RAFAEL VENÂNCIO DE ARAÚJO

MOTILIDADE, VELOCIDADE E FERTILIDADE DO SÊMEN DE SURUBIM-DO-PARAÍBA *Steindachneridion parahybae* (Siluriformes) CRIOPRESERVADO EM DIFERENTES DILUIDORES

LAVRAS - MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção Animal, para a obtenção do título de Doutor.

Orientadora

Dra. Ana Tereza de Mendonça Viveiros

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APROVADA em 31 de outubro de 2011.

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2011

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RESUMO

O surubim-do-paraíba *Steindachneridion parahybae* (Siluriformes) é uma espécie nativa e endêmica da bacia do rio Paraíba do Sul. Ao longo dos anos, esta bacia tem sofrido grande impacto ambiental, decorrente do desmatamento da mata ciliar, da introdução de espécies de peixes exóticas, do represamento e da poluição dos rios, contribuindo para o desaparecimento de muitas espécies, incluindo o *S. parahybae*. A criopreservação de sêmen pode ser uma ferramenta útil na fertilização artificial e em programas de recuperação de estoques ameaçados. Os siluriformes brasileiros estão distribuídos em 11 famílias, em um total de 1.056 espécies, entretanto, estudos relacionados à criopreservação do sêmen dessa ordem têm recebido atenção reduzida. O presente estudo foi realizado com os objetivos de avaliar diferentes meios de congelamento para o sêmen de *S. parahybae*; avaliar o efeito da lactose na qualidade do sêmen pós-descongelamento; comparar a motilidade espermática após o descongelamento avaliada pelo método subjetivo ao microscópio de luz, bem como pelo método computadorizado de avaliação espermática (CASA); determinar as velocidades espermáticas após o descongelamento pelo método computadorizado CASA e estabelecer correlação entre as velocidades espermáticas e a taxa de fertilização para a espécie. O sêmen foi diluído em 5 meios: 0,9% NaCl, *Ginsburg Fish Ringer* modificado, 5% glicose, 10% glicose e BTS™ (Minitub™; Alemanha), combinados ou não com 10% de lactose. O DMSO foi utilizado como crioprotetor (10%). Em uma etapa anterior à criopreservação, um teste de toxicidade dos meios diluidores foi realizado. Após um equilíbrio de 15 minutos a 15 ºC entre sêmen e meio diluidor, a motilidade e o vigor espermático (escore 0-5) foram subjetivamente avaliados a 0 e a 30 segundos após a ativação. O sêmen diluído foi envasado em palhetas de 0,5 ml, congelado em botijão de vapor de nitrogênio e descongelado, a 25 °C, por 20 segundos ou 35 ºC, por 15 segundos. A motilidade foi estimada subjetivamente e objetivamente utilizando CASA. Três meios diluidores avaliados no primeiro estudo (5% glicose, 10% glicose e BTS™) foram selecionados e combinados a duas concentrações de lactose (0 e 10). Após o congelamento do sêmen, metade das amostras foi avaliada quanto à motilidade e à velocidade dos espermatozoides, e a outra metade utilizada para fertilização. Os resultados do teste de toxicidade demonstraram que a motilidade espermática avaliada imediatamente após a ativação foi similar à motilidade avaliada 30 segundos depois. O sêmen diluído em NaCl, 5% glicose e 10% glicose (66-73%) e em 5% glicose-lactose e BTS™-lactose (53-63%) apresentou maior motilidade espermática. Maiores valores de vigor espermática foram observados para o sêmen diluído em 10% glicose (3,4), 5% glicose-lactose, 10% glicose-lactose e BTS™-lactose (2,7-3,2). Para o sêmen criopreservado, não houve diferença

entre a motilidade espermática avaliada subjetivamente ou no CASA. Não houve diferença na motilidade quando o sêmen foi congelado em um dos cinco meios sem adição de lactose (57-64%) e foi maior quando congelado em 5%-glicose e BTS™, com lactose (53-54%). Entre os meios que receberam lactose em sua composição, a motilidade foi maior para o sêmen criopreservado em 5% glicose e BTS™ (53-54%) e não houve diferença entre os meios que não receberam lactose (57-64%). Não houve diferença entre as duas temperaturas de descongelamento testadas (25 °C e 35 °C). A presença de lactose teve efeito positivo, quando combinada com 5% glicose, efeito negativo, quando combinada com 10% glicose e indiferente quando combinada com o BTS™, para a velocidade e a taxa de fertilização. Uma correlação positiva entre a velocidade dos espermatozoides e a taxa de fertilização foi observada. Em geral, os melhores resultados foram obtidos quando o sêmen foi congelado em 5% glicose-lactose: 64% espermatozoides móveis, 69 μm s^{-1} de VCL, 48 μm s^{-1} de VSL, 58 μm s^{-1} de VAP e 45 % de taxa de fertilização. O meio de congelamento 5%-glicose-lactose-DMSO proporciona altos rendimentos na qualidade do sêmen criopreservado e, assim, pode ser empregado como uma ferramenta para a conservação de material genético dessa espécie.

Palavras-chave: CASA. Catfish. Diluidor. Congelamento. Qualidade seminal.

ABSTRACT

The surubim-do-paraíba *Steindachneridion parahybae* (Siluriformes) is a native and endemic species of the Paraíba do Sul river basin. Over the years, the basin has suffered major environmental impacts resulting from the deforestation of riparian vegetation, introduction of exotic fish species, pollution and damming of rivers, contributing to the disappearance of many species, including *S. parahybae*. Sperm cryopreservation can be a useful tool in artificial fertilization programs, and recovery of threatened stocks. The Brazilian Siluriformes are distributed in 11 families with a total of 1056 species. However, studies on sperm cryopreservation of this order have received reduced attention. The aims of this study were to evaluate different freezing media of *S. parahybae* sperm; evaluate the effects of lactose on post-thaw sperm quality; compare post-thaw sperm motility subjectively evaluated under light microscope and computer-assisted sperm analyzer (CASA); determine post-thaw sperm velocities by CASA and establish a correlation between sperm velocities and fertilization rate. Sperm was diluted in 5 extenders: 0.9% NaCl, Ginsburg fish Ringer modified, 5% glucose, 10% glucose and BTS™ (Minitub™; Germany), combined with lactose at 10% or 0%. DMSO was used as cryoprotectant (10%). In a stage prior to cryopreservation, a toxicity test was conducted of freezing media. Sperm was diluted in each freezing media, equilibrated for 15 min at 15ºC and subjectively evaluated for motility and quality motility score (score, 0-5) 0 and 30 s post-activation. Then diluted sperm was loaded into 0.5 ml straws, frozen in a nitrogen vapor vessel and then thawed at 25°C for 20 s or 35ºC for 15 s. Post-thaw sperm motility was estimated subjectively and objectively using CASA. Three extenders evaluated in the first study (5% glucose, 10% glucose and BTS™) combined with two concentrations of lactose (0 and 10) were used to freeze sperm. Half of the samples was evaluated for sperm motility and velocity, and the other half was tested for fertility. The toxicity test showed that sperm motility assessed immediately after activation was similar to that measured 30 s later. Sperm diluted in NaCl, 5% glucose and 10% glucose (66-73%) and 5% glucose-lactose and BTS™-lactose (53-63%) yielded higher sperm motility. Highest quality scores were observed for sperm diluted in 10% glucose (3.4), 5% glucose-lactose, 10% glucose-lactose e BTS™-lactose (2.7-3.2). There was no difference on post-thaw sperm motility evaluated subjectively or by CASA. Among the freezing media that possessed lactose, motility was higher for sperm cryopreserved in 5% glucose and BTS™ (53-54%) and that did not differ from sperm cryopreserved in all lactose-free media (57-64%). There was no difference between the two thawing temperatures tested (25 and 35°C). The presence of lactose had a positive effect when combined with 5% glucose, negative effect when combined with 10% glucose and

indifferent when combined with BTS™, regarding velocities and fertilization rate. There were positive correlations between sperm velocities and fertilization rate. In general, the best results were achieved when sperm was frozen in 5%-glucose-lactose: 64% motile sperm, 69 μm s^{-1} of VCL, 48 μm s^{-1} of VSL, 58 μm s^{-1} of VAP and 45% fertilization rate. Sperm frozen in this medium can be used as a tool for conservation of genetic material of this species.

Keywords: CASA. Catfish. Extender. Freezing. Semen quality.

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PRIMEIRA PARTE

1 INTRODUÇÃO

A ictiofauna de peixes de água doce do Brasil é a mais rica do mundo, com cerca de 2.587 espécies nativas (BUCKUP; MENEZES; GHAZZI, 2007). Entretanto, o conhecimento sobre a diversidade desta ictiofauna é ainda incompleto, como atestam as dezenas de espécies de peixes descritas anualmente no Brasil e, portanto, é de se prever que a riqueza total efetiva seja ainda muito maior.

Algumas espécies de peixes têm a característica de migrar em determinados meses do ano, quando passam por estímulos hormonais e ambientais, até chegarem às calhas dos rios ou aos grandes afluentes, onde ocorre a desova. Muitas dessas espécies, conhecidas como peixes de piracema, têm grande importância comercial e são produzidas em grande quantidade, enquanto outras, devido à sobrepesca ou a outras ações antropogênicas (assoreamento de rios, construções de barragens, diminuição de matas ciliares, aumento da poluição e introdução de espécies exóticas), têm seu número cada vez mais diminuído. Entre essas ações, a construção de barragens nos rios causa profundas modificações no ambiente aquático (SALE, 1985).

A Instrução Normativa 05/04 e suas alterações (BRASIL, 2004) listam mais de 135 espécies nativas de peixes de água doce ameaçadas de extinção e outras sete na categoria de sobre-explotadas ou ameaçadas de sobre-explotação (ROSA; LIMA, 2008). De acordo com o Livro Vermelho da Fauna Ameaçada de Extinção, publicado em 2008 pelo Ministério do Meio Ambiente, pelo menos nove espécies que pertencem à bacia do rio Paraíba do Sul estão listadas como ameaçadas. Entre estas espécies, pode-se destacar o surbim-do-paraíba, que é encontrado raramente apenas em alguns pontos do canal principal do rio Paraíba do Sul (RJ), nos rios Pomba e Paraibuna, sendo considerado comercialmente

extinto no estado de São Paulo, devido à falta de registros comprovados de sua captura na região (CANEPPELE; POMPEU; GARAVELLO, 2008).

A bacia do rio Paraíba do Sul vem, sofrendo ao longo dos anos, represamentos, desmatamento da mata ciliar e poluição, em razão da industrialização e da atividade agrícola regional. O impacto ambiental dessas ações antropogênicas tem resultado numa diminuição da diversidade da fauna pesqueira e, consequentemente, de espécies endêmicas da região, acarretando danos consideráveis sobre ecossistemas terrestres e aquáticos (BRUTON, 1995). O rio Paraíba do Sul, cujo percurso se inicia no município de Paraibuna (SP), a partir da confluência dos rios Paraitinga e Paraibuna, e atravessa o Rio de Janeiro de sul a norte, desaguando em Atafona (RJ), percorrendo um total de cerca de 1.000 km, espalhando-se pelos estados de São Paulo (38%), Rio de Janeiro (38%) e Minas Gerais (24%), em uma área total de aproximadamente 57 mil quilômetros quadrados.

Na década de 1950, o rio Paraíba do Sul e seus afluentes foram considerados dos mais piscosos do estado de São Paulo. Apesar da grande diversidade de peixes, poucos tinham valor comercial e, entre estes, se destacavam, principalmente, a piabanha, *Brycon insignis*; o surubim-do-paraíba, *Steindacneridion parahybae*; a piava, *Leporinus* sp.; a piapara, *Leporinus* sp. e o robalo, *Centropomus* sp. (MACHADO; ABREU, 1952). Ao longo do rio são encontradas 12 espécies exóticas, como dourado (*Salminus brasiliensis*), pacu (*Piaractus mesopotamicus*), mandi-guaçu (*Pimelodus maculatus*) e 115 espécies nativas, como lambari (*Astyanax* sp.), piabanha (*Brycon insignis*), curimbatá (*Prochilodus* sp.), bagre (*Rhamdia parahybae*) e a pirapitinga-do-sul (*Brycon opalinus*) (ROSA; LIMA, 2008).

Além de um trabalho voltado para conservação do hábitat natural, como a revitalização de rios e a conservação da flora ao seu entorno, o desenvolvimento de técnicas que proporcionem a conservação de espécies

passíveis de extinção se torna uma ferramenta muito eficaz. As estratégias para se evitar a redução dos recursos pesqueiros são aplicáveis por meio de estudos relacionados à biologia das espécies, ao desenvolvimento de técnicas de reprodução artificial, de larvicultura e alevinagem e de coleta e à preservação de gametas.

Nos programas de repovoamento e expansão da piscicultura, a criopreservação de sêmen é uma ferramenta que pode ser bastante útil para reprodução artificial, pois podem auxiliar em programas de melhoramento genético e na formação de um banco para a conservação dos recursos genéticos (ÓRFÃO, 2006). Outras vantagens da preservação de sêmen são permitir a troca de material genético entre os laboratórios de reprodução e reduzir o número de reprodutores, diminuindo, assim, os custos de produção e eliminando problemas de assincronia da maturidade gonadal, quando machos e fêmeas não estão preparados simultaneamente, além do estabelecimento de programas de hibridização, utilizando espécies com períodos reprodutivos diferentes (VIVEIROS, 2005). Diante dessas circunstâncias, há uma demanda crescente por técnicas e práticas de preservação de gametas que facilitem a fertilização artificial desses animais para repovoamento dos rios.

A qualidade do sêmen após o descongelamento, usualmente, é avaliada em função da percentagem de células móveis observada em microscópio de luz. Contudo, durante a última década, o uso de um sistema de análise espermática assistida por computador, conhecido como CASA (do inglês *computer-assisted sperm analysis*), tem se tornado cada vez mais popular em laboratórios de tecnologia de sêmen por permitir uma avaliação mais precisa da motilidade, além de outros parâmetros de qualidade espermática. Esse tipo de avaliação objetiva foi relatada primeiramente em peixes, por Cosson et al. (1985), com iluminação estroboscópica e gravação de vídeos de movimentos ativos do espermatozoide de truta. Somente nos últimos anos o sistema CASA foi

adaptado para o estudo de espermatozoides de algumas espécies de peixes (CHRIST et al., 1996; KIME et al., 1996, 2001; RAVINDER et al., 1997; RURANGWA et al., 2001; SANCHES et al., 2010; TOTH et al., 1977, 1995).

