatozoa ($r = 0.16$, $P = 0.52$), but was correlated with the velocity of spermatozoa ($r = -0.47$, $P = 0.05$).

The percentage motility in frozen/thawed sperm ranged from 8 to 62%, when sperm was stored in IS for 5 h before freezing (Table 3). The percentage motility in the fresh sperm (control) was 96%. Ten percentage of Me_2SO yielded the highest percentage motility in post-thawed sperm (62%). A mixture of Me_2SO/propandiole and pure Me_2SO preserved post-thaw motility better than did methanol (Table 3). In cryopreserved sperm, motility rate significantly correlated with the hatching rate ($r = 0.76$, $P = 0.0002$), but not with the velocity of spermatozoa ($r = 0.07$, $P = 0.79$).

The velocity of frozen thawed spermatozoa ranged from 37 to 85 μm/s, when sperm was stored in IS for 5 h before freezing. Methanol at concentrations of 7.5–10% yielded the highest velocity in frozen thawed sperm (105 μm/s) which was similar to the velocity of the control (Table 4). The post-thaw sperm velocity was not correlated with the hatching rate ($r = 0.03$, $P = 0.90$).

Equilibration time

The highest hatching rates were obtained in the control (nonfrozen sperm) and in sperm equilibrated for 5 min (Table 5). Equilibration time had no significant effect on velocity of spermatozoa or on percentage of post-thawed sperm motility. Longer equilibration time resulted in a significantly higher percentage of live spermatozoa.

Sperm volume used for cryopreservation

Hatching rate was better when sperm was frozen in volumes of 1 or 1.8 ml than in 4 ml (Table 6). Percentage of live spermatozoa in post-thawed sperm was significantly higher when 1.8 and 4 ml volumes were frozen than 1 ml volumes. No effect on velocity or percentage of post-thawed sperm motility was observed.

Discussion

When fertilization assays with cryopreserved sperm are conducted to detect the optimal freezing protocol, it is necessary to use a low or medium number of spermatozoa per egg. A number of 8000–80,000 fresh spermatozoa per egg is required for artificial insemination in European catfish but the practical quantity in hatcheries is recommended around 100,000–200,000 spermatozoa per egg [25]. If a high number of spermatozoa per egg is used, low viability and bad quality of post-thawed sperm could be masked. Moreover, using a high number of spermatozoa is not pragmatic for artificial propagation, as males of European catfish generally have a low volume of sperm and so sperm wastage should be avoided. The dilution ratio of sperm and egg with water is another parameter which must be adjusted well. One part sperm to 25 parts of water to 25 parts eggs was suitable for artificial propagation of European catfish in a previous study [25].

The freezing procedure applied was modified from cryopreservation studies on common carp [22] but the same procedure was also previously tested successfully in European catfish with cryo-extender containing 10% Me_2SO as cryoprotectant [19]. The first step of the freezing programme was a slow cooling from +4 to −9 °C at a rate of 4 °C/min. When ice started to form in the external medium, the freezing rate was increased to 11 °C/min from −9 to −80 °C. The temperature of −80 °C was held for 6 min and then the samples were transferred into LN_2. The cooling procedure agreed with data published for African catfish [33,40,41], where a 3-step programme showed better survival in post-thawed sperm compared with a 2-step programme. A cooling rate from 5 to 11 °C/min was specified as optimal. All authors observed that the final temperature and its duration (holding period) just before plunging the frozen sperm into LN_2 were very important. During the holding period, the temperature in the freezer remains constant, but the temperature of freezing sperm still drops until equilibration. Most of the freezing protocols in catfish cool sperm to a low temperature before plunging it into LN_2. Steyn et al. [34] used −65 °C, and Rurangwa et al. [31] and Horváth and Urbanyi [14] −80 °C in African catfish; in European catfish Linhart et al. [21] used −85 °C; in striped catfish Kwantong and Bart [16] used −80 °C, as did Tiersch et al. [36] and Christensen and Tiersch [5] in channel catfish. The warming temperature during thawing of the frozen sperm was 40 °C in the present experiments, in the range of 25–40 °C used by the above-mentioned authors.

The sperm motility of European catfish lasts 1–2 min depending on the osmolality of the medium used to initiate the motility [2]. The low osmolality of urine is the key factor for spontaneous activation of European catfish spermatozoa. The osmolality of urine is eight times lower than that of seminal fluid. The negative effects of urine on European catfish sperm quality are well documented [2,20,25]. Therefore, in the present study the sperm–urine mixture was collected directly into IS and stored at 0 °C in aerobic conditions (see Fig. 2). This study demonstrated that storage of sperm in IS for 5 h before freezing increased the hatching rates, the percentage sperm motility, and the percentage live spermatozoa. When sperm are spontaneously activated with urine, ATP may be lost before dilution of sperm in IS. The relation

between motility, respiration, and ATP production was investigated in turbot (*Psetta maxima*) [9] and in common carp (*Cyprinus carpio*) spermatozoa [29], and usually 50% of ATP was exhausted during the first 5–10 s. Long storage times at optimal osmotic, ionic, and pH conditions could enable the reconstitution of the ATP content in sperm. The assessment of spermatozoa viability by means of dual staining with fluorescent dyes [12] has been widely utilized for fish sperm [8,11,30,32]. As this assay is based upon testing the membrane integrity by synchronously labelling the spermatozoa either with membrane-permeant, or membrane-impermeant fluoresceins [12], only cells with intact membrane integrity are considered "viable" or "live." Trying to correlate the proportion of live spermatozoa with motility and/or fertilization rates presents a potential source of bias. The category of live spermatozoa may not only involve live spermatozoa which are motile but also those which have their ATP content already exhausted [7] and therefore are immotile. This could explain why the proportion of live spermatozoa is higher than that of motile ones.

It was found that Me_2SO, and a mixture of Me_2SO and propandiole resulted in a significantly better hatching rate and percentage of live spermatozoa. Such cryoprotectant composition was not used before in catfish sperm cryopreservation. Despite the vast range of cryoprotectants tested in fish, the most common ones for protection of catfish sperm during freezing are: Me_2SO, glycerol, methanol, dimethyl acetamide (DMA), ethylene glycol, and propylene glycol [14,5,21,31,34,36]. Me_2SO was recommended only for striped catfish [16], *Pangasius gigas* [27], blue catfish [1], and in some papers for African catfish [14] and channel catfish [13]. According to the present results, 8 or 10% of Me_2SO can be used in European catfish as well. Recent studies on African catfish [40] and channel catfish [5,36] showed improved sperm motility and fertility in the post-thawed sperm when methanol was applied.

A 5 min equilibration of sperm in IS containing 10% Me_2SO increased the hatching rates in European catfish. Equilibration time of catfish or common carp sperm in the cryoprotectant is usually not recommended. The slight increase in hatching rates could be explained by an effect of equilibration with a slow shock process at the sperm plasma membrane, when intracellular water was replaced by Me_2SO. The penetration is not triggered by sperm motility, as was observed with sperm of common carp, turbot [7], and sea urchin [35]. Ogier de Baulny et al. [28] obtained interesting results when freezing testicular sperm of European catfish with methanol, Me_2SO, and DMA. DMA was determined as the best cryoprotectant. It increased the ATP level in sperm up to three times within 30 s either as DMA was used in sperm energy metabolism or stimulated energy metabolism of DMA on ATP synthesis.

The freezing volume is generally an important factor for freezing procedure in cryopreservation of fish sperm. All of the volumes tested (1, 1.8, and 4 ml) were suitable for cryopreservation of European catfish sperm. In another study [5], evaluating the sperm motility in 0.25 ml straws was better than 0.5 ml straws in channel catfish. For the prospective European catfish farming, it is more convenient to freeze larger volume of sperm in one tube. This procedure enables the total storage capacity of sperm in one container to be increased as the empty space among tubes is eliminated. Manipulation with bigger tubes is more practical, too.

Cryopreservation of sperm appears to be a useful and reliable technique not only for conservation of gene resources in the European catfish but also for wide-ranging artificial propagation (see Fig. 2).

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Optimization of artificial propagation in European catfish, *Silurus glanis* L.

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Abstract

The present work optimizes procedures for artificial insemination of European catfish, *Silurus glanis* L., with goals to increase collection of sperm and hatching rate. We address technical aspects of immobilizing solution (IS), activation solution (AS), process of insemination, activation of gametes and elimination of eggs stickiness with a practical solution for artificial propagation in hatcheries. Spermiation was stimulated and could be sustained over a period of 1 month after weekly carp pituitary (CP) injection of 5 mg kg^{-1} b.w. Males produced significantly the largest quantity of sperm, 0.12–0.13·10^9 spermatozoa kg^{-1} b.w., after third and fourth injections. The best IS for sperm was determined as solution containing 170–200 mM NaCl, 30 mM Tris–HCl, pH 7 and activation solution for activation of gametes containing 17 mM NaCl, 5 mM Tris–HCl, pH 7. The ratio of 1:2 rather than ratio of 1:1 in the control batch (volume of activation solution in milliliters/grams of eggs) increased the hatchability up to 68%. The highest hatching success of 82–88% from four individual females were found for sperm number of 800–80,000 spermatozoa egg^{-1}, in comparison to 26% hatching at 80 spermatozoa egg^{-1}. The best time for application of alcalase enzyme for elimination of egg stickiness was 3 min after gamete activation at concentration of 20 ml enzyme l^{-1} with exposure of 2 min.

