UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE MORFOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR

Duração da espermatogênese e transplante xenogênico de células germinativas de jundiá (*Rhamdia quelen*)

DOUTORANDA:

Mariana de Araújo da Silva

ORIENTADOR:

Prof. Dr. Luiz Renato de França

Universidade Federal de Minas Gerais

Instituto Nacional de Pesquisas da Amazônia

Belo Horizonte / Manaus

COORIENTADOR:

Prof. Dr. Guilherme Mattos Jardim Costa

Universidade Federal de Minas Gerais

Belo Horizonte

BELO HORIZONTE/MG

MARÇO/2016

MARIANA DE ARAÚJO DA SILVA

Duração da espermatogênese e transplante xenogênico de células germinativas de jundiá (*Rhamdia quelen*)

Tese de doutorado apresentada ao Programa de Pós-graduação em Biologia Celular da Universidade Federal de Minas Gerais como requisito parcial para a obtenção do título de Doutor em Biologia Celular.

Área de concentração: Biologia Celular

Orientador: Luiz Renato de França

Coorientador: Guilherme Mattos Jardim Costa

BELO HORIZONTE

MARÇO/2016

Dedido este trabalho aos meus pais, Gilberto e Ivaneide,
e ao meu irmão Gilberto Jr., por todo amor, compreensão, orações
e por sempre acreditarem em mim, e me apoiarem,
seja lá qual caminho eu escolha seguir.

“O caos é necessário.”

Órbita

Agradecimentos

Agradeço à Deus, pela força dada a cada dia.

Ao Prof. Dr. Luiz Renato de França por ter aceitado me orientar, e ter se mostrado compreensivo quando precisei. Gratidão pelo ensinamento, dedicação e orientação.

Aos meus co-orientadores, ou melhor, anjos da guarda, Guilherme Mattos e Samyra Nassif, que me guiaram pelas mãos durante essa caminhada, com sabedoria, amizade, doação e carinho. Gratidão eterna à vocês.

Á Prof. Dra. Gleide Avelar, pelo conhecimento, amizade, abraços, conversas e trocas de experiências. À Mara Lívia, por ter sempre sido solícita a cada pedido meu, pelos conselhos, abraços e cuidado. Ao André Figueiredo, Flávia Cappuccio, Carolina Oliveira, Amanda Tavares e Rafael Leite por toda amizade, descontração, troca de experiências e auxílio.

À Bárbara Ramalho, e toda sua família, por terem sempre cuidado de mim. Pelo carinho, e por todos os momentos bons, ou não, que vivemos nessa caminhada.

Ao Alaion Fonsêca e ao Pedro Brandão, por terem me ensinado muito, me auxiliado, e terem tornado esse trabalho possível, e me tirado risadas em momentos de tensão.

Ao Prof. Dr. Ewanguedes Kalapothakis, e ao Dr. Anderson do Carmo, por terem aberto as portas do LBMM sempre que foi necessário, se mostrando solícitos e receptivos.

A Érika Alvarenga e ao Laboratório de Aquacultura da Escola de Veterinária, pelos animais e espaço físico cedidos, e todo auxílio na manutenção dos aminais durante o experimento.

Ao veterinário Marco Túlio, e Túlio, em nome da 3D Aqua e Multifish, pelos animais, parceria, ensinamentos e aventuras durante esse processo. E ao Seu Zezinho, pelos jundiás e todos os “causos” contados e “mergulhos” para capturar os peixes.

Ao Luis Claudio, Filipe, Ivison, Nicole, Nathália, Ewerton, que dividem/dividiram comigo esses anos de pensionato, com muitas conversas, risadas, histórias e cuidando um do outro.

As minhas amigas-irmãs, Tatiana, Nyara e Anne, pelo apoio, força e amizade, mesmo distantes, e aos meus sobrinhos Reuven e Lucas, que nos alegram e unem.

Aos Engenheiros da Alegria, um divisor de águas da minha vida, uma família que só cresce, semeando e espalhando amor por aí. Eles me ensinaram que “ninguém é tão pobre que não possa oferecer nada, nem tão rico, que não possa receber”.....”bom trabalho, bom, bom trabalho!”, em cada coração que se possa alcançar.

A minha família Órbita, que me fez descobrir o meu propósito, me deu amigos, e me tornou piloto dos meus sonhos.

A todos do Laboratório de Biologia Celular, pelo convívio e trabalho nesses quatro anos, por todo apoio sempre oferecido por cada um de vocês.