Parâmetros de motilidade espermática apresentados pelo programa estão diretamente relacionados à taxa de fertilização e poderiam predizer o potencial reprodutivo de um indivíduo. Na conservação de gametas, possibilita a verificação da viabilidade de meios diluentes e crioprotetores para o congelamento de sêmen. O analisador de motilidade captura imagens sucessivas, identificando os pontos onde o espermatozoide se encontra a cada imagem.

Embora exista um número grande de estudos, ainda há ambiguidade quanto aos resultados divulgados, primariamente devido à falta de padronização das metodologias utilizadas e da análise dos dados. A evolução das espécies de peixes na tentativa de se adaptar aos diversos habitats aquáticos resultou em diferenças substanciais em sua morfofisiologia e, consequentemente, os espermatozoides de cada espécie demonstram reações diferentes aos protocolos de conservação (KOPEIKA; KOPEIKA; ZHANG, 2007).

2 REVISÃO DE LITERATURA

2.1 *Steindachneridion parahybae*

Reino: Animália;
Filo: Chordata;
Classe: Actinopterygii;
Ordem: Siluriformes;
Família: Pimelodidae;
Gênero: *Steindachneridion;*
Espécie: *Steindachneridion parahybae*, Steindachner, 1877.

Figura 1 Exemplar de *S. parahybae*, popularmente conhecido como surubim-do-paraíba
Fonte: Foto Caneppele, editada por Honji (2011)

Steindachneridion parahybae (Steindachner, 1877), conhecido como surubim-do-paraíba (Figura 1), é uma espécie nativa e endêmica da bacia do rio Paraíba do Sul (20º 26' e 23º 39'S e 41º e 46º 30'W), cuja extensão compreende os estados de São Paulo, Minas Gerais e Rio de Janeiro (HILSDORF; PETRERE JÚNIOR, 2002) (Figura 2). O gênero *Steindachneridion* pertence à ordem Siluriforme, família Pimelodidae, e compreende aproximadamente 6

espécies de peixes com distribuição geográfica predominantemente nas regiões sul e sudeste do Brasil (FROESE; PAULY, 2010).

O surubim-do-paraíba é um bagre de grande porte, atingindo pelo menos 60 cm de comprimento padrão (OLIVEIRA; MORAES JÚNIOR, 1997). É um peixe de "couro" (sem escamas) que tem corpo achatado com o dorso preenchido por pequenas e alongadas manchas. Além disso, o surubim apresenta olhos pequenos e pouco eficientes, porém, sua percepção do meio ambiente é auxiliada principalmente pelos barbilhões, caracterizando-o como uma espécie com maior atividade noturna (BURGESS, 1989; LUNDBERG; LITTMANN, 2003), podendo, ocasionalmente, ser capturado ao entardecer. Sua ocorrência está, geralmente, associada às porções não muito profundas localizadas nas regiões centrais dos rios e próximos a corredeiras. Quanto ao hábito alimentar, o surubim-do-paraíba é carnívoro bentófago, com a dieta consistindo de peixes (*Rineloricaria* sp. e *Pimelodella* sp.) e crustáceos (*Trichodactylus* sp.) (MORAES JÚNIOR; CARAMASCHI, 1993). Durante a larvicultura, é comum verificar-se canibalismo e fotofobia das larvas, características comuns aos bagres.

O período reprodutivo do surubim-do-paraíba estende-se de novembro a março, a fecundação é externa e a desova ocorre quando o nível das águas está em ascensão, durante as chuvas de verão. Considerada como uma das poucas espécies nobres da bacia do rio Paraíba do Sul, foi outrora importante para a pesca profissional. Machado e Abreu (1952) relatam que a pesca da espécie em dez municípios do Vale do Paraíba paulista, nos anos de 1950 e 1951, totalizou 1.989 kg. Até recentemente (2002), esta espécie era capturada com relativa frequência no rio Pomba, em corredeiras próximas à cidade de Laranjal, MG e constituía parte importante das capturas dos pescadores profissionais (CANEPPELE; POMPEU; GARAVELLO, 2008).

Hoje, o surubim-do-paraíba encontra-se na Lista Nacional das Espécies de Peixes Ameaçadas de Extinção (BRASIL, 2004; HONJI et al., 2009) e no Livro Vermelho da Fauna Ameaçada de Extinção (ROSA; LIMA, 2008), com status de espécie criticamente ameaçada. É encontrado raramente apenas em alguns pontos do canal principal do rio Paraíba do Sul (RJ), nos rios Pomba e Paraibuna, sendo considerado comercialmente extinto no estado de São Paulo devido à falta de registros comprovados de sua captura na região (CANEPPELE; POMPEU; GARAVELLO, 2008).

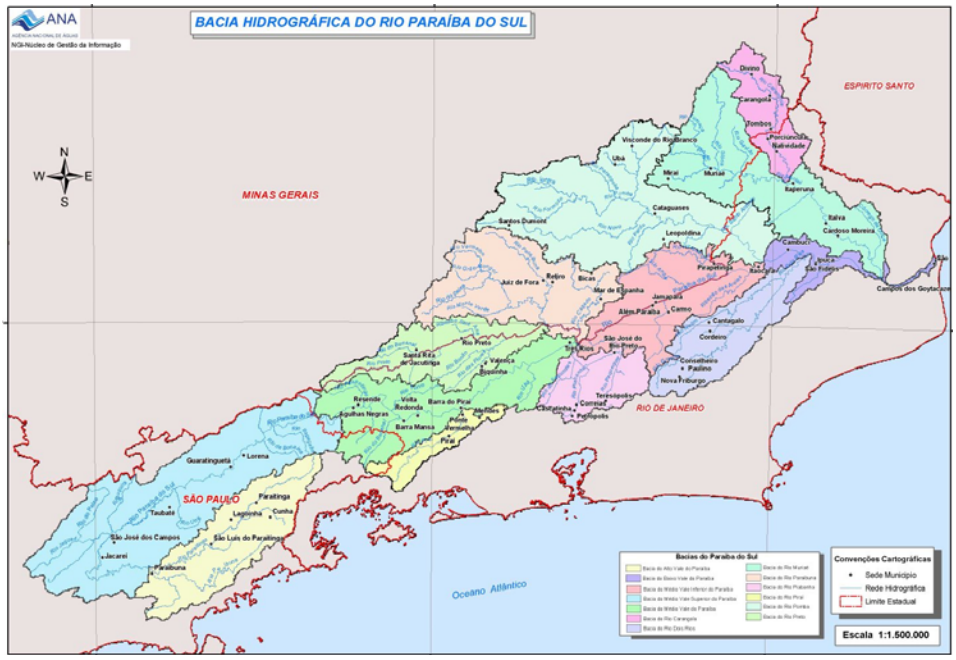

Figura 2 Bacia hidrográfica do rio Paraíba do Sul
Fonte: Comitê das Bacias Hidrográficas do Rio Paraíba do Sul (2011)

2.2 Criopreservação de sêmen

Os trabalhos referentes à criopreservação de sêmen de peixes foram iniciados na década de 1950, com a descoberta dos primeiros crioprotetores (TIERSCH, 2008). Desde o primeiro estudo bem sucedido de criopreservação de sêmen de peixe (BLAXTER, 1953), vários pesquisadores se dedicaram a estudar e aprimorar essa técnica, inclusive no Brasil. Foi empregada, inicialmente, na criopreservação de sêmen de salmonídeos (COSER; GODINHO; TORQUATO, 1987) e a adaptação dessa técnica para as espécies migradoras tropicais da América do Sul foi iniciada na Colômbia e na Venezuela, em 1994 (HARVEY, 2000). No Brasil, estudos sobre a criopreservação de sêmen de espécies nativas haviam sido conduzidos na década de 1980 (KAVAMOTO et al., 1989), mas foi nos anos 1990 que foram intensificados, impulsionados, principalmente, pela preocupação com a preservação da variabilidade genética das populações selvagens devido à construção de barragens de usinas hidrelétricas e à degradação dos habitats (CAROLSFELD et al., 2003). Nos últimos anos, vários protocolos de criopreservação de sêmen foram estabelecidos, experimentalmente aprovados e uma melhoria considerável tem sido alcançada em tecnologia de criopreservação de sêmen de peixes.

A criopreservação do sêmen é um processo que envolve procedimentos que permitem o armazenamento de espermatozoides em nitrogênio líquido a -196 °C, mantendo sua viabilidade por tempo indefinido. A aplicação de tecnologias de criopreservação é essencial quando se busca a formação de bancos de sêmen para a conservação de recursos genéticos e a recuperação de estoques de espécies ameaçados de extinção. Podem-se citar, também, outras vantagens da criopreservação de sêmen, como o estabelecimento de programas de melhoramento genético e hibridização utilizando espécies com períodos reprodutivos diferentes; o fornecimento de materiais genéticos para a

identificação de populações ou estoques por meio de técnicas de biologia molecular; a redução do número de reprodutores (machos), utilizados em programas de propagação artificial com consequente redução de custos; a facilidade de transporte, difusão e troca de material genético entre organizações atuantes na área com risco reduzido de transmissão de patógenos e a diminuição de problemas de assincronia da maturidade gonadal entre reprodutores (principalmente em espécies migratórias), quando machos e fêmeas não estão preparados simultaneamente, entre outros (VIVEIROS; GODINHO, 2009).

No Brasil, diversas instituições de ensino e pesquisa trabalham especificamente com a criopreservação do sêmen de peixes neotropicais e, embora existam muitos protocolos de congelamento de sêmen de peixes já testados e publicados, ainda há muito trabalho a ser feito para melhorar essa tecnologia, sem contar a enorme diversidade de espécies de importância comercial e conservacionista passíveis de estudo. De maneira geral, cerca de 40% a 90% dos espermatozoides de peixes de água doce sofrem algum tipo de danificação durante o processo de congelamento e descongelamento. Os espermatozoides dessas espécies não estão adaptados a um aumento na osmolaridade do meio extracelular e, por isso, estão sujeitos a significante estresse durante o processo de adição de crioprotetores e a subsequente criopreservação (KOPEIKA; KOPEIKA; ZHANG, 2007).

Existe uma grande variabilidade quanto à crioresistência, não somente entre as espécies de peixes, como entre indivíduos da mesma espécie (VIVEIROS; GODINHO, 2009). Devido ao grande número de espécies nesse grupo de vertebrados e suas particularidades com relação às características espermáticas, não existe um único protocolo que permita a criopreservação de sêmen de espécies diferentes de peixes. Para desenvolver protocolos de criopreservação de sêmen, é preciso um conhecimento mínimo sobre criobiologia, velocidades de congelamento e efeito dos crioprotetores.

Um protocolo de criopreservação ideal visa obter um meio de congelamento capaz de prevenir crioinjúrias aos espermatozoides e que não promova à iniciação da motilidade. Além disso, a busca por uma velocidade de congelamento e descongelamento ótima, suficientemente lenta para prevenir a formação de gelo intracelular, e rápida para minimizar o tempo de contato em que as células ficarão expostas aos efeitos de solução, é muito importante para o sucesso no processo de criopreservação e manutenção da viabilidade do espermatozoide pós-congelamento.

2.3 Criobiologia e crioinjúrias

A tecnologia para a criopreservação de gametas de peixes está ainda em desenvolvimento e a sua utilização em produções comerciais ainda é bastante escassa, comparada a algumas espécies de animais domésticos. Entretanto, o progresso alcançado nos últimos anos, com relação ao desenvolvimento de diluentes e técnicas de criopreservação, indica que a criopreservação de gametas de peixes pode vir a ser mais utilizada no futuro. Atualmente, sêmen de várias espécies de peixes é criopreservado com sucesso, alcançando taxas de fertilização cada vez mais semelhantes às obtidas com sêmen fresco, podendo ser utilizados rotineiramente em programas de produção e hibridação. Em revisão sobre criopreservação de gametas, foram listadas mais de 50 espécies de peixes cujos espermatozoides já foram criopreservados (LEUNG; JAMIESON, 1991). Provavelmente, este número tem aumentado de forma considerável nos últimos anos, tendo em vista o grande número de pesquisas relacionadas à criopreservação do sêmen de peixes.

Durante os anos 1980 e 1990, pesquisadores brasileiros utilizaram, principalmente, duas técnicas para congelar sêmen de peixes: pellets e vapor de nitrogênio numa caixa de isopor. A maneira mais antiga de congelar sêmen é em

pellets, que são pequenas gotas formadas ao se gotejar o sêmen diluído diretamente num bloco de gelo seco (-79 °C). Esta técnica foi utilizada em jundiá *Rhamdia quelen* (SILVEIRA; KAVAMOTO; NARAHARA, 1985). A adição de nitrogênio líquido numa caixa de isopor e a exposição ao frio do sêmen já diluído e envasado em palhetas colocado a alguns centímetros acima da superfície do nitrogênio permitem o congelamento do sêmen sem necessidade de equipamentos caros. A tecnologia da criopreservação de sêmen de peixes no Brasil se expandiu grandemente com a utilização de botijões portáteis de vapor de nitrogênio, conhecidos como *dry-shipper* ou botijão canadense. O sêmen, envasado em palhetas (de 0,25 a 4,0 mL), é colocado no interior do botijão e submetido a velocidades entre -30° e -40°C/min (TAITSON; CHAMI; GODINHO, 2008).