Keywords: European catfish *Silurus glanis*; Artificial reproduction; Eggs; Spermatozoa; Aquaculture; Fish

1. Introduction

The European catfish *Silurus glanis* has been cultivated in extensive ponds in Central and Eastern Europe together with carps for more than 100 years. The total

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aquaculture production of European catfish from 10 European countries (Austria, Bulgaria, Croatia, Germany, France, Hungary, Greece, Macedonia, Poland, Czech Republic and Romania) was 602 t in 1993 and presently it is about 2000 t (Linhart et al., 2002).

European catfish suitable for stripping are selected in May–July and kept separately in tanks divided into several compartments (Linhart and Billard, 1995) or if kept together the mouth of fish can be closed with a piece of string passed through holes drilled in the nasal and chin bones after anesthetization to restrict injury of fish during mutual attack. This procedure does not disturb either respiration or sexual maturation (Horvath and Tamas, 1976). Presently, hormonal treatments for induced spawning are practiced in European catfish and are mostly based on the use of carp pituitary (CP). Females ovulate after one or two CP doses of 4–5 mg kg^{-1} b.w. and males after a single injection of 3–5 mg kg^{-1} b.w. (Fijan, 1975; Horvath and Tamas, 1976; Linhart and Billard, 1994; Kouril et al., 1995). Epler and Bieniarz (1989) was successful in spawning females after a single injection of LH-RH analogue (LHRHa), combined with pimozide. The effectiveness of LHRHa was not improved by the addition of domperidone (Kouril et al., 1995), because the best results were obtained with LHRHa alone. Brzuska and Adamek (1999) and Brzuska (2001) successfully stimulated ovulation with LHRHa and pimozide and also with Ovopel (LHRHa, D-Ala^6 manufactured in Hungary) without pimozide. Males are oligospermic and sperm is always contaminated and activated by urine during stripping (Linhart et al., 1986). Sufficient volume of sperm, storage and sperm motility are priorities for successful artificial reproduction of European catfish (Linhart et al., 1987; Legendre et al., 1996). The sperm is stripped into an immobilization solution (IS) (Linhart et al., 1987; Saad and Billard, 1995) or males are sacrificed (Fijan, 1975) or the testes are surgically removed by caesarean section and fish are kept alive (Krasznai et al., 1980). The fertilizing capacity of fresh sperm, sperm in immobilizing solution and testicular sperm was compared by Linhart et al. (1987) with successful improvement of fertilizability when sperm was diluted in immobilizing solution (137 mM NaCl, 67 mM KCl, 133 mM glycine). Nowadays, the sperm is collected in immobilization solution of 200 mM NaCl, 30 mM Tris–HCl, pH 7 (Linhart and Billard, 1994; Saad and Billard, 1995), and stored for 48–72 h at 4 °C. Eggs for artificial insemination were mixed together with sperm and immediately activated in an activating solution (Legendre et al., 1996). Horvath (1977) used an activation solution (AS) of 0.3% NaCl for fertilization. After 5 min, fertilized eggs were incubated in the Weiss' jars or in a net in a tank. The eggs' stickiness is eliminated with a clay suspension or talc suspension added to the fertilized eggs (Kouril et al., 1995), with alcalase enzyme solution after 10–12 h of incubation (Horvath, 1977, 1980) or directly after fertilization using proteolytic trypsin or alcalase (Legendre et al., 1996). Fertilized eggs are incubated in Weiss jars. Hatching is expected 2.5–3 days (60 degree-days) after fertilization at 22–23 °C, and the 1-day-old embryos weigh 2.6 mg each (Kouril et al., 1995; Linhart et al., 2002).

The present work was undertaken to establish optimized procedures for artificial insemination in European catfish, *S. glanis* L. The objectives of this study are to summarize the results of European catfish reproduction concerning: spermiation with

optimization of IS, AS, process of insemination, activation of gametes, elimination of egg stickiness and increased hatch rate with a practical proposal for artificial reproduction in hatcheries.

2. Materials and methods

The experiments were performed during several years at the experimental hatchery of the University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Department of Fish Genetics and Breeding at Vodnany, Czech Republic.

2.1. Broodstock handling and sperm collection

The broodfish were cultivated and prepared for reproduction according to Linhart et al. (1987, 2002) and Linhart and Billard (1994, 1995). Individual broodfish suitable for stripping were selected in May–July and kept isolated in 4000-l tanks divided into three to four compartments with a water flow rate of 0.2 l s^{-1} at 22–23 °C. The spermiation and ovulation were stimulated by CP injected intramuscularly at doses 5 mg kg^{-1} b.w. Females were stripped at 500–510 degree-hours (degree-hours = sum of temperature at each hour) post-stimulation. Sperm was collected 24/48 h after CP stimulation into a 20-ml syringe or tube containing 10 ml of immobilizing solution (200 mM NaCl, 30 mM Tris–HCl, pH 7). Sperm from minimally six individual males was stored at most 12 h in refrigerator at 0–2 °C and pooled prior to fertilization (Experiments 2–7). Ovulated oocytes were stripped from individual females, eggs from a minimum of four females were then pooled (Experiments 2, 3, 4 and 7) or eggs from individual females were used (Experiments 5 and 6), stored at a temperature of 18–20 °C and directly used for experiments. Prior to the fertilization experiments with pooled sperm (Experiments 2–7), sperm concentration was determined with Thoma cell hemocytometer under microscope (400 ×) and mean number was expressed per 20 squares of Thoma cell. Before injection and gamete collection, the males and females were anaesthetized in a solution of 2-phenoxyethanol (1:1000).

2.2. Artificial insemination with test of hatching

Different approaches to artificial insemination were assessed by results of fertilization and hatching rate as followed: 6 g of eggs (160 eggs/1 g; Linhart et al., 2002) were placed into a 50-ml dish; an accurate volume of sperm with estimated number of spermatozoa per milliliter was dropped on them from a micropipette. The dish was then placed on a shaking table with constant 200 rpm and with 10-mm deflection. Six milliliters of activation solution (17 mM NaCl, 5 mM tris, pH 8) or experimental activation solution at 22 °C was added. Three minutes later, around 100 eggs were placed into a special incubator cage of 200 ml supplied with UV sterile recirculated tap water at 23 °C, 9 mg l^{-1} O_2. For each experiment, the procedure

was replicated three to eight times depending on type of experiment. The eggs were counted in each cage and, later during incubation of eggs, the dead eggs and hatched fry were counted, usually up to 3 days of incubation.

The percentage fertilization rate (F_r) was then calculated (Experiments 2, 3, 5, 6 and 7) for each cage from the total number of eggs placed in the cage (E_t) minus dead eggs (E_d) collected up to 24 h after fertilization as followed:

$$F_r = [(E_t - E_d)/E_t] \times 100$$

The percentage of hatching rate (H_r) was also calculated for each cage from the total number of eggs placed in the cage (E_t) and divided with the number of hatched larvae (H_l) as followed:

$$H_r = (H_l/E_t) \times 100$$

2.3. Evaluation of egg incubation quality by comparison fertilization and hatching rate

Differences between fertilization rates measured 24 h after gamete activation and hatching rate 72 h after activation were compared in Experiments 2, 3, 5, 6 and 7 to evaluate quality of egg incubation. Analysis of variance (ANOVA, Statgraphics version 5) showed no significant differences ($P<0.05$) between fertilization and hatching rate. Therefore, only results of hatching rate are subsequently presented.

2.4. Stimulation of spermiation by repeated CP injection (Experiment 1)

Eight males were treated with CP dissolved in Ringer solution (0.5 ml kg^{-1} b.w.) and injected intramuscularly at a dose of 5 mg kg^{-1} b.w. One control group of four males was injected with Ringer solution only. The males were injected four times: in 0th, 6th, 12th and 18th day of experiment with collection of sperm 2 days after each injection. Males were treated after each manipulation in a disinfection bath of 1 g potassium permanganate/100 l water and between stripping were fed with one or two forage fish (100 g). Sperm was collected from the genital papilla into a syringe containing an immobilizing solution of 200 mM NaCl, 30 mM Tris–HCl, pH 7 in order to prevent spontaneous initiation of motility. Sperm was stored on ice until measurement of volume and sperm concentration. Results were averaged from eight replications as volume of sperm per kilogram of male body weight and number of spermatozoa per kilogram of male body weight, according to methods described by Linhart et al. (1995).

2.5. Effect of different NaCl concentration in immobilizing solution (Experiment 2)

Sperm from six different males was collected in IS of 170, 200 and 230 mM NaCl concentration, containing 30 mM tris, pH 7 and for a control without using IS. Collected sperm was stored in aerobic condition at +2 °C for 4 h prior to

experiment. The quantity of sperm per egg was 2000 spermatozoa egg^{-1}. Results are averaged from four replications.

2.6. Determination of NaCl concentration and pH in activation solution (Experiment 3)

An effect of different concentration of 0, 17, 34, 51 and 68 mM NaCl in AS with 5 mM Tris–HCl, pH 8 and different pH levels of 6, 7, 8 and 9 with 17 mM NaCl was tested. The number of spermatozoa per egg was 11,000 and 2000 for testing of NaCl concentration and pH, respectively. Results are expressed from four replications.

2.7. Effect of different ratio between volume of activation solution and egg quantity (Experiment 4)

A rate of 11,000 spermatozoa egg^{-1} was used for insemination of eggs. Immediately after mixing of gametes, 1.5 ml (1:4), 2 ml (1:3), 3 ml (1:2), 6 ml (1:1) and 12 ml (2:1) of AS was added. Results are averaged from three replications.