Aos membros do Programa de pós-graduação em Biologia Celular, do Departamento de Morfologia do ICB-UFMG. Ao Prof. Dr. Hugo Godinho, que acompanhou e aconselhou fases desse trabalho desde o início, sempre solícito. Ao Prof. Dr. José Carlos, sempre amigo, gentil, e que contribuiu para a finalização deste trabalho. A todos os membros da banca, que enriqueceram e enriquecerão ainda mais este trabalho com sua participação.

Aos meus pais, Ivaneide e Gilberto, meu irmão Gilberto Jr., e toda minha família no Rio Grande do Norte, que entenderam e apoiaram minha vontade de cursar o doutorado aqui, e minha perseverança de permanecer em BH. Onde mesmo tão longe, e nos vendo tão pouco ao longo desses anos, nos tornamos mais fortes, mais unidos....amo vocês.

Gratidão a todos que direta ou indiretamente estiveram comigo nessa caminhada, e me ajudaram a chegar até aqui.

Namastê.

Este trabalho foi realizado nos seguintes laboratórios e instituições:

- Laboratório de Biologia Celular, vinculado ao Departamento de Morfologia do Instituto de Ciências Biológicas (ICB), da Universidade Federal de Minas Gerais (UFMG);

- Laboratório de Aquacultura (Laqua), vinculado ao Departamento de Zootecnia (DZOO) da Escola de Veterinária, da Universidade Federal de Minas Gerais;

- Laboratório de Biotecnologia e Marcadores Moleculares, vinculado ao Departamento de Biologia Geral, do Instituto de Ciências Biológicas (ICB), da Universidade Federal de Minas Gerais (UFMG).

Com o auxílio das seguintes agências de fomento:

- CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico
- FAPEMIG: Fundação de Amparo à Pesquisa do Estado de Minas Gerais

LISTA DE ABREVIATURAS

11-KT- 11 cetotestosterona

A_{diff} – espermatogônia diferenciada

A_{und} – espermatogônia indiferenciada

B_{early} – espermatogônia B inicial

B_{int} – espermatogônia B intermediária

B_{late} – espermatogônia B final

BrdU- bromodeoxyuridine

BSA – albumina sérica bovina

CEUA – Comissão de Ética no Uso de Animais

DAPI - *4',6-diamidino-2-phenylindole*

D- diplóteno

DMEM/F12 - *Dulbecco's Modified Eagle Medium/F12*

DNA – ácido desoxirribonucleico

E_1- espermátide inicial

E_2- espermátide intermediária

E_3- espermátide final

FAO – Organização das Nações Unidas para Alimentação e Agricultura

FBS – soro fetal bovino

GFP – *green fluorescent protein*

L/Z – leptóteno/zigóteno

P- paquíteno

PBS – *phophaste buffer solution*

PCR - *Polymerase Chain Reaction*

PGCs – células germinativas primordiais

PKH26 - corante fluorescente que se incorpora na membrana celular

Pl- pré-leptóteno

qPCR - *quantitative Polymerase Chain Reaction*

S – espermatócito secundário

SSCs – células-tronco espermatogoniais

T – testosterona

Resumo

Devido ao seu rápido crescimento, boa adaptabilidade a diferentes condições ambientais e importância econômica, o jundiá (*Rhamdia quelen*) representa um excelente modelo experimental para se investigar a estrutura e a função dos testículos e mesmo biotecnologias reprodutivas em teleósteos. O transplante de espermatogônias é uma destas abordagens biotecnológicas e consiste na remoção de células-tronco do testículo de um animal doador e a transferência das mesmas para o testículo de um animal receptor, onde estas células irão se desenvolver e formar espermatozoides maduros com características genéticas do doador. Em peixes teleósteos, esta técnica foi desenvolvida com sucesso em nosso laboratório, utilizando-se a tilápia-nilótica (*Oreochromis niloticus*) como modelo experimental para o transplante alogênico. No presente estudo, investigamos a viabilidade do transplante xenogênico utilizando-se a tilápia-nilótica como modelo receptor para o desenvolvimento das espermatogônias-tronco de jundiá. Após o transplante, observamos que o microambiente somático da gônada da tilápia-nilótica é capaz de permitir a colonização, proliferação e diferenciação de células-tronco espermatogoniais de jundiá transplantadas. Assim, este estudo foi pioneiro em demonstrar a produção de espermatozoides após o transplante xenogênico entre espécies pertencentes à diferentes ordens taxonômicas, indicando que, diferentemente de mamíferos, os testículos de peixes apresentam grande plasticidade filogenética em relação aos fatores necessários ao desenvolvimento de espermatogênese exógena. Ainda, buscando a melhor compreensão das características reprodutivas do jundiá, realizamos experimentos para descrever a morfologia de suas células germinativas, bem como estimar a duração da espermatogênese nesta espécie. Desta maneira, à semelhança de outras espécies de peixes, demonstramos que drástica redução do volume nuclear das células germinativas ocorre durante a espermatogênese. Esta diminuição volumétrica é observada particularmente na fase espermatogonial (de espermatogônias indiferenciadas a espermatogônias do tipo B), bem como do final das fases meióticas até final da fase espermiogênica (entre diplótenos e espermátides finais). Numa outra vertente desta investigação, através de análises imunohistoquímicas do testículo de jundiá, feitas após a injeção de BrdU em diferentes intervalos de tempos, observamos que a duração conjunta das fases meiótica e espermiogênica nesta espécie foi muito curta, requerendo cerca de 7 dias para a diferenciação de espermatócitos primários iniciais em espermatozoides. No entanto, apesar desta duração ser similar àquela encontrada para tilápia-nilótica mantidas