Algumas informações sobre criobiologia são necessárias para a compreensão das injúrias que acontece inevitavelmente durante o congelamento e o descongelamento, e como preveni-las. O congelamento, tanto muito lento quanto muito rápido, tende a ser prejudicial às células. Quando qualquer célula em meio aquoso é submetida a temperaturas entre -5 °C e -15 °C, ocorre formação de gelo no meio externo (cristalização). Entretanto, o conteúdo celular permanece líquido e supergelado. O calor latente do processo de cristalização eleva a temperatura para o ponto de congelamento do meio, isto é, -5 °C. Como parte da água do meio extracelular é transformada em gelo, o meio se torna cada vez mais concentrado (hiperosmótico) em relação às células. Numa tentativa de manter o equilíbrio, a água do meio intracelular sai das células. Os subsequentes eventos físicos que acontecem na célula dependem da velocidade de congelamento. Se o congelamento é suficientemente lento, a célula é capaz de perder água rapidamente, desidratar e encolher. Entretanto, a exposição prolongada das células ainda não-congeladas a um meio cada vez mais hiperosmótico causará uma desidratação excessiva e a célula murchará, além do

contato com resíduos intra e extracelulares cada vez mais concentrados. Esses efeitos são conhecidos como “efeitos de solução”. Por outro lado, se as células são congeladas muito rapidamente, elas não são capazes de perder água suficientemente rápido para manter o equilíbrio. A água torna-se cada vez mais supergelada e, eventualmente, congela-se internamente, formando cristais de gelo intracelular. Durante o descongelamento, esses cristais podem se recristalizar em cristais maiores e destruir as membranas celulares.

Não existe exatamente uma linha que separe os danos causados pela formação de gelo intracelular dos danos causados pelos efeitos de solução, principalmente porque os efeitos de solução ocorrem durante todo o processo de congelamento. Os danos causados pelo processo de criopreservação podem levar a consequências adicionais, que incluem deformação estrutural das organelas celulares, anormalidades na estrutura da cromatina espermática e alterações no genoma (BILLARD, 1983). Não há como impedir completamente a ocorrência de tais processos, tampouco os danos causados tanto pelos efeitos de solução quanto pela cristalização durante o congelamento. Dessa forma, a velocidade de congelamento ótima é suficientemente lenta para prevenir a formação do gelo intracelular e suficientemente rápida para minimizar o tempo em que as células ficarão expostas aos efeitos de solução. A velocidade de congelamento ótima varia de -1 a -10 °C/min, para sêmen humano (HENRY; NOILES; GAO, 1993) e -100 °C/min, para sêmen bovino (WOELDERS; MATTHIJS; ENGEL, 1997).

O sêmen de várias espécies de peixes neotropicais das ordens Characiformes e Siluriformes já foi congelado com sucesso por meio desse método de congelamento (VIVEIROS; GODINHO, 2009). O uso de congeladores programáveis é uma técnica que tem sido empregada mais recentemente e tem permitido o emprego de velocidades mais lentas, o uso de mais de uma velocidade por programa, a inclusão de tempo de espera entre

etapas, etc. Entretanto, em função do alto custo, esse equipamento ainda não se torna viável para a aplicação em espécies brasileiras.

O sêmen deve ser armazenado submerso em nitrogênio líquido, a -196 °C, independentemente da forma de congelamento. Somente no momento em que será utilizado, o sêmen deve ser descongelado. O descongelamento é feito sempre em banho-maria e as temperaturas podem variar entre 25 °C e 80 °C, por 3 a 60 segundos, dependendo da espécie. Em geral, o sêmen da maioria dos siluriformes tem sido descongelado em temperaturas que variam de 25 ºC a 50 ºC, por 5 segundos a 5 minutos, de acordo com o recipiente utilizado para armazenar o sêmen (VIVEIROS, 2011). Na Tabela 1 observam-se alguns protocolos de congelamento e descongelamento que apresentaram os melhores resultados, segundo seus autores, utilizados em espécies de siluriformes.

Table 1 Protocolos de congelamento de espécies de bagres, em estudos realizados desde 1998

Tipo de congelamento	Freezing Program	Contêiner	Espécies	Referência
Programável	-4°C/min to -9°C; -11°C/min to -80°C	1,8-mL criotubos	*S. glanis*	Linhart et al. (2005)
Programável	-4°C/min to -4°C; -11°C/min to -80°C	0,25-mL	*C. gariepinus*	Horvath e Urbanyi (2000) e Urbanyi et al. (1999, 2000)
Programável	-5°C/min; held at -40°C for 5 min	1,0-mL cryovial	*C. gariepinus*	Viveiros et al. (2000, 2001) e Viveiros e Komen (2008)
Programável	-8°C/min from -5° to -80°C	1,8-mL criotubos	*C. gariepinus*	Rurangwa et al. (2001)
Programável	-10°C/min	2-mL criotubos	*P. gigas*	Mongkonpunya et al. (2000)
Programável	-10°C/min to -80°C	0,25-mL	*P. larnaudii*	Kwantong e Bart (2006)
Programável	- 10°C/min to -80°C	0,25-mL	*P.hypophthalmus*	Kwantong e Bart (2003) Kwantong e Bart (2009)
Programável	-45°C/min; held at -80°C for 10 min	0,5-mL	*I. punctatus*	Christensen e Tiersch (2005)
N2 vapor	3 min at 3-4 cm above LN2	0,25-, 0,5-, 1,2-mL	*C. gariepinus*	Miskolczi et al. (2005)
N2 vapor	3 min at 3 cm above LN2	0,5-mL	*C. gariepinus*	Kovács et al. (2010)
LN2 vapor	15 min at 6.5 cm above LN2	0,5-, 1-mL	*I. furcatus*	Bart et al. (1998)
LN2 vapor	10 min at 6 cm above LN2 surface 5 min on LN2	2-mL criotubos	*P. fulvidraco*	Pan et al. (2008)
N2 vapor	20 min 3 cm above LN2	0,5 mL straw	*S. glanis*	Baulny et al. (1999)
N2 vapor	Until semen reached -80°C	2-,5-mL criotubos	*P. gigas*	Mongkonpunya et al. (2000)
N2 vapor	-16°C/min to -140°C	0,5-mL	*I. furcatus*	Lang et al. (2003)
N2 vapor (*shipping dewar*)	Samples remained in shipping dewa for < 1 wk	0,5-mL	*P. corruscans*	Carolsfeld et al. (2003)

Fonte: Adaptado de Viveiros (2011)

2.4 Crioprotetores e diluidores

A sobrevivência dos espermatozoides ao congelamento e ao descongelamento pode ser maximizada pela otimização dos protocolos, entretanto, essa sobrevivência será baixa se os agentes crioprotetores permeáveis (ou intracelulares) não forem adicionados ao meio de congelamento. A descoberta de que o glicerol poderia prevenir as crioinjúrias causadas durante o congelamento lento e o descongelamento dos espermatozoides e de células vermelhas (POLGE; SMITH; PARKES, 1949) aumentou o interesse pela formação de bancos de células vivas, em temperaturas baixas. A partir daí, vários compostos foram identificados como protetores de células contra os danos causados pelo congelamento. Vários crioprotetores permeáveis têm sido testados em sêmen de peixes, como dimetil sulfóxido (DMSO), glicerol, metanol, dimetil acetamida (DMA), etileno glicol, metil glicol e propileno glicol. Os crioprotetores que têm produzido os melhores resultados nos últimos 10 anos para proteger os espermatozoides de Siluriformes são dimetil sulfóxido (DMSO), metanol e dimetil acetamida (DMA), em sua maioria, 10% (v:v), variando de 5% a 15%. O metanol tem o menor peso molecular e, portanto, mais rápida permeação celular, quando comparado a outros compostos, tais como DMSO, DMA, e glicerol. No entanto, ainda não se sabe se esses compostos precisam permear as células para serem eficazes (VIVEIROS, 2011).

Esses crioprotetores reduzem o ponto de congelamento do meio extracelular, atenuam os efeitos deletérios dos cristais de gelo e regulam a velocidade de desidratação das células, reduzindo os danos causados pela alta concentração de solutos durante o congelamento lento, os efeitos de solução (MAZUR, 1970). Entretanto, a alta concentração dos crioprotetores ou um prolongado tempo de exposição podem desnaturar as proteínas celulares, reduzindo a viabilidade das células antes mesmo do congelamento. Outro grupo

de crioprotetores impermeáveis ou extracelulares tem sido adicionado ao meio de congelamento, em combinação com os agentes internos. Os crioprotetores impermeáveis incluem açúcares (sacarose, glicose), polímeros (dextran) e proteínas (gema de ovo e leite desnatado). Esses agentes recobrem a superfície da célula, estabilizando a membrana. A gema de ovo e o leite em pó desnatado são os mais comumente utilizados nos meios de congelamento.

O meio de congelamento nos quais os espermatozoides são misturados antes do processo de congelamento é composto por crioprotetores internos que são misturados às soluções aquosas (diluidores) para que atinjam o interior e a superfície dos espermatozoides no processo de criopreservação. Para que não haja a ativação da motilidade dos espermatozoides, a solução aquosa deverá conter sais e ou açúcares para manter alta a osmolaridade no meio diluidor. Soluções simples, como NaCl 0,9% e glicose 5%, bem como soluções mais complexas, como o BTS™ (primariamente desenvolvido como diluidor de sêmen de suíno, Minitub™), têm sido utilizadas com sucesso na criopreservação de sêmen

A combinação diluidor e crioprotetor permeável pode ser mais ou menos efetiva na proteção dos espermatozoides durante o congelamento e o descongelamento, de acordo com o protocolo utilizado em cada laboratório. Alguns fatores interferem no sucesso da criopreservação, tais como taxa de diluição, temperatura do meio no momento da adição do sêmen e tempo de exposição dos espermatozoides ao meio antes do congelamento propriamente dito, entre outros. Além disso, diferenças entre exemplares domesticados em relação aos selvagens e diferenças na alimentação e no manejo da criação podem alterar a composição do plasma seminal e, consequentemente, a sensibilidade dos espermatozoides ao processo de criopreservação.

2.5 Avaliação da qualidade do sêmen

Após o descongelamento, na maioria das vezes, a qualidade do sêmen é avaliada em função da percentagem de células móveis observada em microscópio de luz. Este método é subjetivo, mas, quando executado por um técnico treinado, pode ser bastante preciso, prático e útil para aplicação em pisciculturas.

Durante a última década, o uso de um sistema de análise espermática assistida por computador, conhecido como CASA (do inglês *computer-assisted sperm analysis*), tem se tornado cada vez mais popular em laboratórios de tecnologia de sêmen europeu e americano. O sistema CASA quantifica os movimentos das células espermáticas utilizando pelo menos doze características da motilidade calculadas via computador, constituindo um método objetivo de avaliação, de desempenho rápido, fácil e preciso (RURANGWA et al., 2001). O sistema CASA é o mais objetivo método de quantificação da qualidade de sêmen disponível atualmente (WILSON-LEEDY; INGERMANN, 2007).

Estudos têm sido realizados com a finalidade de comparar a avaliação subjetiva da motilidade com o sistema CASA (NASCIMENTO et al., 2010; VIVEIROS et al., 2010). Até o momento, não foi encontrada diferença significativa entre os dois métodos de avaliação. Isso garante a qualidade da avaliação feita por técnicos bem preparados e valida o uso dessa avaliação em laboratórios que não dispõem de recursos para aquisição de tal equipamento, bem como na rotina das pisciculturas.

3 OBJETIVO DO ESTUDO

O presente estudo foi realizado com os seguintes objetivos:

a) avaliar diferentes meios de congelamento para o sêmen de surubim-do-paraíba *Steindachneridion parahybae*;
b) avaliar o efeito da lactose na qualidade do sêmen pós-descongelamento;
c) comparar a motilidade espermática após o descongelamento, avaliada pelo método subjetivo ao microscópio de luz, e pelo método objetivo por meio do CASA;
d) determinar as velocidades espermáticas após o descongelamento pelo método computadorizado CASA;
e) estabelecer uma correlação entre as velocidades espermáticas e a taxa de fertilização para a espécie.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1

Subjective and computer-assisted motility of sperm cryopreserved in DMSO-based media, of the endangered fish species surubim-do-paraíba *Steindachneridion parahybae* (Siluriformes).

Preparado de acordo com as normas da revista Zygote

Abstract

VIVEIROS, A. T. M; ARAÚJO, R. V; CARVALHO, I. C; ISAÚ, Z. A; CANEPPELE, D; LEAL, M. C; Subjective and computer-assisted motility of sperm cryopreserved in DMSO-based media, of the endangered fish species surubim-do-paraíba *Steindachneridion parahybae* (Siluriformes). In:___. **Motilidade, velocidade e fertilidade do sêmen de surubim-do-paraíba *Steindachneridion parahybae* (Siluriformes) criopreservado em diferentes diluidores.** 2011. 93p., p. 38-66. Tese (Doutorado em Zootecnia) - Universidade Federal de Lavras, Lavras, MG.*

The aim of this study was to evaluate the effects of 10 freezing media on the sperm motility of surubim-do-paraíba *Steindachneridion parahybae*. Sperm was diluted in 5 extenders: 0.9% NaCl, Ginsburg fish Ringer modified (a complex saline solution), 5% glucose, 10% glucose and Beltsville Thawing Solution™ (BTS™; a complex saline, glucose, gentamycin solution), combined or not with 10% lactose. DMSO was used as cryoprotectant. In experiment 1, diluted sperm was equilibrated for a 15 min at 15ºC and then subjectively evaluated for motility (expressed as the percentage of motile sperm) and quality motility score (0 = no movement; 5 = rapidly swimming sperm) 0 and 30 s post-activation. In experiment 2, diluted sperm was frozen in a nitrogen vapor vessel and then thawed in water bath at two temperatures: 25°C for 20 s and 35ºC for 15 s. Post-thaw sperm motility was immediately estimated both subjectively and objectively using a computer-assisted sperm analyzer (CASA). After a 15-min equilibration time, sperm motility evaluated immediately after activation was similar to motility evaluated 30 s later. Highest motilities were observed when fresh sperm was diluted in lactose-free media NaCl, 5% glucose and 10% glucose, (66-73% motile sperm) and in media containing lactose 5% glucose and BTS™, (53-63% motile sperm). Highest quality scores were observed when sperm was diluted in 10% glucose lactose-free medium (score 3.4) and in media containing lactose 5% glucose, 10% glucose and BTS™, (score 2.7-3.2). When post-thaw sperm motility was evaluated, there was no difference between the two methodologies (subjective and CASA) or between the two thawing temperatures (25 and 35°C). Post-thaw motility was not different among sperm frozen in the five lactose-free extenders (57-64% motile sperm), and was highest when sperm was frozen in 5% glucose and BTS™, in media containing lactose (53-54% motile sperm). The addition of lactose to the freezing media evaluated here did not improve post-thaw sperm quality. *S. parahybae* sperm can be effectively frozen in any of the five lactose-free media tested.