2.8. Determination of optimal sperm/egg ratio (Experiment 5)

Eggs from four females in triplicate were used. Sperm gathered before stripping of females was at a concentration of $1.5 \cdot 10^9$ spermatozoa ml^{-1} of sperm and IS. The seminal plasma with IS were separated from spermatozoa by centrifugation ($2000 \times g$ for 5 min at room temperature) of the over abundant sperm solution. Supernatant (nonspermic centrifuged seminal plasma with IS) was latter used for dilution of sperm. Before mixing eggs and sperm and their activation with AS, the supernatant at volumes 199.8, 198, 180 and 0 μl diluted the original sperm volume of 0.2, 2, 20 and 200 μl, respectively. Number of 80, 800, 8000 and 80,000 spermatozoa egg^{-1} was achieved by the diluting procedure of sperm and supernatant. Results are expressed together for each 4 female (3 replications) and also as means of all females (12 replications).

2.9. Effect of female on hatching success (Experiment 6)

Eggs from eight individual females were used. The rate of 11,000 spermatozoa egg^{-1} was used for insemination of eggs. Results were averaged from six repetitions for each female.

2.10. Definition of the optimal time for the beginning of the eggs stickiness elimination (Experiment 7)

The rate of 50,000 spermatozoa per egg was used for insemination of eggs. Diluted alcalase enzyme was added at 3, 4 or 5 min after activation of gametes with 4 ml of AS. Alcalase enzyme, Merck EC 3.4.21.14, was diluted 1:50 with dechlorinated tap water at 22 °C. Results are summarized from four repetitions with egg samples pooled from four females.

2.11. Data analysis

Means of the data acquired were evaluated from 3, 4, 6, 8 and 12 repetitions in Experiments 4 and 5, 2–4 and 7, 6, 1 and 5, respectively. Statistical significance was assessed using multiple ANOVA (Statgraphics version 5), followed by multiple comparison Tukey HSD range tests. Probability values <0.05 were considered as significant.

3. Results

3.1. Stimulating of spermiation by repeated CP injection (Experiment 1)

Control group of four males injected with Ringer solution only did not spermiate. The mean volume of sperm collected was in the range of 0.21–0.47 ml kg^{-1} b.w. per sampling (Table 1). During the entire time of CP injection, the males produced sperm significantly with the best quantity of sperm after the third injection with $0.12 \cdot 10^9$ spermatozoa kg^{-1} b.w. and the fourth injection with $0.13 \cdot 10^9$ spermatozoa kg^{-1} b.w., compared to the first and second injections with $0.03–0.06 \cdot 10^9$ spermatozoa kg^{-1} b.w. (Table 1). The ANOVA showed significant ($P<0.014$) effect of males on the collected quantity of sperm per body weight of males.

3.2. Effect of different NaCl concentration in immobilizing solution (Experiment 2)

In the case of different concentration of NaCl in IS (Fig. 1), the percentages of the hatching 71% was the highest with 170 mM NaCl. Significantly lower hatching rate was observed with IS containing 230 mM NaCl.

3.3. Determination of NaCl concentration and pH in activation solution (Experiment 3)

The significantly highest fertilization expressed by percentage of hatching, 90% and 87%, was found for AS of 0 and 17 mM NaCl. The hatching rate was not lower for concentration of 34 and 51 mM NaCl in AS and decreased to the level of 70% in AS of 68 mM NaCl (Fig. 2).

Table 1
Changes in the volume of sperm and number of spermatozoa per kg body weight of male (b.w.) after four repeated injections of 5 mg kg^{-1} b.w. of CP on days 0, 6, 12 and 18 with collection of sperm 2 days after each injection

Males injection	0 day (mean ± S.D.)	6 days (mean ± S.D.)	12 days (mean ± S.D.)	18 days (mean ± S.D.)
Volume of sperm (ml kg^{-1} b.w.)	0.21 ± 0.13^{a}	0.47 ± 0.18^{c}	0.37 ± 0.11^{b}	0.45 ± 0.15^{bc}
Number of spermatozoa ($10^9 \cdot kg^{-1}$ b.w.)	0.03 ± 0.02^{a}	0.06 ± 0.02^{a}	0.12 ± 0.04^{b}	0.13 ± 0.05^{b}

One control group of four males was also injected with Ringer solution only without spermiation. Mean values with standard deviation (S.D.) of eight replicates are shown. Groups with a common superscript do not differ significantly ($P<0.05$).

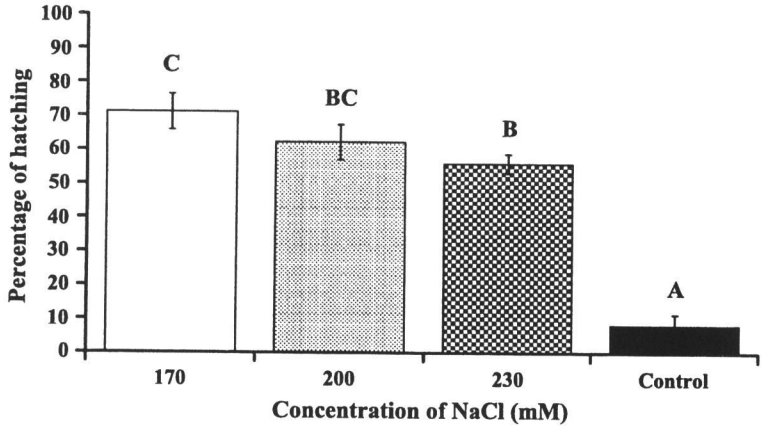

Fig. 1. Comparison of different NaCl concentrations in 30 mM tris, pH 7 in immobilizing solution to sperm without immobilizing solution as control with the same level of 2000 spermatozoa egg^{-1}. Mean values of four replicates are shown; vertical lines are S.D. Groups with a common superscript do not differ significantly ($P<0.05$).

As the optimal pH for AS was obtained pH at 6 and 7 with significantly highest hatching rate of 64% and 61%, respectively. Significantly lower results with 49% and 48% hatching rates were found with pH 8 and 9, respectively (Fig. 3).

3.4. Effect of different ratio between volume of activation solution and egg quantity (Experiment 4)

Significantly, the highest percentage of hatching (68%) was observed at ratio 1:2 (volume of activation solution in milliliters/eggs in grams). Hatching rate was not

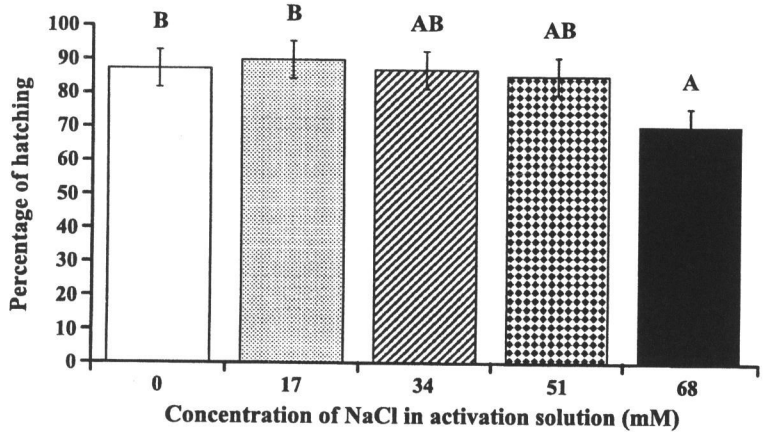

Fig. 2. Hatching success after artificial insemination with different concentrations of NaCl in 5 mM Tris–HCl, pH 8 of activation solution with the same level of 11,000 spermatozoa egg^{-1}. Mean values of four replicates are shown; vertical lines are S.D. Groups with a common superscript do not differ significantly ($P<0.05$).

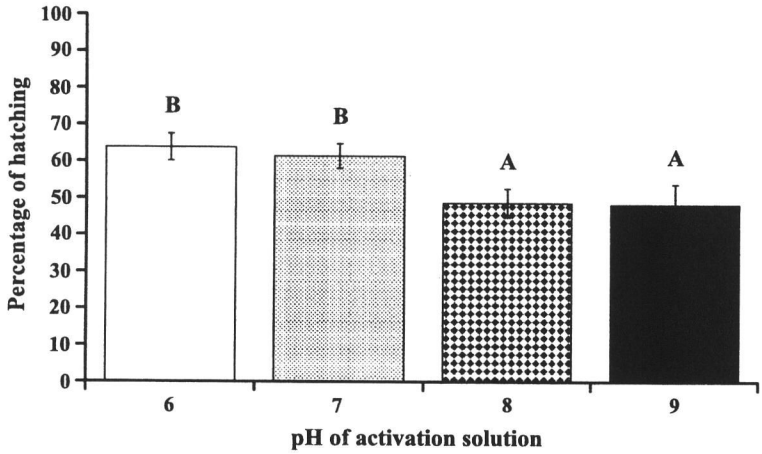

Fig. 3. Hatching success after artificial insemination with different pH of activation solution containing 17 mM NaCl + 5 mM Tris–HCl with the same level of 2000 spermatozoa egg^{-1}. Mean values of four replicates are shown; vertical lines are S.D. Groups with a common superscript do not differ significantly ($P<0.05$).

significantly different at the ratio 1:1 and 1:3. The percentage of hatching significantly dropped to the levels of 45% and 41% at the ratios 1:4 and 2:1, respectively (Fig. 4).