Keywords: CASA, Catfish, Extender, Freezing, Semen quality.

Resumo

VIVEIROS, A. T. M; ARAÚJO, R. V; CARVALHO, I. C; ISAÚ, Z. A; CANEPPELE, D; LEAL, M. C; Motilidade espermática subjetiva e assistida por computados do sêmen criopreservado em meios a base de DMSO de uma espécie ameaçada de extinção, surubim-do-paraíba *Steindachneridion parahybae* (Siluriformes). In:__. **Motilidade, velocidade e fertilidade do sêmen de surubim-do-paraíba *Steindachneridion parahybae* (Siluriformes) criopreservado em diferentes diluidores.** 2011. 93p., p. 38-66. Tese (Doutorado em Zootecnia) - Universidade Federal de Lavras, Lavras, MG.*

O estudo foi realizado com o objetivo de avaliar os efeitos de dez meios de congelamento sobre a motilidade espermática de surubim-do-paraíba, *Steindachneridion parahybae*. O sêmen foi diluído em 5 diluidores: 0,9% NaCl, *Ginsburg Fish Ringer* modificado (solução salina complexa), 5% glicose, 10% glicose e *Beltsville Thawing Solution*™ (BTS™, solução salina complexa, glicose, solução de gentamicina), combinado com lactose, 10% ou 0% (controle). O DMSO foi utilizado como crioprotetor. No experimento 1, o sêmen diluído nos diferentes meios de congelamento foi incubado, por 15 minutos, a 15 ºC e, em seguida, subjetivamente avaliado quanto à motilidade (expressa em porcentagem de espermatozoides móveis) e ao vigor espermático (0 = nenhum movimento; 5 = rapidamente móvel), 0 e 30 segundos após ativação. No experimento 2, o sêmen diluído foi congelado em botijão de vapor de nitrogênio e descongelado em banho-maria, em duas temperaturas: 25 °C, por 20 segundos e 35º C, por 15 segundos. Após o descongelamento, a motilidade espermática foi imediatamente estimada tanto subjetivamente como objetivamente, usando um analisador de sêmen computadorizado (CASA). Depois de 15 de equilíbrio, a motilidade avaliada imediatamente após a ativação foi similar à motilidade avaliada 30 segundos depois. Maiores motilidades foram observadas quando o sêmen foi diluído em NaCl, 5% glicose e 10% glicose, sem adição de lactose (66%-73% de espermatozoides móveis) e em 5% glicose e BTS ™, em meios contendo lactose (53%-63% espermatozoides móveis). Os mais altos valores para o vigor espermático foram observados para o sêmen diluído em 10% glicose em meio sem lactose (score 3,4) e em 5% glicose, 10% glicose e BTS ™, em meios contendo lactose (score 2,7-3,2). Quando a motilidade foi avaliada após o descongelamento, não houve diferença entre as duas metodologias utilizadas (subjetiva e CASA) ou entre as temperaturas de descongelamento testadas (25 °C e 35 °C). A motilidade pós-descongelamento não foi diferente quando o esperma foi congelado em um dos cinco meios sem adição de lactose (57-64% espermatozoides móveis) e foi maior quando o esperma foi congelado em 5%-glicose e BTS ™, nos meios contendo lactose (53%-54% de espermatozoides móveis). A adição da lactose aos meios de congelamento aqui

avaliados não melhorou a qualidade espermática pós-descongelamento. O sêmen de *S. parahybae* pode ser efetivamente congelado em qualquer um dos cinco meios testados, sem adição de lactose.

Palavras-chave: CASA, Bagre, Diluidor, Congelamento, Qualidade espermática.

1. Introduction

In Brazil, the siluriforms are distributed into 11 families with 1056 species. The family Pimelodidae contains 83 species, and 6 of these species belong to the genus *Steindachneridion* (Buckup *et al.,* 2007). The surubim-do-paraíba *Steindachneridion parahybae* (Steindachner, 1876) is a Brazilian freshwater catfish species, endemic to the Paraíba do Sul River Basin (Oliveira & Moraes Jr, 1997), in the states of Minas Gerais, Rio de Janeiro and São Paulo (Southeastern Brazil). During the 1950's, *S. parahybae* was a important commercial fishery resource (Machado & Abreu, 1952), but nowadays this species is critically threatened in nature and there is very few information about its reproductive behavior (Caneppele *et al.*, 2009).

The knowledge of reproductive biology and physiology is critical for conservation and species management, allowing the prevention of species extinction and the utilization of males in natural and artificial reproductive programs (Wildt, 2005). The cryopreservation of fish sperm provides a tool by which reproduction is optimized during the reproductive period thereby larvae production is increased. Cryopreserved sperm serves as genetic bank or germplasm which may help ensure genetic diversity and reproductive success for population management strategies (Viveiros *et al.*, 2011). Despite the great number Brazilian siluriforms, studies on sperm cryopresevation of these species have received reduced attention. Only three species of the family Pimelodidae were subject of such studies: *Pseudoplatystoma corruscans* (Carolsfeld *et al*., 2003), *Rhamdia quelen* (Fogli da Silveira *et al.,* 1985) and *Steindachneridion melanodermatum* (Bombardelli *et al.,* 2011).

For storage in liquid nitrogen, sperm must be diluted in a freezing medium containing an extender and a permeable cryoprotectant agent (CPA). As teleost sperm is immotile in seminal plasma, motility is triggered when the

sperm comes in contact with a hyposmotic medium. Thus any solution tested as sperm extender must have an appropriate osmolality, mostly above 300 mOsm kg^{-1} (Alavi & Cosson, 2006). Simple saline solutions such as NaCl (Kwantong & Bart, 2006), complex saline solutions as Ginsburg fish Ringer (Viveiros *et al.*, 2000), sugars as fructose (Kovacs *et al.*, 2010; Miskolczi *et al.*, 2005) or glucose (Carolsfeld *et al.*, 2003) and the combination of salts and sugar as Hanks' balanced salt solution (HBSS; Christensen & Tiersch, 2005; Lang *et al.,* 2003) have been successfully tested as sperm extender of siluriforms. Over the last decade, dimethyl sulfoxide (DMSO), methanol, and dimethyl acetamide (DMA), mostly at 10% (v/v), have been used as CPA for catfish sperm (Viveiros, 2011).

In Brazilian Siluriformes, the freezing medium that yielded the highest post-thaw sperm quality was glucose-powdered milk-DMSO for *P. corruscans* (Carolsfeld *et al.*, 2003), a combination of salts, glucose (named V2E) and DMSO for *R. quelen* (Fogli da Silveira *et al.,* 1985) and glucose-powdered milk-methanol for *S. melanodermatum* (Bombardelli *et al.,* 2011). Recently, we evaluated the effects of DMSO, methanol and methyl glycol on the sperm motility of *S. parahybae* after a 30-min equilibration (Isaú & Viveiros unpublished data). Sperm diluted in DMSO yielded the highest motility (54%) compared to the methanol (37%) and methyl glycol (33%), regardless extender composition.

Post-thaw sperm quality in fish is mostly evaluated as the percentage of motile cells observed under light microscope. This method is subjective, but reliable when performed by a well-trained technician. The use of a computer-assisted sperm analyzer (CASA) has become increasingly popular in laboratories worldwide. During the last two decades, the CASA system has been adapted to fish sperm (Toth *et al.*, 1995, 1997; Christ *et al.,* 1996; Kime *et al.*, 1996, 2001; Ravinder *et al.*, 1997; Lahnsteiner, 2000; Rurangwa *et al.*, 2001 and Wilson-Leedy & Ingermann, 2007). In Brazilian fish species, the use of CASA system to

analyze sperm quality is modest and has been used in few species of Characiformes (Viveiros *et al.,* 2010 and Nascimento *et al.,* 2010) only in two species of Siluriformes, *R. quelen* (Sanches *et al.,* 2010a) and *S. melanodermatum* (Bombardelli *et al.,* 2011).

The aim of this study was to evaluate the effects of some extenders, combined or not with lactose, on sperm motility of surubim-do-paraíba *Steindachneridion parahybae*. Sperm motility was evaluated after a 15-min equilibration time (subjectively using a light microscope) and after thawing (both subjectively and objectively using CASA).

2. Materials and methods

2.1 Fish handling, sperm collection and initial evaluation

All fish were handled in compliance with published guidelines for animal experimentation (Van Zutphen *et al.,* 2001). *S. parahybae* broodfish F1 males (originated from wild parents kept in captivity) with 400-570 of body weight and 36-40 cm of total length were selected from concrete tanks at the Hydrobiology and Aquaculture Station of the Energy Company of São Paulo (CESP) in the city of Paraibuna, state of São Paulo State, Brazil (20°26' and 23°39'S, and 41° and 46°30'W), during the spawning season (February). Males with detectable running sperm in response to soft abdominal pressure were selected. The urogenital papilla was carefully dried and sperm was hand-stripped directly into test tubes. Sperm was collected without hormone induction at room temperature (25–27°C). Immediately after collection, tubes containing sperm were placed in crushed ice (~10°C). An aliquot of 2 µl of each sample was placed on a slide and observed under a light microscope (Model L1000, Bioval, Jiangbei, China) at 400 x magnification. As fish sperm in seminal plasma should be immotile, any sperm motility observed was attributed to urine or water

contamination and the samples discarded. In immotile samples (n = 8 males), sperm motility (expressed as percentage of motile sperm) was subjectively estimated immediately after the addition of 30 µl of 0.29% NaCl (~98 mOsm kg^{-1}) as activating agent (Viveiros & Godinho, 2009). All samples possessed at least 80% motile sperm and were used in the subsequent analysis. Volume, concentration (hemacytometer/ Neubauer chamber) and pH (YSI meter 660, Lake Village, AK, USA) of each sperm sample were determined. The sperm from each male was centrifuged at 2000 g for 30 min at room temperature and osmolality was measured cryoscopically (Digital MicroOsmometer Roebling, Type 13/13, Messtechnik TM, Berlin, Germany).

2.2 Experiment 1: Subjective motility after a 15-min equilibration

Ten extenders comprising the combination of 5 extender compositions and 2 concentration of lactose (0 and 10%) were prepared. The extenders tested were a simple saline solution (NaCl); a complex saline solution (Ginsburg fish Ringer modified); two simple sugar solutions (5 and 10% glucose) and a complex sugar-saline solution (Beltsville Thawing Solution™ = BTS™). BTS™ (Minitub™, Tiefenbach / Landshut, Germany) is a commercial boar sperm extender and has been successfully used in some Brazilian Characiformes species (Viveiros & Godinho, 2009). For extender formula please refer to Table 1. Dimethyl sulfoxide (DMSO – $(CH_3)_2SO$; Cromato Produtos Químicos LtdaTM, Diadema, SP, Brazil) was used as cryoprotectant, according to our previous experiment (Isaú & Viveiros unpublished data). Sperm samples (n = 3 males) were diluted in each media at a final proportion of 10% sperm, 80% extender and 10% DMSO. After a 15-min equilibration at 15ºC, an aliquot of 2 µl of each sample was placed on a glass slide and observed under a light microscope (Model L1000, Bioval, Jiangbei, China) at 400 x magnification. Sperm motility and quality motility score (an arbitrary grading system ranging

from 0 = no movement to 5 = rapidly swimming spermatozoa; Viveiros *et al.,* 2011) were subjectively determined 0 and 30 s post-activation.

Table 1 Chemical composition of the media tested as sperm extender of surubim-do-paraíba *Steindachneridion parahybae*

Chemicals (g)[a]	Extenders				
	1.17% NaCl	Ginsburg [c]	5% Glucose	10% Glucose	BTS™ [b]
NaCl	1.17	1.170	--	--	--
Glucose	--	--	5.0	10.0	4.00
$NaHCO_3$	--	0.036	--	--	0.13
KCl	--	0.045	--	--	0.08
Sodium citrate	--	--	--	--	0.63
EDTA	--	--	--	--	0.13
Gentamycin sulfate	--	--	--	--	0.02
$CaCl_2.2H_2O$	--	0.072	--	--	--
mOsmol kg^{-1}	387	405	288	638	345

[a]All chemicals were diluted in deionized water to a final weight of 100 g, and pH was set at 7.6. [b]BTS™: Beltsville Thawing Solution™ . [c]Ginsburg fish Ringer modified
*Lactose was added at 10g in each extender when tested

2.3 Experiment 2: Subjective and CASA motility after thawing

Sperm (n = 5) was diluted in each of the same 10 extenders combined with DMSO used in experiment 1 and equilibrated for 15 min. The samples were then loaded into 0.5 ml straws (n = 6 replicate straws / male) and frozen in a nitrogen vapor vessel (Cryoporter™ LN2 *dry vapor shipper*, Cryoport Systems, Brea, CA, USA) at approximately -170°C. Using this methodology, straws reach -170ºC after ~ 329 seconds at a freezing rate of approximately – 35.6ºC min^{-1} between 21ºC and -170ºC (Maria *et al.,* 2006a). Then, straws were transferred to liquid nitrogen (M.V.E XC 34/18, CHART/MVE, Marietta, GA 30066, USA) at -196°C within 20–24 h. In total, 300 straws (6 replicate straws x 10 media x 5 males) were cryopreserved. Two weeks later, straws were placed back into dry-shipper and transported 370 km by car from CESP to the Department of Animal

Sciences at the Federal University of Lavras (UFLA), Lavras, MG, Brazil, where straws were stored in liquid nitrogen (M.V.E. Millenium, XC 20, Chart, Minessota, USA) until sperm motility analysis. Approximately one year later, straws were thawed in water bath at two temperatures: 25°C for 20 s and 35ºC for 15 s. Post-thaw sperm motility was immediately estimated, both subjectively as described for fresh sperm and objectively using CASA. One microliter of post-thaw sperm was activated in 20 µl of 0.29% NaCl. Motility was first triggered in eppendorf tubes and the sample was than transfered to Leja™ counting chamber (chamber depth: 20 µm) slide placed under a phase contrast microscope (Nikon™ E200, Japan), at 100 x magnification, whit a green filter, at pH1 position. The microscope was connected to a video camera (Basler Vision Technologies™ 602FC, Ahrensburg, Germany) generating100 frames s^{-1}. Video recording started ~10 s post-activation. Each image (n = 25) was analyzed using the standard settings for fish by Sperm Class Analyzer™ software (SCA™, Microptics, S.L. Version 5.1, Barcelona, Spain). Sperm was considered immotile when curvilinear velocity (VCL) was < 20 µm s^{-1}. Although the SCA™ assesses move than 15 sperm motility and points, for brevity only curvilinear velocity (VCL), straight line velocity (VSL), and average path velocity (VAP) were considered for analyses. To determine these velocities each individual sperm cell (n = at least 150 sperm / field) was followed throughout the 25 images and a sperm trajectory was calculated.