3.5. Determination of optimal sperm/egg ratio (Experiment 5)

The highest hatching success (82%, 88% and 86%) were found for 800, 8000 and 80,000 spermatozoa egg^{-1}, respectively, in all four females (Fig. 5). The fertilization significantly decreased to 26% at the level of 80 spermatozoa egg^{-1}. ANOVA showed significant effect

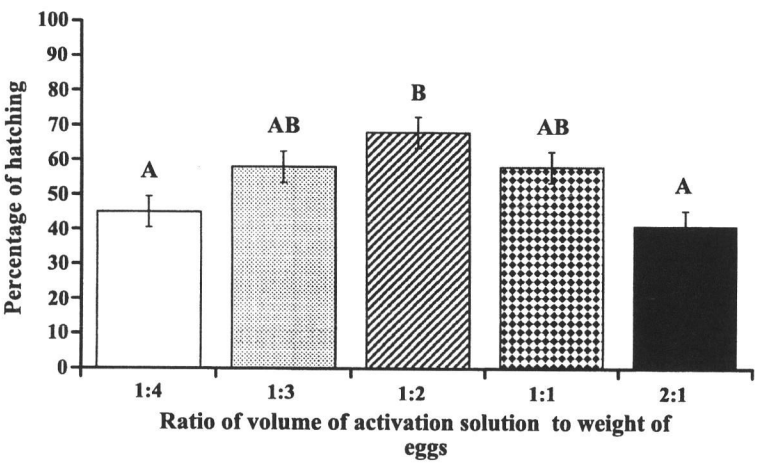

Fig. 4. Percentage of hatching at different ratios of volume of activation solution (17 mM NaCl, 5 mM Tris–HCl, pH 8) to weight of eggs with the same level of 10,000 spermatozoa egg^{-1}. Mean values of three replicates are shown; vertical lines are S.D. Groups with a common superscript do not differ significantly ($P<0.05$).

4. Discussion

4.1. Optimal artificial propagation of European catfish

The results of this research focused on optimization of the artificial insemination of European catfish under hatchery conditions are summarized in Fig. 6. Four- to eight-year-old European catfish broodfish taken from growing ponds in April are stored separately by sex in two ponds and stocked with forage fish, e.g. *cyprinids* at the quantity of 4 kg/kg b.w. of catfish (Steffens et al., 1994). Broodfish suitable for stripping are selected in May–July and kept separately in 4000-l tanks divided into three to four compartments. Males and females are treated minimally by 4–5 mg kg^{-1} b.w. of CP or by 40 μg kg^{-1} b.w. of LHRHa or Ovopel (LHRHa, D-Ala^6 manufactured in Hungary) (Brzuska and Adamek, 1999; Brzuska, 2001). Male spermiation can be sustained by weekly CP injection during a period of 1 month (Table 1). The sperm should be collected in the IS (170 or 200 mM NaCl, 30 mM Tris–HCl, pH 7) at a ratio of <0.9:1 (sperm: IS), to prevent activation of spermatozoa by urine and can be stored for 3 days at +4 °C (Linhart et al., 1986; Linhart and Billard, 1994). The ovulated oocytes and sperm are mixed together and activated by AS of 17 mM NaCl, 5 mM Tris–HCl, pH 7 at an optimum temperature of 21–23 °C. The in vitro storage of ova should not exceed a few hours at stable temperature of 18–20 °C, and it is advised to carry out the insemination and activation steps as soon as possible after ovulation. Ova contaminated by urine during stripping should be discarded (Linhart and Billard, 1995). Two milliliters of sperm + IS (usually light white colour) with a minimum concentration of $0.08 \cdot 10^9$ ml^{-1} spermatozoa plus 50 ml of AS are optimal to fertilize 100 g of eggs. These are mixed together during 10 s and, 2 min later, another 25 ml of AS is added in. For elimination of egg stickiness, alcalase enzyme is used 3 min after fertilization. Optimum rates between eggs and diluted enzymes (20 ml of alcalase enzyme, Merck EC 3.4.21.14, diluted in 980 ml of hatchery water or AS) is 1:1 (grams of eggs/milliliters of enzymes) with stirring for 2 min. After 2 min of exposure in enzyme solution, the eggs are rapidly rinsed with hatchery water and transferred to Weis jars. Hatching is expected to start 2.5–3 days (60 D°) after fertilization at 22–23 °C.

4.2. Comparison with other Siluriformes and other fishes

The artificial propagation of European catfish in Czech traditional conditions has become comparable to the common carp (Linhart et al., 2003a), well established. The use of optimal procedures during stripping, activation of gametes, fertilization and elimination of egg stickiness results in stable hatching rates of 90–100%. Nowadays, the European catfish is entirely produced artificially in the Czech Republic and France (Linhart et al., 2002) and other methods such as half-artificial or natural propagation (Horvath et al., 1984) are not used. In other *Siluriformes* species, such simplicity and repeatability in artificial propagation has not been yet achieved (Legendre et al., 1996). Individual steps with application of single or multiple CP injection of males, use of immobilizing and activation solutions and enzyme treatment for elimination of egg

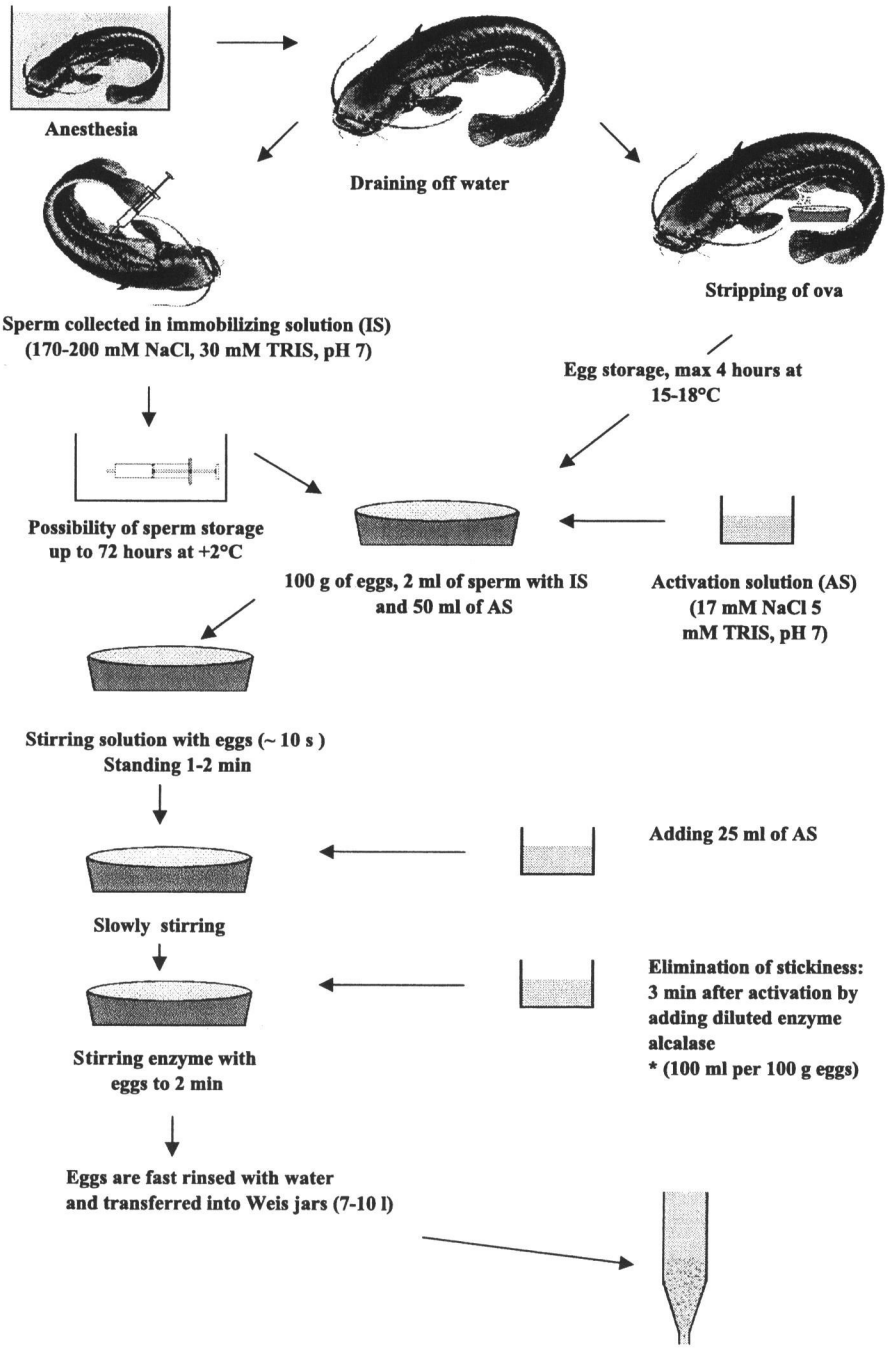

Fig. 6. Procedure for the artificial insemination of European catfish (*20 ml of alcalase enzyme, Merck EC 3.4.21.14, was diluted with 980 ml of hatchery water) after stripping of hormonally treated broodstock.