2.4 Statistical analyses

Values are expressed as means ± standard deviation (SD). Statistical analyses were conducted with the SISVAR computational program (Ferreira, 1999). Sperm motility was tested for normal distribution using the univariate procedure. When data did not have a normal distribution, an arc sin transformation was performed. Data were tested for significant differences using

ANOVA, followed by the Scott-Knott test, when applicable. The level of significance for all statistical tests was set at 5% ($P < 0.05$).

3. Results

The following mean sperm values were found for *S. parahybae* males (n=8): 94% motile rate, volume of 7 ml, 12 x 10^9 spermatozoa ml^{-1} and seminal plasma pH of 7.5 (Table 2).

Table 2 Male body weight and fresh sperm features of surubim-do-paraíba *Steindachneridion parahybae*

Features	Mean ± SD*	Range (min – max)
Body weight (g)	484 ± 50	400 - 570
Total length (cm)	38 ± 1.3	36 - 40
Semen volume (ml)	7 ± 2.2	13.4 - 20.7
Concentration (spermatozoa x 10^9 ml^{-1})	12 ± 2	10 - 15
Subjective motility (% motile sperm)	94 ± 5	90 - 100
Semen pH	7.5 ± 0.2	7.1 - 7.7
Seminal plasma osmolality (mOsmol kg^{-1})	275 ± 4.5	265 - 280

* SD - Standard deviation

3.1 Experiment 1: Subjective motility after a 15-min equilibration

Sperm motility (% motile sperm and score) evaluated immediately after activation produced similar results ($p > 0.05$) to motility evaluated 30 s later, regardless extender composition or the presence of lactose. Thus, the average between data collected at 0 and 30 s pos-activation was used to describe and discuss the effects of extender and lactose (Table 3).

An interaction between extender and lactose was observed. When sperm was diluted in lactose-free media, highest motilities (66-73% motile sperm) were observed in NaCl, 5% glucose and 10% glucose. When lactose was added, motility significantly decreased in samples diluted in NaCl, Ginsburg and 10%

glucose (4-27% motile sperm). Motility of sperm diluted in 5% glucose and in BTS™ was not affected by the addition of lactose.

The highest quality motility score (3.4) among the lactose-free media, was observed when sperm was diluted in 10% glucose. When lactose was added, quality motility score increased from 2.5 to 3.2 in samples diluted in 5% glucose, and decreased in samples diluted in NaCl, Ginsburg and in 10% glucose from 2.8-3.4 to 0.9-2.7. Quality motility score of samples diluted in BTS™ was not affected by the addition of lactose.

Table 3 Subjective motility and quality motility score (mean ± SD; n = 3 males x 3 replicate straws) of *Steindachneridion parahybae* fresh sperm diluted in five different extenders combined or not with lactose and equilibrated for 15-min. Motility was evaluated immediately (0 s) and 30 s post-activation with 0.29% NaCl as activating agent

Extender[2]	Seconds post activation [1]	Motility (% motile sperm)		Quality motility (score 0-5*)	
		Lactose (%)		Lactose (%)	
		0	10	0	10
NaCl	0	71 ± 11	9 ± 15	3.3 ± 0.3	1.4 ± 1.7
	30	68 ± 7	8 ± 14	2.6 ± 0.2	1.0 ± 1.2
	Average	**69 ± 9 aA**	**8 ± 14 cB**	**2.9 ± 0.3 bA**	**1.2 ± 1.4 bB**
Ginsburg	0	58 ± 14	4 ± 7	3.3 ± 0.3	1.0 ± 1.7
	30	51 ± 14	4 ± 7	2.3 ± 0.3	0.9 ± 1.5
	Average	**54 ± 14 bA**	**4 ± 7 cB**	**2.8 ± 0.3 bA**	**0.9 ± 1.6 bB**
5% Glucose	0	67 ± 10	64 ± 17	2.8 ± 0.2	3.6 ± 0.4
	30	64 ± 7	62 ± 17	2.2 ± 0.2	2.9 ± 0.4
	Average	**66 ± 8 aA**	**63 ± 17 aA**	**2.5 ± 0.2 bB**	**3.2 ± 0.4 aA**
10% Glucose	0	76 ± 12	29 ± 19	3.8 ± 0.4	3.0 ± 0.7
	30	71 ± 12	24 ± 17	3.0 ± 0.0	2.3 ± 0.6
	Average	**73 ± 12 aA**	**27 ± 18 bB**	**3.4 ± 0.2 aA**	**2.7 ± 0.6 aB**
BTS™	0	60 ± 16	58 ± 17	3.1 ± 0.2	3.3 ± 0.6
	30	57 ± 14	49 ± 18	2.7 ± 0.3	2.9 ± 0.2
	Average	**58 ± 15 bA**	**53 ± 17 aA**	**2.9 ± 0.3 bA**	**3.1 ± 0.4 aA**
Fresh sperm	0	93 ± 3 a		5 ± 0 a	
	30	89 ± 4 a		4 ± 1 a	
	Average	**91 ± 3.5**		**4.5 ± 0.5**	

[a,b; A,B]Means followed by different superscripts (lowercase for columns and uppercase for lines) within the same parameter, are different (Scott-Knott; $p < 0.05$). *Qualitative motility scores were assigned ranging from 0 (no movement) to 5 (rapidly swimming spermatozoa). [1]Motility evaluated either 0 or 30 s post-activation did not differ (Scott-Knott $p > 0.05$). [2]For chemical compositions of these extenders please refer to Table 1

3.2 Experiment 2: Subjective and CASA motility after thaw

Post-thaw sperm motility subjectively evaluated using light microscope produced similar results ($p > 0.05$) to motility evaluated by the CASA, in all ten freezing media. Thus, the data obtained with CASA was used to describe and discuss the results. Similarly, there was no significant difference ($p > 0.05$) of sperm motility when straws were thawed at 25ºC or 35ºC, and thus the average was used to describe and discuss the results (Table 4). An interaction between extenders and lactose was observed. When sperm was frozen in lactose-free media, post-thaw motility varied from 57 to 64% motile sperm in all five media. When lactose was added, motility decreased to16-36% motile sperm in sampled frozen in NaCl, Ginsburg and in 10% glucose. Motility of sperm samples diluted in 5% glucose and in BTS™ was not affected by the addition of lactose.

Table 4 Subjective motility rate and computer assisted sperm analyzer (CASA), (mean ± SD; n = 5 males x 3 replicates) of *Steindachneridion parahybae* sperm cryopreserved in five different extenders combined or not with lactose. Motility was triggered in 0.29% NaCl

Extender [2]	Thawing temperature [1]	Subjective motility (% motile sperm)*		CASA motility (% motile sperm)*	
		Lactose (%)		Lactose (%)	
		0	10	0	10
NaCl	25°C/20 s	64 ± 9	21 ± 19	63 ± 4	27 ± 15
	35°C/15 s	64 ± 6	19 ± 16	61 ± 4	25 ± 15
	Average	**64 ± 7 aA**	**20 ± 17 cB**	**62 ± 4 aA**	**26 ± 15 cB**
Ginsburg	25°C/20 s	63 ± 8	9 ± 12	65 ± 5	16 ±13
	35°C/15 s	62 ± 6	10 ± 11	62 ± 5	15 ±14
	Average	**63 ± 7 aA**	**10 ± 11 dB**	**64 ± 5 aA**	**16 ± 13 dB**
5% Glucose	25°C/20 s	63 ± 3	57 ± 13	64 ± 4	55 ± 9
	35°C/15 s	59 ± 4	54 ± 5	63 ± 6	52 ± 7
	Average	**61 ± 3 aA**	**56 ± 9 aA**	**64 ± 5 aA**	**53 ± 8 aA**
10% Glucose	25°C/20 s	67 ± 7	27 ± 21	58 ± 7	37 ±10
	35°C/15 s	64 ± 6	29 ± 22	56 ± 7	35 ± 8
	Average	**65 ± 6 aA**	**28 ± 21 bB**	**57± 7 aA**	**36 ± 9 bB**
BTS™	25°C/20 s	67 ± 6	55 ± 8	63 ± 2	53 ± 6
	35°C/15 s	63 ± 8	55 ± 5	58 ± 4	55 ± 4
	Average	**65 ± 7 aA**	**55 ± 6 aA**	**61 ± 3 aA**	**54 ± 5 aA**
Fresh sperm		97 ± 5		---	

[a,b; A,B] Means followed by different letters (lowercase for columns and uppercase for lines) are different (Scott-Knott; $p < 0.05$)

*Post-thaw sperm motility evaluated by either subjectively or CASA did not differ (Scott-Knott; $p > 0.05$)

[1] There was no significant difference of sperm motility when straws were thawed at 25ºC or 35ºC (Scott-Knott; $p > 0.05$)

[2]For chemical compositions of these extenders please refer to Table 1

4. Discussion

In this study, some parameters of fresh sperm collected during the reproductive season, as well as the effects of some freezing media on sperm motility evaluated after a 15-min equilibration and after thawing of *S. parahybae* were evaluated.

4.1 Fresh sperm features

Fresh sperm motility (94% motile sperm), volume (7 ml or 14.5 ml kg^{-1}), concentration ($12x10^9$ spermatozoa ml^{-1}), pH (7.5) and seminal plasma osmolality (275 mOsmol kg^{-1}) from the eight males used in this study were all within the range previously reported for this species (Sanches *et al.,* 2010b, 2011; Caneppele *et al.,* 2010 and Romagosa *et al.,* 2011) and other Siluriformes (Viveiros, 2011). It is interesting to observe that in catfish species, seminal plasma osmolality it is below the range 300 - 350 mOsmol kg^{-1} observed for the majority of teleost fish species (Alavi & Cosson, 2006).

In *S. parahybae* the osmolality of seminal plasma is 275 mOsmol kg^{-1} (present study), in catfish *Silurus glanisel* it is 280 mOsmol kg^{-1} (Billard *et al*., 1997), in *R. quelen* it is 300 mOsmol kg^{-1} (Mojica, 2004) and in bagre rayado *Pseudoplatystoma metaense*, it ranges from 226 to 270 mOsmol kg^{-1} (Ramirez-Merlano *et al.*, 2011). These differences may be related to the ionic and biochemical composition of the seminal plasma which, besides being species-specific, also varies according to the advancement in the reproductive season and other factors such as aging of spermatozoa and spermatozoa concentration (Alavi *et al.,* 2010).

4.2 Motility evaluated: 0 and 30 s post-activation

The duration of sperm motility is highly variable between different species of fish and may be related to several factors such as the reproductive season (Rana, 1995) or the rate at which energy reserves of sperm depleted after the activation (Cosson, 2004). In the present study, sperm motility subjectively evaluated 30 s post-activation was similar to the motility evaluated immediately after activation. The fresh sperm motility of *S. parahybae* was assessed 10 and 20 s post-activation in distilled water and, different from our results, a significant decrease in sperm motility assessed 10 s (89% motile sperm) was observed compared to 20 s (67% motile sperm) post-activation (Sanchez *et al.,* 2011). Similarly, fresh sperm motility of *R. quelen* significantly decreased from 65% motile sperm when evaluated 15s post-activation to 41% motile sperm when evaluated 25s post-activation using distilled water (Sanches *et al*., 2010a). This fast decrease in sperm motility may be associated with several factors such as the activating agent used, the osmolality of the surrounding medium (Alavi & Cosson, 2006), the concentration of some ions, pH, temperature and dilution rate (Cosson, 2010). In *Esox lucius*, sperm motility was triggered in media ranging from 0 to 400 mOsmol kg^{-1}. In general, the greatest percentage of motile spermatozoa and highest spermatozoa velocity were observed between 125 and 235 mOsmol kg^{-1}. When spermatozoa were exposed to distilled water (0 mOsmol kg^{-1}), an hypo-osmotic shock was observed leading to flagellar damages which in turn limited the duration of motility (Alavi *et al.*, 2009). It is possible that the two studies cited previously, where distilled water was used as activating agent for the *S. parahybae* sperm, the sperm has suffered some sort of flagellar damage due to the lower osmolality of distilled water, as observed for *E. lucius*, limiting flagellar movement some time after activation. In this present study, the osmolality of the 0.29% NaCl medium (~98 mOsmol kg^{-1}), used as

activating agent, was higher than the osmolality of the distilled water, which could explain the maintenance of sperm motility 30 s post-activation.

4.3 Extenders

Ten extenders comprising the combination of 5 extender compositions and 2 concentration of lactose (0 and 10%) were assessed after a 15-min equilibration and post-thaw. When sperm was frozen in lactose-free media, there was no difference on post-thaw sperm motility among all five extenders tested: NaCl, Ginsburg, BTS™, 5 and 10% glucose.