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8. Genofond a kryokonzervace spermatu u jeseterovitých:

8.1 Genofond u jeseterovitých

Z původních jeseterovitých ryb se na území nynější ČR vyskytovaly: jeseter velký (*Acipenser sturio*) v Labi (poslední úlovek byl zaznamenán v roce 1917 – Baruš a Oliva 1995) a jeseter malý (*Acipenser ruthenus*) v povodí řeky Moravy. V současnosti se u nás mimo původního jesetera malého v akvakulturních chovech vyskytují: jeseter sibiřský (*Acipenser baerii*), jeseter ruský (*Acipenser gueldenstaedtii*), jeseter hvězdnatý (*Acipenser stellatus*), vyza velká (*Huso huso*) a blízký příbuzný jeseterovitých – z čeledi veslonosovitých veslonos americký (*Polyodon spathula*). Akvakulturní chov jeseterovitých není pro svá specifika v podmínkách ČR rozšířen; úspěšný umělý výtěr byl v ČR proveden u jesetera malého, jesetera sibiřského, jesetera ruského a jesetera hvězdnatého. Produkce je finalizována jako okrasné ryby ve věku 1-2 roky a je limitována především omezeným počtem dospělých a reprodukce schopných generačních ryb a jejich cenou. A právě omezený počet reprodukovaných ryb může být důvodem k využívání technik kryokonzervace spermatu pro zachování variability v rámci chovaných populací.

8.2 Kryokonzervace spermatu veslonosa amerického

Experimenty týkající se kryokonzervace spermatu jeseterovitých byly prováděny u veslonose amerického na experimentální rybí líhni kentucké univerzity ve Frankfortu, USA. Cílem bylo otestovat použití různých kryoprotektantů a extendorů. Různé koncentrace kryoprotektantu (metanolu nebo DMSO) byly přidávány k neředěnému nebo extendery 1 a 2 (ext.1: 20 mM TRIS pH8 + 30 mM sacharóza + 0,5 mM KCl; ext.2: 20 mM TRIS pH8 + 50 mM sacharóza + 0,5 mM KCl) ředěnému (v poměru 1:1) spermatu veslonosa, sperma bylo rozpipetováno po 1 ml do 2 ml kryozkumavek (kryotub) a mraženo v přeprogramovaném mrazícím automatu PLANER Kryo 10 serie III podle programu: z 0°C do -5°C rychlostí 3°C za minutu, z -5°C do -15°C rychlostí 5°C za minutu, z -15°C do -25°C rychlostí 10°C za minutu, z -25°C do -80°C, teplota -80°C byla držena 5 min, potom byly kryotuby přeneseny do kapalného dusíku (a uchovány do příštího dne). Sperma bylo

rozmrazováno na vodní lázni teploty 40°C po dobu 105s a spolu s nemraženým použito ke kontrole pohyblivosti (z videozáznamu mikroskopického obrazu pohybu spermií) a testu oplozenosti. Oplozenost nemraženého spermatu uchovaného 24 hodin při 3°C byla 81,5%, což potvrzuje kvalitu použitých gamet. Nejlepších výsledků oplozenosti u rozmraženého spermatu (64-75%) bylo dosaženo použitím spermatu bez extenderu nebo s extenderem 1 s 8% metanolu v počtu $3,6.10^5$ spermií na jikru. Tyto výsledky se statisticky průkazně nelišily od výsledků v kontrole. Při použití DMSO jako kryoprotektantu bylo dosaženo jen velmi nízké úrovně oplozenosti 8-15%.

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Abstract

A sperm cryopreservation method using different cryoprotectants and sperm from different males was developed. Different percent of pure cryoprotectant (methanol and DMSO) was added in extender 1 or 2 (20 mM tris pH 8 + 30 mM sucrose + 0.5 mM KCl or 20 mM tris pH 8 + 50 mM sucrose + 0.5 mM KCl, dilution 1:1) or non-extended sperm and every 1 ml of mixture was transferred to a 2-ml cryotube. The cryotubes were directly transferred to a pre-programmed PLANER Kryo 10 series III at 0 °C and cooled from 0 °C to –5 °C at a rate of 3 °C.min^{-1}, from –5 °C to –15 °C at a rate of 5 °C.min^{-1}, from –15 °C to –25 °C at a rate of 10 °C .min^{-1}, from –25 °C to –80 °C at a rate of 20 °C .min^{-1}, then held for 5 min at -80 °C and finally transferred into LN_2 until next morning. The sperm was thawed in a water bath at 40 °C for 105 s. Fertilization rate of control sperm (unfrozen) samples after 24-h storage at 3 °C was 81.5 % it indicated that the gametes were of good quality. Percentage and the velocity of motile sperm from video frames using image analysis were evaluated in fresh and post-thawed sperm. The results of hatching rate significantly correlated with post-thawed sperm motility (r=0.49, P=0.035) and velocity (r=0.55, P=0.014) and none correlated with velocity of post-thawed spermatozoa (r=0.32, P=0.177). The best fertilization rates 64-75 % in post-thawed sperm ($3.6.10^5$ spermatozoa per egg) were obtained, when sperm either without any extender or with both extenders was treated with methanol in concentration 8 or 10 %. These results were not significantly different compared with the fresh sperm control sample. Hatching rate was very low, only 8-15 %, when sperm was frozen with 8 or 10 % DMSO. ANOVA showed a significant effect of males on sperm motility, velocity and fertilization rate in post-thawed sperm.

Key words:

Aquaculture, Fish, Motility, Reproduction, Spermatozoa, Cryopreservation, *Polyodon spathula*

At present time, several sperm banks were established in Russia, Ukraine, Hungary and Czech Republic for genetics conservation of sturgeon species (Billard et al., 2004; Flajshans et al., 1999).

Spermatozoa of chondrostean fish (*Chondrostei*) are different then teleostean fish (*Teleostei*). These differences concern morphology with presence of acrosome (Ginsburg et al., 1968; Dettlaff et al., 1993), physiology with long duration of sperm motility (Cosson et al., 2000, Linhart et al., 2002; Allavi et al., 2004) and biochemistry with presence of acrosin during acrosome reaction (Piros et al., 2002). Spermatozoa of sturgeons and paddlefish are essentially immotile in the seminal plasma (Linhart et al., 1995; Cosson and Linhart 1996; Cosson et al., 2000; Linhart et al., 2003; Alavi et al., 2004). Ionic concentration in seminal fluid of paddlefish and sturgeons are lower than for teleosts (Linhart et al., 1991; Piros et al., 2002). The level and ratio of ions in seminal fluid is very important for the maintenance of energetic content. When the quantity or ratio between Na^+ and K^+ change, the sperm motility can be initiated (Cosson et al., 1999, Linhart et al., 2002, Alavi et al., 2004). Osmolality of seminal fluid in paddlefish (33.0 - 62.7 $mOsmol.kg^{-1}$, Linhart et al., 2003) was higher than that for Siberian sturgeon (33.0-46.3 $mOsmol.kg^{-1}$, Gallis et al., 1991).

In this study, a sperm cryopreservation method using different cryoprotectants and sperm from different normal males (not sex-inverted males) was developed. Velocity, percentage of post-thawed sperm motility and fertilization yield were used to evaluate the success of each tested protocol.

Material and Methods

The experiments were conducted at the Aquaculture Research Center, Kentucky State University

(KSU), Frankfort, Kentucky, USA. Paddlefish males from 4.0 to 8.0 kg were captured in Ohio River, Kentucky. Broodfish were transported to the hatchery of the Aquaculture Research Center at KSU. Males were selected for good condition and held in circular metal tanks (1 000 l) with a water flow rate of 12 $l.min^{-1}$, dissolved oxygen of 9.0 mg $0_2.l^{-1}$ and at water temperature between 15 and 19^0C. Maturity of males was judged presence of milt after abdominal compression. The spermiation and ovulation were stimulated with LHRH analogue, des-Gly^{10} (D-Ala^6) LHRH ethylamide (Sigma Chemical Company) injected intramuscularly at the dose of 50 $\mu g.kg^{-1}$ b.w. Sperm was collected 24 h after stimulation. Ovulated oocytes were stripped from individual females 30 hours post stimulation. Eggs from 3 females were then pooled, short-term stored at a temperature of 17^oC and directly used for experiments.

Sperm Collection and Dilution

For stripping, males were fished out from the tank, fixed in dorsal position and urogenital pore was wiped. A 10-mL plastic syringe with 5 cm of Tygon tubing was used to collect sperm. The tube was inserted into the urogenital pore to fill up with sperm, and then sperm was transferred to 100 ml containers which were stored on ice for 1.5 hour until examination. Quality of the extended sperm was checked for the percentage of sperm motility from video records. Only sperm with motility higher than 80% was used for cryopreservation. Sperm concentration was counted in Thoma cell haemocytometer under Nicon microscope (400x) and the value was expressed as mean number of spermatozoa counted in 20 squares of the Thoma cell.

Cryopreservation and Thawing

Different percents of pure cryoprotectants (methanol and DMSO) were added in extender 1 (20 mM tris pH 8 + 30 mM sucrose + 0.5 mM KCl, dilution 1:1), extender 2 (20 mM tris pH 8

+ 50 mM sucrose + 0.5 mM KCl, dilution 1:1) or non-extended sperm and every 1 ml of mixture without equilibration time was transferred to a 2-ml cryotube. The cryotubes were directly transferred to a pre-programmed PLANER Kryo 10 series III at 0 °C and cooled from 0 °C to –5 °C at a rate of 3 °C.min^{-1}, from –5 °C to –15 °C at a rate of 5 °C.min^{-1}, from –15 °C to –25 °C at a rate of 10 °C .min^{-1}, from –25 °C to –80 °C at a rate of 20 °C .min^{-1}, then held for 5 min at -80 °C and finally transferred into LN_2 until next morning. The sperm was thawed in a water bath at 40 °C for 105 s and used for fertilization and video recording of sperm motility.