Simple saline solutions such as NaCl (Kwantong & Bart, 2006), and glucose are commonly used as extender of Siluriformes sperm, not only because of the simple composition but also because they are commercially available. Complex saline solutions such as Ginsburg fish Ringer (Viveiros *et al.,* 2000) and the combination of salts and sugar such as Hanks' balanced salt solution (HBSS; Christensen & Tiersch, 2005; Lang *et al.,* 2003) have also been successfully used as sperm extender the catfish species. Glucose is commonly used as fish sperm extender, both in simple formulae (5% glucose) and in more complex formulae (combined with egg yolk or with ions and antibiotics such as BTS™ and M III™). The addition of energetic substrates, such as glucose or lactate, to the African catfish *Clarias gariepinus* sperm was found to prevent the decrease in intracellular ATP concentration during storage (Zietara, *et al.,* 2004). On the other hand, a recent review states that spermatozoa from species with external fertilization, such as the *S. parahybae*, cannot rely on the medium to provide their energy supply (Bobe & Labbé 2010). Glucose is also added to extenders due to its stabilization effects on the sperm liposomal membrane (Quinn, 1985). The BTS™ is a commercial extender for boar sperm and has been successfully used as fish sperm extender in some Brazilian Characiformes species (Viveiros & Godinho, 2009) as pacu *P. mesopotamicus* (Órfão *et al.,*

2008), piracanjuba *B. orbignyanus* (Maria *et al.,* 2006a, 2006b), Streaked prochilod *P. lineatus* (Viveiros *et al.*, 2009a; Miliorini, 2006), pirapitinga *Brycon nattereri* (Oliveira *et al.,* 2007), dourado *S. brasilienses* (Viveiros *et al.,* 2009b) and piapara *L. obtusidens* (Koch *et al.,* 2007). This is the first time the BTS™ is tested as extender for catfish sperm. In the present study, sperm frozen in the BTS™ produced 61 % post-thaw motility.

4.4 Lactose

The addition of lactose to the media NaCl, Ginsburg and 10% glucose had a negative effect on sperm motility rate and had no effect when combined with the media BTS™ and 5% glucose. These results were similar when assessed before (15-min equilibration) and after the freezing process, leading us to believe that the decrease in sperm quality may be related the formulation of the media used rather than to the cryopreservation process itself. As with glucose, the lactose is also added to extenders due to its stabilization effects on the spermatozoa liposomal membrane. In the cyprinid *Chalcalburnus chalcoides*, the effect of lactose as cryoprotectant of membrane was assessed. The sperm was diluted and cryopreserved in a saline solution (BSMIS: NaCl 4.38 g, KCl 5.22 g, $CaCl_2$ 0.22 g, $MgSO_4$ 0.12 g, Tris 2.42 g), DMSO and two levels of lactose: 0.5% and 1%. There was a higher rate of sperm motility in the freezing media consisting of 0.5% lactose (43% motile sperm) compared to medium 1% lactose (12% motile sperm). According to the authors, concentrations above 1% lactose probably caused dehydration cell (Lahnsteiner *et al.*, 2000). However in another study, 5% lactose was added to BTS™ and DMSO in cryopreservation of sperm of *P. lineatus*, resulting in 64% motile sperm (Felizardo *et al.*, 2010). In this present study, the addition of 10% lactose to the media freezing had a negative effect or had no effect on sperm motility of *S. parahybae*. However, when the sperm was assessed after 15-min equilibration

was observed an increase in the quality motility score for the sperm diluted in 5% glucose-lactose (increased 2.5 for 3.2 score), when compared the same solution that have not received lactose. The quality motility score is a subjective assessment of sperm quality, however, is directly related to the velocity of sperm. Sperm quality variables such as the velocity and beat /cross frequency of flagellum may be correlated to the potential fertilization rate and allow the prediction of individual reproductive potential (Rurangwa *et al.*, 2004).

The osmolarity of the media BTS™ and 5% glucose increased by lactose was 664 and 700 mOsm kg^{-1} respectively, since media NaCl, 10% glucose and Ginsburg, who were the means which of sperm motility decreased after addition of lactose, osmolarity ranged from 741 to 900 mOsm kg^{-1} (Table 1). Apparently, freezing media with osmolality above 741 mOsm kg^{-1} may cause damage to sperm of *S. parahybae* since it solutions with high osmotic pressure in contact with sperm cells promotes efflux of water and increased the internal osmotic concentration. In mammalian sperm this process causes denaturation of macromolecules and cell plasmolysis, and there may be total collapse of the cell membrane (Medeiros *et al.*, 2002). Similar studies using lactose should be performed to evaluate other parameters of sperm quality, as such sperm velocity and fertilization capacity of *S. parahybae* cryopreserved sperm.

4.5 Thawing temperatures

The thawing temperatures tested in the present study (25 and 35ºC), did not affect post-thaw sperm motility. In general, frozen catfish sperm has been mostly thawed in a water bath at 25-50 ºC from 5 sec to 5 min (Viveiros, 2011). The ideal thawing temperature varies from species to species, to the volume and size of the straws and the cooling rate used during the freezing of sperm.

4.6 Subjective motility x CASA motility

The CASA system has been used to assess sperm quality in several species of catfish (Ramirez-Merlano *et al.,* 2011; Sanches *et al.,* 2010a; Rurangwa *et al.,* 2001 and Kime *et al.,* 1996). The subjective analysis of sperm motility using a light microscope, traditionally used in most studies as a parameter for assessing sperm quality (Viveiros & Godinho, 2009), was compared to the CASA system in the present study. There was no significant difference between the two methods of evaluation used. Similarly, in two other Brazilian freshwater Characiformes, *P. brachypomus* (Nascimento *et al.,* 2010) and *P. lineatus* (Viveiros *et al.,* 2010), there was no significant difference when post-thaw sperm motility was analyzed subjectively or using CASA.

For indicators analyses of sperm quality, CASA is the most objective and comprehensive quantification currently available (Wilson-Leedy and Ingermann, 2007). The use of CASA as a method to evaluate sperm quality is important, however, the great equipment cost has restricted its use to few laboratories, especially in developing countries (Nascimento *et al.*, 2010), furthermore there is a great necessity for standardization of CASA settings in order to allow comparison among studies and fish species. Based on the present study, a well-trained technician using a light microscope could easily and reliably assess sperm motility, even under field conditions.

5. Conclusions

The addition of lactose to the freezing media evaluated here did not improve post-thaw sperm quality. *S. parahybae* sperm can be effectively frozen in any of the five lactose-free media tested. Although the CASA system provides precise data on sperm motility, the subjective evaluation is very practical and

can be conducted by well-trained personnel in commercial fish farms as an acceptable and reliable evaluation method of sperm quality.

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ARTIGO 2

Fertility, velocities and motility of Surubim-do-paraíba *Steindachneridion parahybae* (Siluriformes) sperm cryopreserved in lactose and lactose-free media

Preparado de acordo com as normas da revista Journal of Applied Ichthyology

Abstract

ARAÚJO, R. V; CARVALHO, I. C; SANCHES, E. A; CANEPPELE, D; LEAL, M. C; VIVEIROS, A. T. M. Fertility, velocities and motility of Surubim-do-paraíba *Steindachneridion parahybae* (Siluriformes) sperm cryopreserved in lactose and lactose-free media. In:___. **Motilidade, velocidade e fertilidade do sêmen de surubim-do-paraíba *Steindachneridion parahybae* (Siluriformes) criopreservado em diferentes diluidores.** 2011. 93p., p. 67-93. Tese (Doutorado em Zootecnia) - Universidade Federal de Lavras, Lavras, MG.*

The surubim-do-paraíba *Steindachneridion parahybae* is a freshwater catfish species that inhabits the Paraíba do Sul river Basin. The use of cryopreserved sperm has been considered to facilitate procedures during the artificial reproduction and conservation of a specie. The aim of this study was to evaluate the effects of lactose addition to the freezing media on post-thaw sperm quality. Post-thaw sperm quality was determined in terms of motility (subjective and computer-assisted sperm analyzer = CASA), velocities and fertility. Sperm samples were diluted in 6 freezing media prepared as a factorial of 3 extender compositions (5% glucose, 10% glucose and Beltsville Thawing Solution™ = BTS™) and 2 concentration of lactose (0 and 10%); DMSO was used as cryoprotectant. Sperm diluted in each medium was loaded into 0.5 ml straws, frozen in nitrogen vapor (vessel), and stored in liquid nitrogen (-196 °C). Half of the samples were evaluated for sperm motility and velocities, and the other half for fertility. There was no difference ($P>0.05$) between subjective or CASA assessment of post-thaw sperm motility. Post-thaw motility varied between 59 and 66% motile sperm in all samples, except for samples frozen in 10% glucose-lactose medium (36% motile sperm). The presence of lactose was positive when combined with 5% glucose, malefic when combined with 10% glucose and indifferent when combined with BTS™, regarding velocities and fertilization rate. There were positive correlations between sperm velocities (VCL, $r = 0.41$; VAP, $r = 0.28$) and fertilization rate. In general, the best results were achieved when sperm was frozen in 5% glucose-lactose medium: 64% motile sperm, 69 $\mu m\ s^{-1}$ of VCL, 48 $\mu m\ s^{-1}$ of VSL, 58 $\mu m\ s^{-1}$ of VAP and 45% fertilization rate. Based on these results, lactose should be added to the freezing media, only when 5% glucose is used as sperm extender of *S. parahybae*. Sperm cryopreservation in 10% glucose-DMSO or 5% glucose-lactose-DMSO yields high post-thaw sperm quality and thus can be used as a tool for artificial reproduction and conservation of genetic resources as this is a threatened species.

Keywords: CASA, Catfish, Extender, Freezing, Semen quality.

Resumo

ARAÚJO, R. V; CARVALHO, I. C; SANCHES, E. A; CANEPPELE, D; LEAL, M. C; VIVEIROS, A. T. M. Fertilidade, velocidade e motilidade do sêmen de Surubim-do-paraíba *Steindachneridion parahybae* (Siluriformes) criopreservado em meio contendo ou não lactose. In:___. **Motilidade, velocidade e fertilidade do sêmen de surubim-do-paraíba *Steindachneridion parahybae* (Siluriformes) criopreservado em diferentes diluidores.** 2011. 93p., p. 67-93. Tese (Doutorado em Zootecnia) - Universidade Federal de Lavras, Lavras, MG.*

O surubim-do-paraíba, *Steindachneridion parahybae*, é uma espécie de bagre nativo da bacia do rio Paraíba do Sul. A criopreservação do sêmen tem sido utilizada para facilitar os procedimentos durante a reprodução artificial e a conservação das espécies. Este estudo foi realizado com o objetivo de avaliar os efeitos da adição de lactose ao meio de congelamento na qualidade do esperma criopreservado. A qualidade dos espermatozoides após o descongelamento foi determinada em termos de motilidade (avaliada subjetivamente e em um analisador de esperma computadorizado, CASA), velocidades e fertilidade. As amostras de esperma foram diluídas em seis meios de congelamento preparados por uma combinação fatorial de três diluidores (5% glicose, 10% glicose e *Beltsville Thawing Solution*™ = BTS™) e duas concentrações de lactose (0% e 10%); DMSO foi usado como crioprotetor. O sêmen diluído em cada um dos meio foi envasado em palhetas de 0,5 ml, congelados em vapor de nitrogênio (vessel) e, posteriormente, armazenado em nitrogênio líquido (-196 °C). Metade das amostras foi avaliada quanto à motilidade e à velocidade dos espermatozoides e a outra metade foi utilizadas para avaliar a fertilidade. Não houve diferença ($P>0,05$) entre o método de avaliação subjetivo e o CASA para a motilidade dos espermatozoides após o descongelamento. A motilidade variou entre 59% e 66% espermatozoides móveis em todas as amostras, exceto para as congeladas em 10% glicose-lactose (36% espermatozoides móveis). A presença de lactose teve efeito positivo quando combinada a 5% glicose, negativo quando combinada a 10% glicose e foi indiferente quando combinada ao BTS™, para a velocidade e a taxa de fertilização. Houve correlação positiva entre a velocidade dos espermatozoides (VCL, $r = 0,41$; VAP, $r = 0,28$) e a taxa de fertilização. Em geral, os melhores resultados foram obtidos quando o esperma foi congelado em 5% glicose-lactose: 64% espermatozoides móveis, VCL = 69 $\mu m\ s^{-1}$, VSL = 48 $\mu m\ s^{-1}$, VAP = 58 $\mu m\ s^{-1}$ e taxa de fertilização de 45%. Com base nos resultados, a lactose deve ser adicionada ao meio de congelamento, somente quando 5% glicose for utilizado como diluidor de esperma de *S. parahybae*. A criopreservação do sêmen em 10% glicose-DMSO ou 5% glicose-lactose-

DMSO proporciona altos rendimentos na qualidade do esperma após o descongelamento e, assim, pode ser empregado como uma ferramenta na reprodução artificial e para a conservação dos recursos genéticos, já que esta é uma espécie ameaçada.

Palavras-chave: CASA, Bagre, Diluidor, Congelamento, Qualidade espermática.

1. Introduction

The surubim-do-paraíba *Steindachneridion parahybae* (Steindachner, 1876) is a catfish species which belongs to the order Siluriformes, family Pimelodidae, and is endemic to the Paraíba do Sul River Basin (20°26' and 23°39'S, and 41° and 46°30'W), located in Minas Gerais, Rio de Janeiro and São Paulo States in the southeastern Brazil (Oliveira and Moraes Jr., 1997). Until 1950, *S. parahybae* was a very important species for fisheries (Machado and Abreu 1952), however, due to pollution, river damming, sand extraction from riverbeds and floodplains, predatory fishing, and the introduction of exotic fish species, the status of this species is currently set as endangered (Caneppele et al., 2008).

Fish sperm cryopreservation provides a tool by which reproduction is optimized thereby larvae production is increased. In addition, cryopreserved sperm serves as genetic bank, which may help ensure genetic diversity and reproductive success for population management strategies (Viveiros et al., 2011). The use of sperm cryopreservation as a technique to store genetic material of endangered species has long been used. However, this technique has received little attention concerning Brazilian species siluriformes (Viveiros and Araújo et al., 2011 unpublished data). The order Siluriformes, also known as catfishes, contains 33 families and during the past decade, 6 families and 9 species of this order were subjects of cryopreservation studies (Viveiros, 2011). Although Brazil has more than 1.056 catfish species distributed in 11 families (Buckup et al., 2007), only four species were subject of studies on sperm cryopresevation: surubim-pintado *Pseudoplatystoma corruscans* (Carolsfeld et al., 2003), jundiá *Rhamdia quelen* (Fogli da Silveira et al., 1985), surubim-do-iguaçu *Steindachneridion melanodermatum* (Bombardelli et al., 2011) and *S. parahybae* (Viveiros and Araújo, 2011 unpublished data).

For storage in liquid nitrogen, sperm must be diluted in a medium containing an extender and a permeable cryoprotectant agent (CPA). Over the last decade, dimethyl sulfoxide (DMSO), methanol, and dimethyl acetamide (DMA), mostly at 10% (v/v), have been used as CPA for catfish sperm (Viveiros, 2011). Recently, we tested 5 extenders combined or not with lactose, and DMSO during cryopreservation of *S. parahybae* sperm. When sperm was frozen in lactose-free media, post-thaw motility varied from 57 to 64% motile sperm in all five media. When lactose was added, motility decreased (16-36% motile sperm) in sampled frozen in NaCl, Ginsburg fish Ringer modified and in 10%-glucose. The addition of lactose did not affect post-thaw motility of samples frozen in 5% glucose and BTS™ (Viveiros and Araújo, 2011 unpublished data).

The aim of this study was to further investigate the effects of lactose on post-thaw sperm quality of surubim-do-paraiba *Steindachneridion parahybae,* determined in terms of motility (subjective and CASA), velocities and fertility.

2. Materials and methods

2.1 Fish and sperm collection

All fish were handled in compliance with published guidelines for animal experimentation (Van Zutphen et al., 2001). The *S. parahybae* males utilized as sperm donors were captured from the wild and kept in captivity in Hydrobiology and Aquaculture Station of the Hydroelectric Company of São Paulo (CESP) in Paraibuna city, São Paulo state, Brazil. Males were selected during the spawning season (January and February). Males with detectable running sperm in response to soft abdominal pressure were selected. The urogenital papilla was carefully dried and sperm was hand-stripped directly into test tubes. Sperm was collected without hormone induction and at room

temperature of 27–29°C. Immediately after collection, tubes containing sperm were placed in crushed ice (~10 °C). An aliquot of 2 μl of each sample was placed on a slide and observed under a light microscope (Model L1000, Bioval, Jiangbei, China) at 400 x magnification. Any sperm motility (auto-activation) observed was attributed to urine or water contamination and the sample discarded, as fish sperm in seminal plasma is immotile. In immotile samples (n = 3 males), sperm motility (expressed as percentage of motile sperm) was subjectively estimated immediately after the addition of 30 μl of 0.29% NaCl (~98 mOsm kg^{-1}) as activating agent (Viveiros and Godinho, 2009). All samples possessed at least 70% motile sperm and were used in the subsequent analysis. Volume, concentration (hemacytometer/ Neubauer chamber) and pH (YSI meter 660, Lake Village, AK, USA) of each sperm sample were determined. The osmolality of the seminal plasma was measured cryoscopically (Digital MicroOsmometer Roebling, Type 13/13, Messtechnik TM, Berlin, Germany) after centrifugation of sperm at 2000 g for 30 min at room temperature. Sperm characteristics of all samples were evaluated by the same technician and at room temperature.

2.2 Sperm cryopreservation

Sperm was diluted in one of the six freezing media comprising the combination of 3 extenders (5% glucose, 10% glucose and BTS™) and 2 concentration of lactose (0 and 10%) according to our previous study (Viveiros and Araújo, 2011 unpublished data). For extender formulae please refer to Table 1. Extenders were adjusted to a pH of 7.6. Dimethyl sulfoxide (DMSO – $(CH3)_2SO$; Cromato Produtos químicos LtdaTM, Diadema, SP, Brazil) was used as cryoprotectant. Sperm samples were diluted in each freezing media at a final proportion of 10% sperm, 80% extender and 10% DMSO. After dilution, sperm samples were immediately aspirated into 0.5-ml straws (n = 6 replicate straws)

and frozen in a nitrogen vapor vessel (Cryoporter™ LN2 *dry vapor shipper*, Cryoport Systems, Brea, CA, USA) at approximately -170°C, which gives a freezing rate of approximately -35.6°C min^{-1} between -21°C and -170°C (Maria et al., 2006). Then straws were transferred to liquid nitrogen (M.V.E XC 34/18, CHART/MVE, Marietta, GA 30066, USA) at -196°C within 20–24 h. In total, 108 straws (6 replicate straws x 6 media x 3 males) were cryopreserved. Half of the frozen replicate straws were used for the fertilization trial (see below). The other half was placed back into a nitrogen vapor vessel and transported ~370 km by car from CESP to the Laboratory of Semen Technology at the Department of Animal Sciences at the Federal University of Lavras (UFLA), Lavras, Minas Gerais state, Brazil, where straws were stored in liquid nitrogen (M.V.E. Millenium, XC 20, Chart, Minessota, USA) until sperm motility and velocities analysis.

Table 1 Chemical composition of the media tested as sperm extender of surubim-do-paraíba *Steindachneridion parahybae*

Chemicals (g)[a]	Extenders		
	5% Glucose	10% Glucose	BTS™ [b]
Glucose	5.0	10.0	4.00
$NaHCO_3$	--	--	0.13
KCl	--	--	0.08
Sodium citrate	--	--	0.63
EDTA	--	--	0.13
Gentamycin sulfate	--	--	0.02
mOsmol kg^{-1}	288	638	345

[a] All chemicals were diluted in deionized water to a final weight of 100 g
[b] BTS™: Beltsville Thawing Solution™ (Minitub)
*Lactose was added at 10g to each extender when tested

2.3 Sperm motility and velocities evaluation

Straws were thawed in a 25°C water bath for 20 s (Viveiros and Araújo, 2011 unpublished data), and post-thaw sperm motility was immediately estimated, both subjectively as described for fresh sperm and objectively using CASA. One microliter of post-thaw sperm was activated in 20 μl of 0.29% NaCl and placed in a Leja™ counting chamber (chamber depth: 20 μm) on a phase contrast microscope (Nikon™ E200, Japan), 100 x magnification, green filter, and pH1 position. The microscope was connected to a video camera (Basler Vision Technologies™ 602FC, Ahrensburg, Germany) which generated 100 frames s^{-1}. Video recording was started at ~10 seconds post-activation. Each image (n = 25) was analyzed using the standard settings for fish by Sperm Class Analyzer™ software (SCA™, Microptics, S.L. Version 5.1, Barcelona, Spain). Sperm was considered immotile when curvilinear velocity (VCL) was < 20 μm s^{-1} Although SCA™ simultaneously assessed more than 15 sperm motility end points, for brevity only VCL, straight line velocity (VSL), and average path velocity (VAP) were considered for further analysis, as similar effects were observed for all end points. To determine these velocities, each individual sperm cell (n = at least 150 sperm /field) was followed throughout the 25 images and a sperm trajectory was calculated.

2.4 Artificial fertilization

The other half of the straws (n = 3 replicate straws x 6 media x 3 males) were thawed as described for motility analysis and used to fertilize fresh oocytes. To harvest oocytes, females (n = 3) received two doses (0.5 and 5 mg kg^{-1} body weight) of carp pituitary extract (Argent Chemical Laboratories, Redmond, WA, USA) at a 12-h interval, and were hand-stripped 5 h after the second dose. All females responded positively to the hormonal treatment and all oocytes were pooled together. The pool of oocytes was divided in 54 aliquots of

0.5 g (~140 oocytes). Each aliquot was fertilized with post-thaw sperm of one replicate straw at an approximate ratio of ~1 x 10^5 spermatozoa per oocyte, according to Sanches et al., 2010b. Fertilization was initiated by the addition of 5 ml tank water, and mixed for 1 min. Subsequently, 10 ml tank water was added and samples were mixed for another 2 min. Finally, eggs were transferred to 150 ml funil-type incubation units with 10 cm in diameter, and incubated in a flow-through system (pH of 7.53 ± 0.47, dissolved oxygen of 6.64 ± 0.27 mg l^{-1}, electrical conductivity of 24.00 ± 5.00 μS cm^{-1} and temperature of 23.81 ± 0.51°C). As control, freshly collected sperm from another three males was pooled and used to fertilize three aliquots of the same pool of oocytes, using the same ratio of 1 x 10^5 spermatozoa per oocyte. The fertilization rate was determined 10 h later and expressed as the percentage of fertilized eggs at blastopore closure stage, over the total number of eggs.

2.5 Statistical analyses

Values are reported as means ± standard deviation (SD). Statistical analyses were conducted with the SISVAR computational program (Ferreira, 1999). Sperm motility, velocities, and fertility were tested for normal distribution using the univariate procedure. When data did not have a normal distribution, an arcsin transformation was performed. Data were tested for significant differences by ANOVA, followed by the Scott-Knott test, when applicable. A Pearson test was used to determine the correlation between sperm velocities and fertilization rates. The level of significance for all statistical tests was set at 0.05.

3. Results

Fresh sperm of the *S. parahybae* males used in this study possessed a mean volume of 4 ml, with 7.8 x 10^9 spermatozoa ml^{-1}, pH of 7.5 and 80% motile sperm. Data of the body weight and fresh sperm features are depicted in Table 2.

Table 2 Body weight and fresh sperm features of surubim-do-paraíba *Steindachneridion parahybae* (*n* = 3 males)

Features	Mean ± SD	Range (min-max)
Body weight (g)	1219 ± 372	856 - 1600
Total length (cm)	47.7 ± 4.5	43 - 52
Semen volume (ml)	4.0 ± 0.9	3 - 4.5
Concentration (spermatozoa x 10^9 ml^{-1})	10.4 ± 1.5	9 - 10.3
Subjective motility (% motile sperm)	80 ± 10	70 - 90
Semen pH	7.5 ± 0.3	7.2 - 7.8
Seminal plasma osmolality (mOsmol kg^{-1})	283 ± 7	278 - 291

There was no difference ($P > 0.05$) between the two methods of post-thaw sperm motility evaluation (subjectively and under CASA). Thus data obtained from CASA was used to describe and discuss the results.

An interaction between extenders and lactose for both sperm motility and velocities was observed. The addition of lactose did not affect post-thaw motility when sperm was cryopreserved in 5% glucose (64-66% motile sperm) or BTS™ (59-62% motile sperm), but decreased motility from 65% to 36% when samples were frozen in 10% glucose (Table 3).

Table 3 Sperm motility determined both subjectively under light microscope and by computer-assisted sperm analyzer (CASA), and fertilization rate (*n* = 3 males x 3 replicate straws x 3 females) of sperm cryopreserved in glucose or in BTS™, combined or not with lactose, of surubim-do-paraíba *Steindachneridion parahybae*. As control, freshly collected sperm of three males were evaluated for motility, pooled and used to fertilize the same oocytes (n = 1 pool of sperm x 3 females)

Extender	Lactose (%)	Subjective motility* (% motile sperm)	CASA motility* (% motile sperm)	Fertilization (%)
5% Glucose	0	54 ± 9^{b}	66 ± 10^{a}	34 ± 9^{c}
	10	60 ± 10^{b}	64 ± 6^{a}	45 ± 7^{b}
10% Glucose	0	68 ± 7^{b}	65 ± 3^{a}	48 ± 5^{b}
	10	33 ± 5^{c}	36 ± 5^{b}	35 ± 11^{c}
BTS™	0	61 ± 6^{b}	59 ± 11^{a}	30 ± 10^{c}
	10	61 ± 8^{b}	62 ± 7^{a}	22 ± 8^{d}
Fresh sperm	--	80 ± 10^{a}	--	74 ± 6^{a}

[a-d] Mean ± SD within the same column, followed by different superscripts are different (Scott-Knott; $P<0.05$)

*Post-thaw sperm motility evaluated by either CASA or subjectively did not differ ($P>0.05$)

The highest post-thaw sperm velocities were achieved when sperm was cryopreserved in 5% glucose-lactose: 69 μm s^{-1} of VCL, 48 μm s^{-1} of VSL and 58 μm s^{-1} of VAP (Figure 1). The addition of lactose was always positive when combined with 5% glucose, negative when combined with 10% glucose and indifferent when combined with BTS™, regarding velocities. VAP ($r = 0.28$; $P = 0.039$) and VCL ($r = 0.41$; $P = 0.002$) were positively correlated with fertilization rate (Figure 2). The correlation between VSL and fertilization rate was not significant ($r = 0.26$; $P = 0.053$).

The highest fertilization rate was observed when sperm was cryopreserved in 5% glucose-lactose (45%) and in 10% glucose (48%). The addition of lactose was benefic only when sperm was cryopreserved in 5% glucose.