Experimental Design

Pooled eggs (the same number of eggs per one gram of eggs) was used for all experiments. For each treatment or different experimental conditions (different extenders, croprotectants and males), six cryotubes with sperm were frozen and after thawing of the sperm (next morning) the following parameters were evaluated for each: velocity of sperm, % of sperm motility, % of fertility and % of hatching. The same parameters were measured in the control sample of unfrozen fresh sperm.

In the first series of experiment, effect of two different cryoprotectants of extended or non-extended sperm were studied. The cryoprotectants studied were DMSO at final concentrations of 8 and 10 and methanol at final concentration of 8 and 10 %. One ml of extended or non-extended sperm with cryoprotectants were always gently homogenized and transferred into cryotubes of 2 ml and frozen.

In the second series of experiment, methanol as cryoprotectant was tested. Eight and 10 % of DMSO was added to the non-extended sperm and 8 % of extended sperm with extender 1 (20 mM tris pH 8 + 30 mM sucrose + 0.5 mM KCl, dilution 1:1), the solution was gently homogenized. Latter, one ml of the solution was transferred into cryotubes of 2 ml and frozen.

In the third series of experiment, three different males were tested. Eight % of Me_2SO was added to extended sperm with extender 1 and 2, respectively. The mixture was gently homogenized and volumes of 1 ml were transferred into cryotubes and frozen. Unfortunately for last fertilization at male no. 3, extender 2, was not sufficient quantity of eggs. It was the reason why there is no fertilization rate at male 3 in Fig. 3..

Fertilization and Hatching Trial

The suitability of different methodologies of cryopreservation was assessed by results of fertilization and hatching rates (additionally to other parameters of sperm quality as mentioned above) as followed: twenty grams of eggs (80 eggs per 1 g) were placed into a 50 ml dish; an accurate volume of sperm (thawed or non frozen as control) with 360,000 spermatozoa per one egg was dropped on them from a micropipette. Twenty ml of hatchery water at 17 °C was added. One minute later additional 20 ml water was added and 2 minutes later around 200 eggs were placed into a glass dish of 200 ml supplied with UV-sterilized recirculated tap water at 17 °C, 9 $mg.l^{-1}$ O_2. For each experiment, the procedure was replicated three times. The fertilization was counted in each dish at embryogenesis stage of 32 blastomers.

The percentage of fertilization rate (F_r) was calculated for each dish from the total No. of eggs placed in the dish (E_t) minus eggs without cleavage (E_d) as followed:

$$F_r = [(E_t - E_d)/E_t]x100$$

Observation of sperm motility and velocity

Spermatozoa were evaluated for the percentage of motility and velocity. Measurements used dark field microscopy and a Nikon camera setup as described by Cosson et al. (2000) and Linhart et al. (2002). Percentage of motility and velocity was examined under 200x magnification

immediately after mixing 0.5 µl of sperm with 49.5 µl of swimming medium (SM = 20 mmol.l^{-1} NaCl + 20 mmol.l^{-1} TRIS-HCl, pH 8.2), on a glass slide previously prepositioned on the microscope stage. The final dilution was 1:100. Within 10 s after mixing, a video recording was started for 1-min to be used in the evaluation of spermatozoa swimming activity. The focal plane was always positioned near the glass slide surface. The movements of spermatozoa were recorded at 60 frames.s^{-1} using a 3CCD video camera (SONY DXC-970MD) mounted on a dark-field microscope (NIKON Optiphot 2, Japan) and visualized on a video monitor illuminated with stroboscopic lamp of Strobex (Chadvick-Helmut, 9630, USA). The adjustable frequency stroboscopic flash illumination was set in automatic register with video frames (60 Hz) for sperm velocity measurement.

Evaluation of the velocity and percentage of motility

Velocity and motility were assessed at 20 s after activation: the successive positions of the video - recorded sperm heads (thawed or non frozen as control) were analyzed from video frames by means of Olympus MicroImage software (Version 4.0.1. for Windows with a special macro by Olympus C&S). The velocity and percentage of motility were measured by evaluating spermatozoa head positions on five successive frames with three different colours (frame 1 - blue, frames 2 - 4 - green and frame 5 - red). The analyses were repeated 3 times from 3 records at 20-second intervals, i.e. frames 1-5. Thirty to forty spermatozoa were evaluated from each frame. Motile spermatozoa were visible in three colours, while non-moving spermatozoa were white. Percentage of motile spermatozoa was easily calculated from white versus red cells. Velocity of spermatozoa was calculated as µm.s^{-1} based on length traces of spermatozoa from blue to green and red heads, calibrated for magnification. Excel 97 automatically calculated both values.

Data analysis

Average values of the acquired parameters and SD were counted from 3 replicates for fertilization, velocity and percentage of sperm motility. Statistical significance was assessed using multiple analysis of variance (ANOVA, Statgraphics version 5), followed by Tukey HSD multiple range test comparisons. Correlation was evaluated from the nine average values of the following parameters: velocity of post-thawed sperm, % of post-thawed sperm motility and % of fertility assessed with Microsoft Excel 97. Probability values < 0.05 were considered as significant.

Results

Fertilization rate of control sperm (unfrozen) samples after 24-h storage at 3 °C ranged from 65 to 90 %. It indicated that the gametes were of good quality. The best cryoprotectant for having live post-thawed sperm ($3.6.10^5$ spermatozoa per egg) was methanol (8 %) that gave fertilization rates from 61 to 76%. ANOVA showed a significant effect of male on post-thawed sperm velocity and fertilization rate.

Effect of extender and cryoprotectant

The results of individual parameters (percentage of motile sperm and average velocity of spermatozoa at 20 s after activation as well fertilization rates) in extended or non extended frozen/thawed sperm with using two different cryoprotectants (either the product or concentration) are shown in Figures 1-2 with comparison to the control sperm (non frozen). Fertilization rate of control sperm (unfrozen) samples after 24-h storage at 3 °C was 81.5 % (Figs 1-3).

The excellent fertilization rates 64-75 % were obtained on post-thawed sperm when sperm was either without dilution or diluted with extender 1 and treated by methanol in concentrations 8

or 10 % (Fig. 1). These results were not significantly different compared with the fresh sperm control sample. Hatching rate was very low, only 8-15 %, when sperm was frozen with 8 or 10 % DMSO (Fig. 1). At second test, 8 % of methanol with Extender 1 was the best combination for storage fertilization rate in thawed sperm. That combination was slightly but not significantly different with non-extended fresh sperm (Figure 2). ANOVA showed a significant influence of males on fertilization rate oscillating from 38 to 64 %, when fertilization rate of thawed sperm in all males were not statistically different from the fresh sperm.

No significantly different percentage of motility at 20 sec (70-86 %) after activation in frozen/thawed sperm was obtained, when sperm was treated with DMSO or methanol (Figure 1) and also when different sperm of three males were tested (Figure 3). The average value in the fresh sperm (control) was 98 %. Solution of 8 % methanol used as the cryoprotectant with extender 1 yielded in the highest percentage of motility in post-thawed sperm (82 %, Figure 2). ANOVA showed no influence of post-thawed sperm from different males on sperm motility. The results of post-thawed sperm motility (without fresh sperm) significantly correlated with hatching rate of post-thawed sperm ($r=0.49$, $P=0.035$) and did not correlate with velocity of post-thawed spermatozoa ($r=0.32$, $P=0.177$).

High velocity of post-thawed spermatozoa ranged from 118 to 157 $\mu m.s^{-1}$ was obtained, when sperm was frozen with methanol of 8 or 10 % (Figures 1 and 2). Methanol of 8 % with extender 1 was the best cryoprotectant for saving the high velocity (157 $\mu m.s^{-1}$) in post-thawed sperm. Also when sperm was stored only without extender with 8-10 % of methanol, the velocity of post-thawed spermatozoa was on the level 118-135 $\mu m.s^{-1}$ (Figure 2). Similar insignificant velocity of spermatozoa was found between the fresh none extended sperm and thawed sperm extended with methanol (Figures 1 and 2). ANOVA showed a significant influence of males on

sperm velocity of post-thawed sperm. Post-thawed sperm velocity correlated with hatching rate (r=0.55, P=0.014).

Regarding all parameters it can be concluded that methanol of 8 or 10 % with extenders 1 or 2 are useful for keeping of good quality of frozen/thawed sperm.

Discussion

A complete freezing procedure to preserve the sperm of paddlefish consisted of four different cooling rates, four steps during cooling and the temperature at which the super-cooled sperm were plunged into LN_2 (see figure 4). This methodology was highly modified from cryopreservation studies on common carp (Linhart et al., 2000) and European catfish (Linhart et al., 2006). The first step of the freezing program was a slow cooling from 0 °C to –5 °C at a rate of 3 °C.min^{-1}. When ice started to form in the external medium, the program was accelerated at the rate of 5°C .min^{-1} from –5 °C to –15 °C, then at rate of 10 °C from –15 °C to –25 °C and finally at the rate of 20 °C .min^{-1} from –25 °C to –80 °C. The sperm was at the temperature of -80°C held for 6 min and then transferred into LN_2. In programmable freezer Jahnichen et al. (1999) used a two step freezing regime at the rate of 3.5- 5°C .min^{-1} from +2 °C to –14 °C, then at rate of 10-20 °C from –14 °C to –70 °C and direct transfer to liquid nitrogen. The freezing rate was also controlled according to different size of straws by the height above the surface of liquid nitrogen at the level 3-5 cm (Horvath and Urbanyi, 2000; Glogowski et al., 2002).