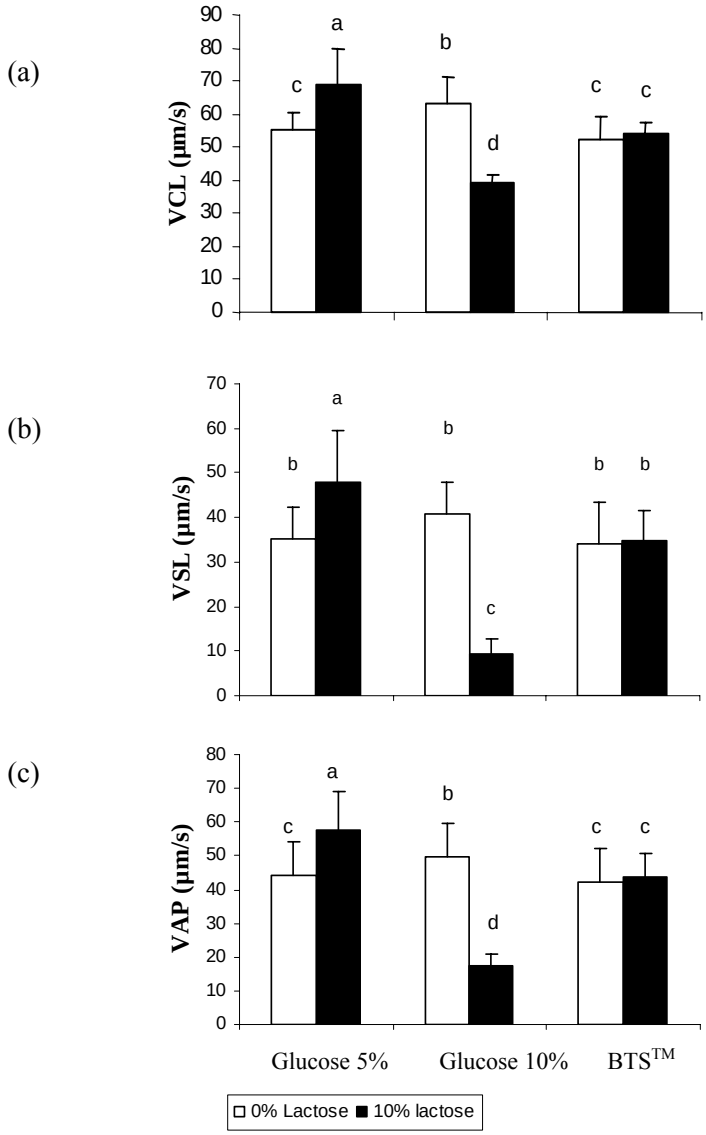

Figura 1 (a) Curvilinear velocity (VCL, µm s^{-1}), (b) straight-line velocity (VSL, µm s^{-1}) and (c) average path velocity (VAP, µm s^{-1}) of sperm cryopreserved in glucose or in BTS™, combined (black bars) or not (white bars) with lactose, of surubim-do-paraíba *Steindachneridion parahybae*. Bars represent mean values and vertical lines represent standard deviation (SD). (a–e) Means followed by different superscripts differ (Scott-Knott, $P < 0.05$)

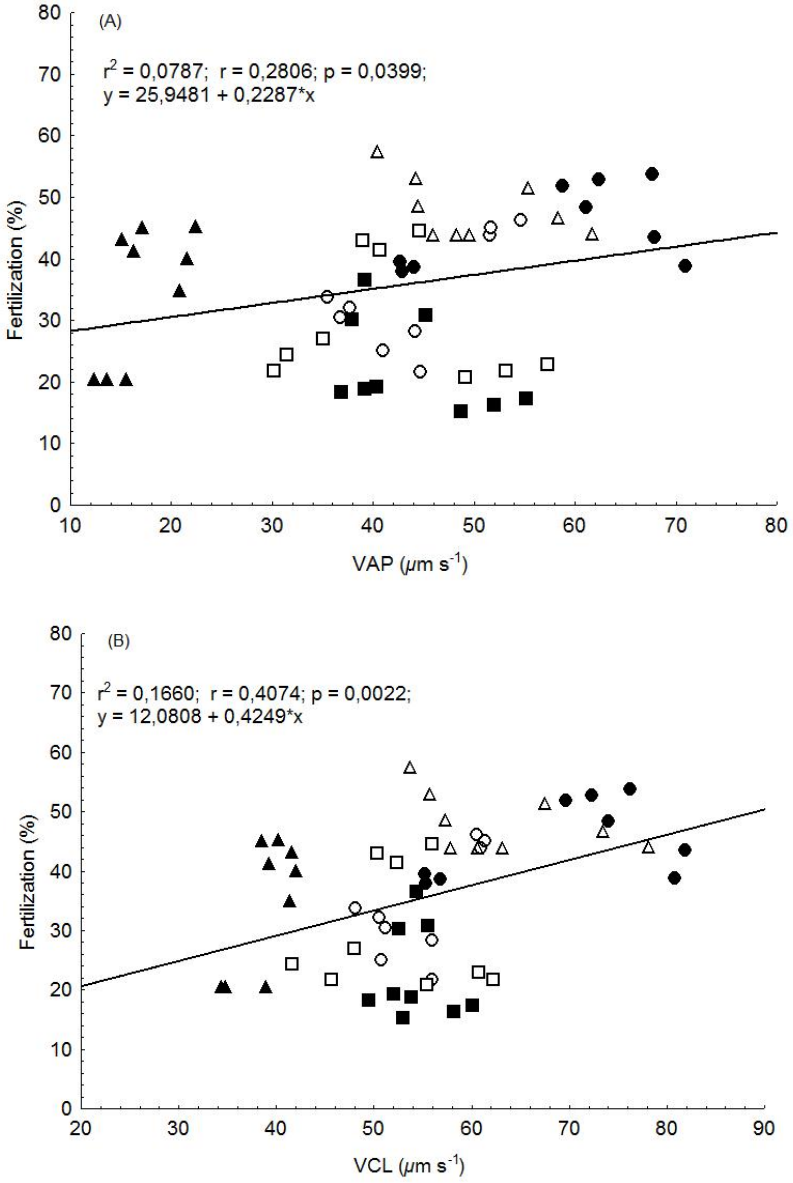

Figura 2 Correlations between sperm velocities parameters and (A) average path (VAP) and (B) curvilinear (VCL), and fertilization rates of *Steindachneridion parahybae* eggs fertilized with sperm cryopreserved in (○) 5% glucose; (△) 10% glucose; (□) BTS™; (●) 5% glucose-lactose (▲) 10% glucose-lactose or (■) BTS™-lactose

4. Discussion

In this study, some parameters of fresh sperm quality, collected during the reproductive season (February) without hormone induction, as well as the effects of different freezing media on post-thaw *S. parahybae* sperm were evaluated. This is the first report using cryopreserved sperm in the fertilization to oocytes of *S. parahybae.*

4.1 Fresh sperm features

Fresh sperm collected during the reproductive season presented 80% motile sperm, an average volume of 4 ml (3.3 ml kg^{-1}), concentration of 10.4 × 10^9 spermatozoa ml^{-1} and pH of 7.5. All these parameters are within the range described for the same species (Sanches et al., 2010b, 2011; Caneppele et al., 2010; Romagosa et al., 2011; Viveiros and Araújo et al., 2011 unpublished data).

The seminal plasma osmolality of *S. parahybae* ranged from 278 to 291 mOsmol kg^{-1}. It is interesting to observe that, although the osmolarity of the seminal plasma ranges from 300 to 350 mOsmol kg^{-1} in most of the teleost species (Alavi and Cosson, 2006), in catfish species, seminal plasma osmolality does not exceed 300 mOsmol kg^{-1}. In *Silurus glanisel* seminal plasma osmolality was 280 mOsmol kg^{-1} (Billard et al., 1997), in *R. quelen* 300 mOsmol kg^{-1} (Mojica, 2004), *Clarias macrocephalus* 269 mOsmol kg^{-1} (Tan-Fermin et al., 1999), and in bagre rayado, *Pseudoplatystoma metaense*, a catfish found in South America (Orinoco River in Colombia and Venezuela) ranged from 226 to 270 mOsmol kg^{-1} (Ramirez-Merlano et al., 2011). The values of seminal plasma osmolality are mainly related to the ionic and biochemical composition of the seminal plasma which, besides being species-specific, also varies according to

the advancement in the reproductive season, aging of spermatozoa and sperm concentration (Alavi et al., 2010).

4.2 Extenders

In the present study, glucose was tested as sperm extender both as a simple solution (at 5 and 10%) and combined with salts and antibiotics (BTS™). There was no difference on post-thaw sperm motility when sperm was cryopreserved in 5% glucose, 10% glucose or BTS™ (59-66% motile sperm). Fertility and VCL, however, were higher when sperm was cryopreserved in 10% glucose (48%, 63 μm s^{-1}), compared to 5% glucose (34%, 55 μm s^{-1}) or BTS™ (30%, 52 μm s^{-1}). Glucose is commonly used as extender due to its stabilization effects on the spermatozoa liposomal membrane (Quinn, 1985). Furthermore, the use of commercial extenders such as 5% glucose and BTS™, facilitates the procedures for cryopreservation, as no laboratory equipments such as scale, chemicals and pH meter, are necessary. The glucose and BTS™ have been used with great success as sperm extender of several Brazilian characiforms (Viveiros and Godinho, 2009) as well as of *S. parahybae* (Viveiros and Araújo et al., 2011 unpublished data).

4.3 Lactose

In the present study, the addition of lactose to the freezing media as an extra protection of sperm membrane was evaluated. The addition of lactose decreased motility when sperm was frozen in 10% glucose, but did not affect motility of samples frozen in 5% glucose or in BTS™, when compared to the same lactose-free media. The addition of lactose improved sperm velocities and fertility when sperm was frozen in 5% glucose and decreased the same parameters when sperm was frozen in 10% glucose, when compared to the same lactose-free media. The better post-thaw sperm quality after the addition of

lactose to the 5% glucose medium was probably caused by the increase on the global osmolality. We have observed in our previous study that freezing media containing DMSO as CPA frequently trigger sperm motility (Orfão et al., 2011). By increasing the global osmolality the DMSO induced motility may have been suppressed. On the other hand, when lactose was combined with 10% glucose, a negative effect was observed in all parameters evaluated, probably due to a hyperosmotic shock. Apparently, freezing media with high osmolality (above 700 mOsm kg^{-1}) may cause damage to sperm of *S. parahybae* as with high osmotic pressure in contact with sperm cells promotes efflux of water and increases the internal osmotic concentration (Alavi and Cosson., 2006). This process causes denaturation of macromolecules and cell plasmolysis, and there may be total collapse of the cell membrane (Medeiros et al., 2002). Similar results were observed in our previous studies where sperm of *S. parahybae* was cryopreserved in five extenders combined or not with lactose. The addition of lactose decreased motility when sperm was frozen in NaCl, Ginsburg Fish Ringer and 10% glucose, but did not affect motility of samples frozen in 5% glucose or in BTS™, when compared to the same lactose-free media (Viveiros et al., 2011 unpublished data).

4.4 Subjective and CASA motility.

There was no significant difference between the subjective analysis of sperm motility using a light microscope, traditionally used in most studies as a parameter for assessing sperm quality, and the CASA system. Similar results were observed for the *S. parahybae* (Viveiros and Araújo et al., 2011 unpublished data) and for the Brazilian freshwater Characiformes, *P. brachypomus* (Nascimento et al., 2010) and in *P. lineatus* (Viveiros et al., 2010). Thus, a well-trained technician using a light microscope could easily and reliably assess sperm motility, even under field conditions.

The CASA systems, quantify the sperm movement using at least a dozen computer calculated motility characteristics, provides objective methods of assessment and is easy to perform, precise and rapid (Rurangwa et al., 2001). For analyses of these indicators of sperm quality, CASA is the most objective and comprehensive quantification currently available (Wilson-Leedy and Ingermann, 2007). However, there is a great necessity for standardization of CASA setting, particularly the equipment characteristics, the chamber used, the image acquisition rate, resolution, lens magnification, track sampling time, smoothing algorithm, number of cells, slides and fields examined, and temperature of analysis (Verstegen et al., 2002) in order to allow comparison among studies and fish species (Viveiros et al., 2011 unpublished data).

Table 4 describes the parameters used to evaluate the quality sperm of four Siluriformes species using the CASA. Among these four studies, we can observe that there is a difference between the parameters used for evaluation of Siluriformes sperm quality. Each species has a particular characteristic and without standardization of CASA settings, it is impossible to compare sperm quality among different studies.

Table 4 Freezing sperm methods and CASA settings used in four Siluriformes species

Species	Freezing Method	CASA Settings	Results				Reference
			Motile sperm (%)	VCL ($\mu m\ s^{-1}$)	VAP ($\mu m\ s^{-1}$)	VSL ($\mu m\ s^{-1}$)	
S. parahybae	Glucose-Lactose-DMSO 0.5 ml straw	100 frames s^{-1} Leja™ (20 µm)	64%	69 µm s^{-1}	58 µm s^{-1}	48 µm s^{-1}	Present study
C. gariepinus	Mounib-egg yolk-DMSO 1.8-ml cryotube	25 frames s^{-1}	71%	23 µm s^{-1}	15 µm s^{-1}	10 µm s^{-1}	Rurangwa et al., 2001
R. quelen	Fresh sperm	60 frames s^{-1} Neubauer chamber (100 µm)	65%	77 µm s^{-1}	59 µm s^{-1}	47 µm s^{-1}	Sanches et al., 2010a
P. metaense	Fresh sperm	Frames s^{-1} not specified Makler™ chamber (10 µm)	80%	51.2 µm s^{-1}	47 µm s^{-1}	43.7 µm s^{-1}	Ramirez-Merlano et al., 2011

Mounib solution (g/L): Reduced glutathione 2.0; $KHCO_3$ 10.0; sucrose 42.0. Ginzburg fish Ringer (g/L): NaCl 6.5; KCl 0.25; $CaCl_2$ 0.3; NaHCO3 0.2

4.5 Velocities and fertility

The motility rate and sperm velocities have been associated with reproductive success in fish (Rurangwa et al., 2001). In the present study, there were positive correlations between fertilization rates and sperm velocity (VCL, r = 0.41 and VAP, r = 0.28) of *S. parahybae*. Similarly, positive correlations between sperm velocities and fertilization rate were also reported for curimba, *P. lineatus*, African catfish *Clarias gariepinus*, Pleuronectiformes turbot *Psetta maxima*, carp *Cyprinus carpio*, and rainbow trout *Oncorhynchus mykiss* (Viveiros et al., 2010; Rurangwa et al., 2001; Dreanno et al., 1999; Linhart et al., 2000; Lahnsteiner, 2000). There is, however, some evidence from subjective motility scores that there are changes in the sperm towards the end of the spermiating period which may decrease its fertilising capability despite an undiminished motility (Munkittrick and Moccia, 1987; Moccia and Munkittrick, 1987). With this possible exception, the data so far strongly suggest that CASA can reliably predict the fertilising ability of sperm, and could provide a very useful method for aquaculture (Kime et al., 2001).

The indirect measurement of sperm quality through fertilizing capacity depends on egg quality, which is variable and affects the success of fertilization. In fish farms, it is necessary to assess sperm quality to ensure the success of artificial fertilization. Fertilization may be dependent on the number of motile sperm and their velocity. If sperm quality may be related to fertilizing ability, then it is possible to use the CASA routinely in a wide range of sperm freezing protocols, without involving female fish and therefore, without the variability in egg quality among female fish (Rurangwa et al., 2004).

5. Conclusions

Based on the present study, lactose should be added to the freezing media of *S. parahybae*, only when 5% glucose is used as sperm extender. Sperm frozen in 10% glucose-DMSO or 5% glucose-lactose-DMSO yields high post-thaw sperm quality and thus can be used as a tool for artificial reproduction and conservation of genetic resources as this is a threatened species.

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