When experiments with hatching rate in post-thawed sperm are conducted to detect the optimal freezing protocol, it is necessary to use low or medium number of spermatozoa per egg. Number of fresh spermatozoa ranging from 10,000 to 100,000 per egg is required for artificial insemination in sturgeon's species (Ginsburg, 1968; Dettlaff et al., 1993). If high number of

spermatozoa per egg (millions) is used, low viability and bad quality of post-thawed sperm could be masked (Linhart et al., 2006). Moreover, using high number of thawed spermatozoa is not pragmatic for artificial propagation. Dilution rate of sperm and egg with activating medium (water, activation solution etc.) during the process of fertilization is another parameter which must be adjusted well. We used dilution rate of sperm 1:100 (sperm: water) and eggs 1: 1-2. Both rates were found suitable for artificial propagation of paddlefish in previous study (Mims, 2000). Our idea was to use "practical" quantity of sperm, which could reveal the best procedure for freezing of sperm, but also to simulate methods of artificial propagation for future practical use of frozen paddlefish sperm at large scale.

Extenders with sacharose or sucrose or NaCl eventually with KCl in solution buffered at pH 7-8 are used for sperm conservation sturgeon and paddlefish (Billard et al., 2004, Horvath and Urbanyi; Glogowski et al., 2002). Dimethylsulfoxide (DMSO), ethyleneglycol (EG) or methanols are usually used as extenders in sturgeon and paddlefish for protection of sperm during crystallization (Horvath and Urbanyi, 2000; Glogowski et al., 2002; Tsvetkova et al., 1996). The results from last period of five years including our results confirmed, that methanol is the best cryoprotectant for spermatozoa protection of chondrostea species (Horvath and Urbanyi, 2000; Glogowski et al., 2002). Our study showed also good parameters of motility and velocity of spermatozoa when methanol was applied. The thawed sperm extended with DMSO surprisingly showed decreased fertilization rates in paddlefish. The dramatic decrease of hatching rates could be explained by effect of acrosomal reaction with dramatic shock process according to rapid penetration of acrosomal membrane with releasing of acrosin enzyme (Ciereszko et al., 1996a, b, 2000). As DMSO can interact with the phospholipids membranes (Anchordoguy et al., 1991), a change in the lipid bi-layer structure with DMSO remains possible.

Spermatozoa of sturgeons and paddlefish are essentially immotile in the seminal plasma (Linhart et al., 1995; Cosson and Linhart, 1996; Cosson et al., 2000; Linhart et al., 2003; Alavi et al., 2004). Sperm is immediately activated when transferred into a swimming medium, usually fresh water or low salt concentration solutions (Drabkina, 1961), similar to freshwater teleosts. Motility is the greatest immediately after activation. All sperm motility parameters decrease rapidly during the post-activation period and the percentage of motile cells also gradually decrease (Cosson et al., 2000). During the earliest period of motility, spermatozoa of sturgeons and paddlefish move in fresh sperm at velocities of 175-250 $\mu m.s^{-1}$ (Cosson et al., 2000, Linhart et al., 2002). Also in the thawed sperm, similar velocity around 140-160 $\mu m.s^{-1}$ with 70-90 % of motile spermatozoa was found when optimal freezing procedure was applied.

Cryopreservation of paddlefish sperm appears to be a useful and reliable technique not only for conservation of gene resources of the species but also for wide-ranging artificial propagation (see figure 4).

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Legends

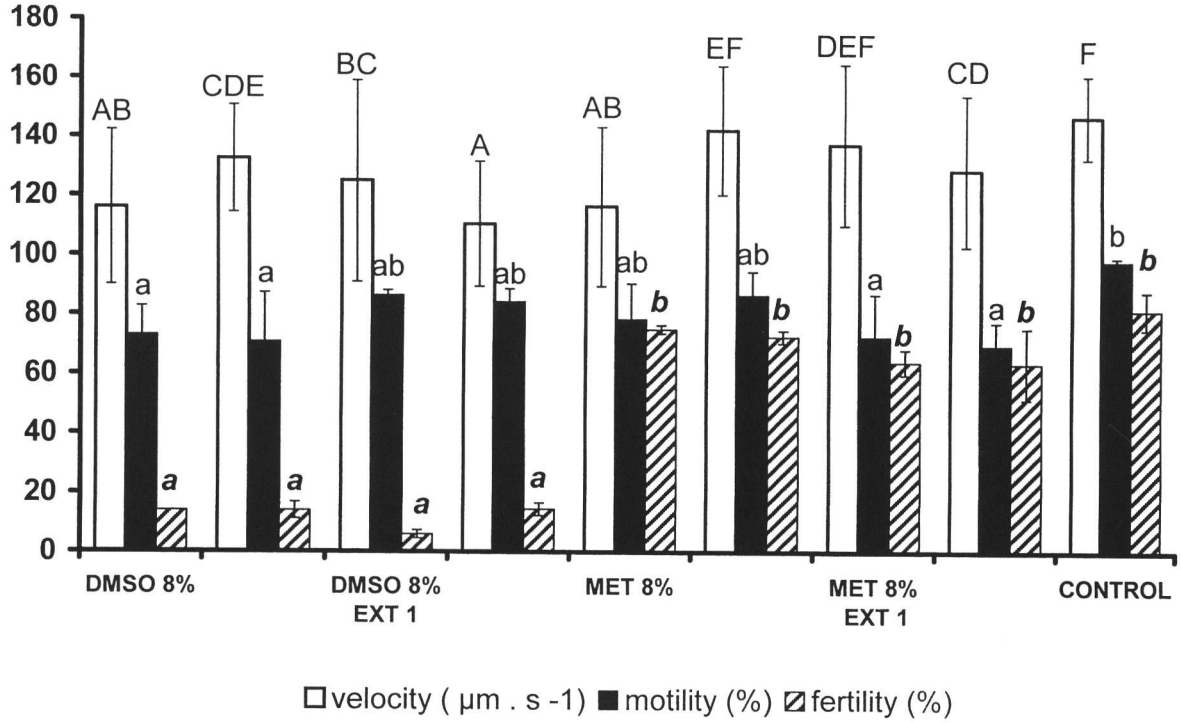
Fig. 1. Effect of 2 different types of cryoprotectants was evaluated. The columns express average values of data found in post-thawed sperm and all different concentration for DMSO and methanol (8 and 10 %) with or without extender 1 in fertilization rates, percentage of sperm motility at 20 s after activation and velocity of spermatozoa at 20 s after activation. Fresh non-extended sperm was used as control. Groups with a common superscript for each parameter do not differ significantly ($P<0.05$).

Fig 2. Effect of methanol as cryoprotectant was evaluated. The columns express average values of data found in post-thawed sperm and all different concentration for methanol (8 and 10 %) with or without extender 1 in fertilization rates, percentage of sperm motility at 20 s after activation and velocity of spermatozoa at 20 s after activation. Fresh non-extended sperm was used as control. Groups with a common superscript for each parameter do not differ significantly ($P<0.05$).

Fig. 3. Effect of 3 different males on cryopreservation success of sperm was evaluated. The columns express average values of data found in post-thawed sperm of three different males with extenders 1 and 2, and methanol (8 %) in fertilization rates, percentage of sperm motility at 20 s after activation and velocity of spermatozoa at 20 s after activation. As control was used fresh not extended sperm. Groups with a common superscript for each parameter do not differ significantly ($P<0.05$).

Fig. 4. Procedure of sperm cryopreservation in paddlefish after stripping of hormonally treated broodstock.

Fig. 1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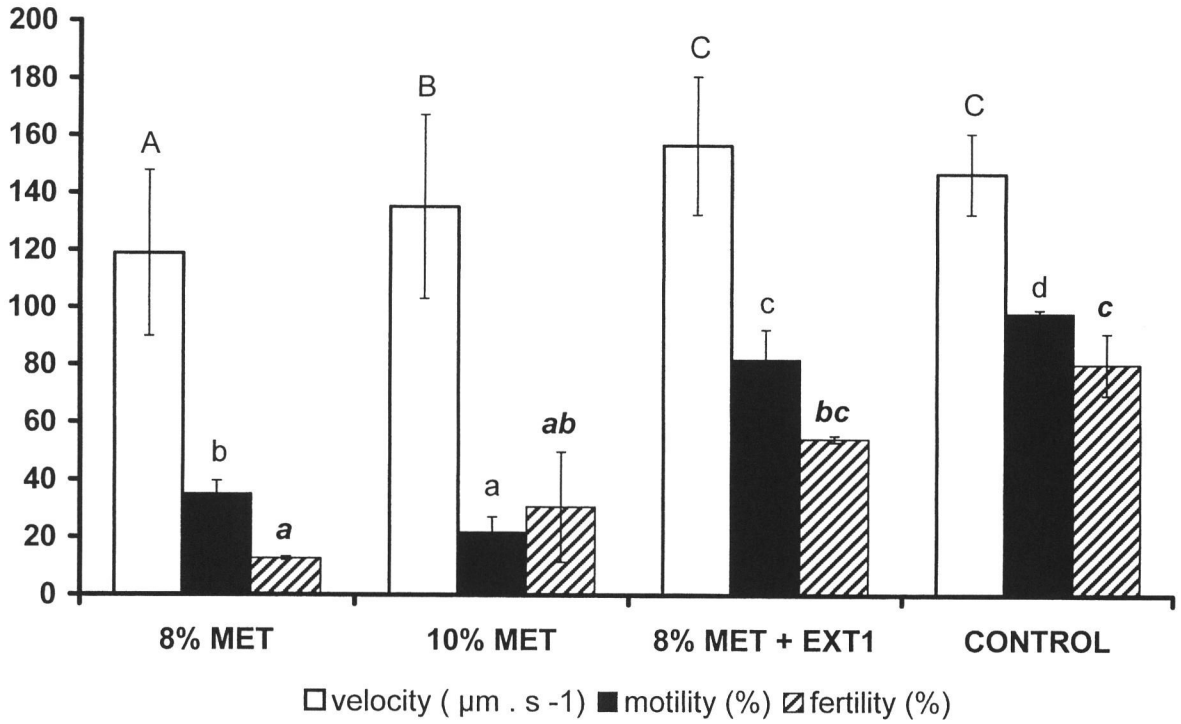
velocity (µm . s -1)
motility (%)
fertility (%)

Fig.2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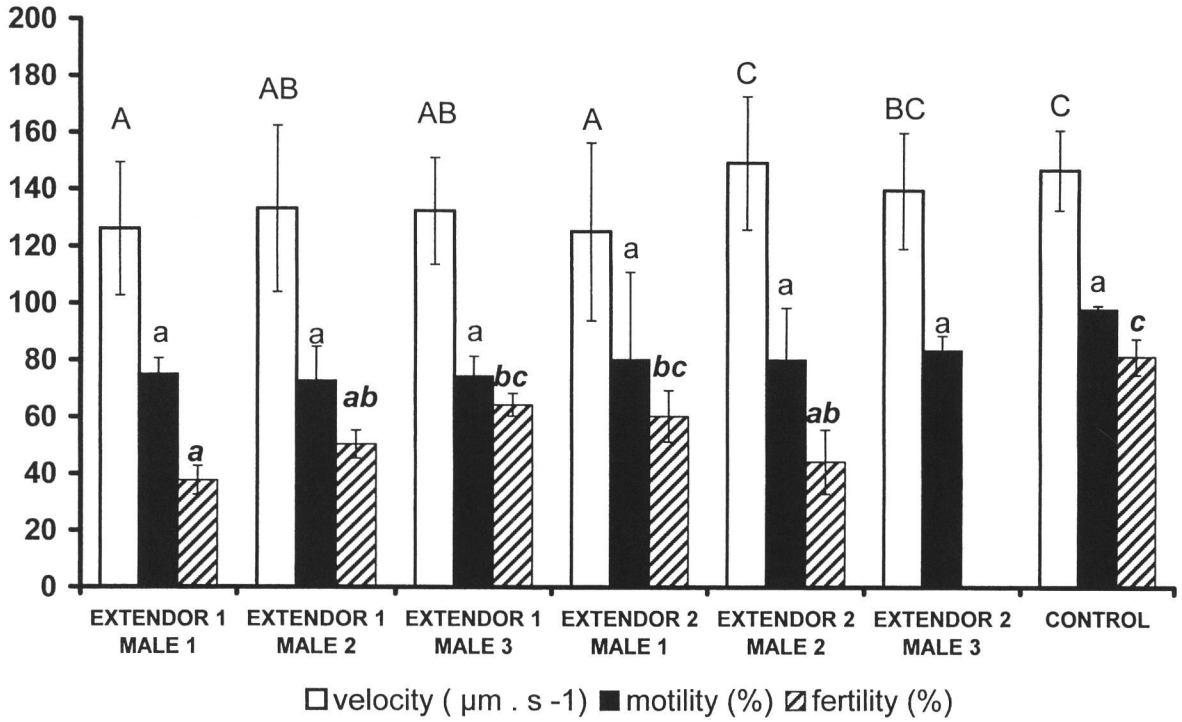
8% MET + EXT1
CONTROL
velocity (µm . s -1)
motility (%)
fertility (%)

Fig. 3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EXTENDOR 1 MALE 1
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EXTENDOR 1 MALE 3
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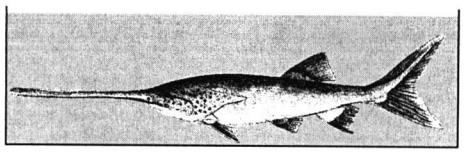
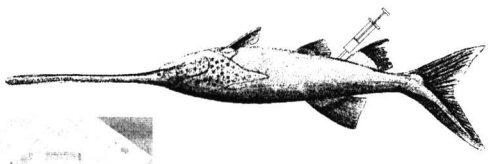
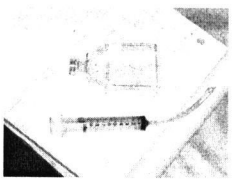
EXTENDOR 2 MALE 3
CONTROL
velocity (µm . s -1)
motility (%)
fertility (%)

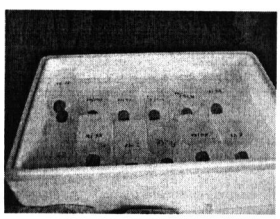
Anesthesia





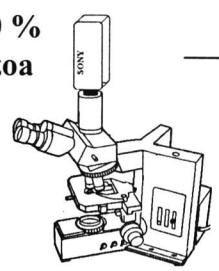


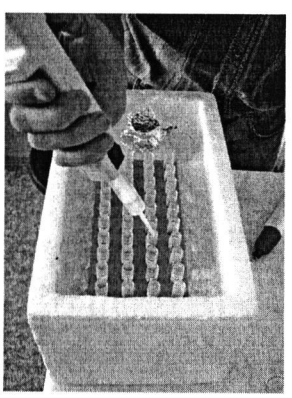
Collection of sperm



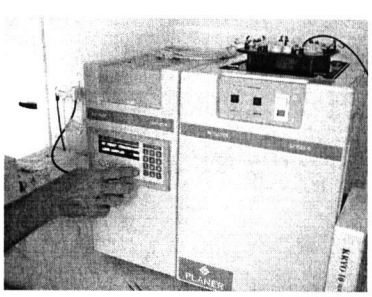
1.5 hours storage sperm in plastic contejners on ice at temperature 0-4°C.

Minimum 80 % of spermatozoa motility



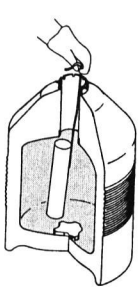


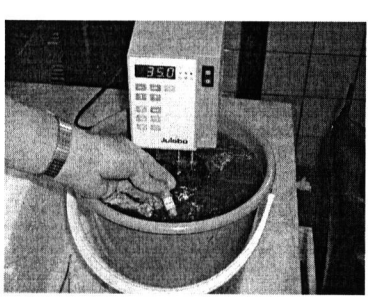
Methanol (8%) was added in extender 1 (20 mM tris pH 8 + 30 mM sucrose + 0.5 mM KCl, dilution 1:1) or extender 2 (20 mM tris pH 8 + 50 mM sucrose + 0.5 mM KCl, dilution 1:1) and every 1 ml of mixture without equilibration time was transferred to a 2-ml cryotube



Freezing in the PLANER KRYO 10 III at 0 °C to –5 °C at a rate of 3 °C .min-1, from –5 °C to –15 °C at a rate of 5 °C .min-1, from –15 °C to –25 °C at a rate of 10 °C .min-1, from –25 °C to –80 °C at a rate of 20 °C .min-1, then held for 5 min at -80 °C ,

Finally, transfer to liquid nitrogen (LN2)





Frozen sperm were thawed in a water bath 40 °C for 105 s.

9. Závěr:

V rámci prezentované práce byly navrženy a testovány postupy kryokonzervace spermatu představitelů kostnatých (kapr, lín, sumec) a chrupavčitých (veslonos) ryb především z důvodu ověřit možnost využití pro ochranu genofondu (rozmanitosti genofondu) formou banky zamraženého spermatu.

Dosažené a publikované výsledky naznačují, že kryokonzervaci je možno k uvedenému účelu použít, je však nutné zohlednit druhová specifika. Z hlediska dosavadních zkušeností se jako kritické předpoklady úspěšné aplikace kryokonzervace jeví: primární kvalita získaného spermatu a správný postup použití zmrazeného/rozmrazeného spermatu.

Rozšíření používání zmraženého spermatu u ryb v produkčním rybářství v obdobném rozsahu jako např. u skotu či drůbeže brání pro potřeby provozu nízká úroveň dosahované oplozenosti, cena technického vybavení a materiálu. V neposlední řadě je třeba k nepříznivým faktorům přičíst i vlastní reprodukční strategii našich hospodářsky významných druhů ryb (velké množství potomstva, ale během odchovu vysoké ztráty).

Jako hlavní parametr úspěšnosti je třeba považovat testy oplozenosti na jikrách, ostatní sledované parametry, jako motilita zjišťovaná mikroskopicky, rychlost pohybu spermií, osmolalita spermatu, podíl živých spermií zjišťovaný diferenciálním fluorescenčním barvením jako parametry doplňkové.

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