na mesma temperatura (30ºC), observamos que, em comparação ao transplante entre tilápias, maior intervalo de tempo é requerido para a produção de espermatozoides exógenos após o transplante xenogênico de jundiá para tilápias. Neste sentido, mais estudos se fazem necessários para compreender os aspectos fisiológicos envolvidos na adaptação e colonização de espermatogônias-tronco de jundiás no ambiente testicular (nicho espermatogonial) de tilápias-niloticas após o transplante. Finalmente, além de melhor compreender os aspectos referentes a fisiologia testicular e a biologia reprodutiva de jundiás, os estudos por nós aqui desenvolvidos propiciam um cenário bastante alvissareiro em relação a utilização da tilápia-nilótica para se produzir espermatozoides de outras espécies de teleósteos que estejam ameaçadas de extinção e/ou que apresentem interesse comercial em aquacultura.

Palavras chaves: Jundiá (*Rhamdia quelen*), transplante xenogênico de espermatogônias-tronco, células germinativas, duração da espermatogênese.

SUMÁRIO

1. INTRODUÇÃO

1.1 Peixes teleósteos

Os peixes teleósteos correspondem à cerca de 50% das aproximadamente 50 mil espécies de vertebrados conhecidas até hoje, exibindo grande variedade em sua biologia, morfologia e em habitats ocupados (Pough et al., 2008). Dotados de características aperfeiçoadas tais como respiração, locomoção, nutrição e, principalmente, a reprodução, que se caracteriza pela ampla variação de estratégias reprodutivas (LeGac e Loir, 1999; Nakatani et al., 2001; Moyle e Cech, 2003). Dessa forma, a liberação dos gametas para a fertilização externa, os níveis de cuidado parental, e a migração reprodutiva, seja por piracema, ou acompanhadas por grandes modificações osmóticas, exemplificam a complexidade reprodutiva dos teleósteos.

Filogeneticamente, os teleósteos (infraclasse Teleostei) abrangem 96% de todos os peixes existentes, incluindo-se mais de vinte mil espécies em 4.064 gêneros, 426 famílias e 38 ordens, sendo conhecidos como “peixes ósseos modernos” (Nelson, 2006). Todos os grupos incluídos nesta infraclasse possuem esqueleto ósseo, importante na evolução dos padrões de locomoção do grupo, e modificações na musculatura da mandíbula que os distinguem dos outros Actinopterígios. Neste contexto, os teleósteos constituem o maior e provavelmente mais diversificado grupo de vertebrados do nosso planeta.

Algumas espécies de teleósteos são modelos biológicos experimentais largamente utilizados na reprodução, genética e melhoramento, como por exemplo o zebrafish (*Danio rerio*), o medaka (*Oryzias latipes*), a truta arco-íris (*Oncorhynchus mykiss*) e a tilápia nilótica (*Oreochromis niloticus*) (Feitsma et al., 2007; Lacerda et al., 2013). Essas espécies propiciam importantes investigações envolvendo a biologia comparada dos

vários sistemas constituintes dos vertebrados. Dessa forma, devido ao grande potencial econômico da piscicultura no mercado mundial de alimentos, se faz necessário uma melhor compreensão das peculiaridades relacionadas a biologia reprodutiva dos peixes.

1.2 A tilápia-nilótica

A tilápia-nilótica (*O. niloticus*) é um teleósteo pertencente à ordem Perciformes, família Cichlidae e à sub-família Tilapiinae. Cerca de 70 espécies de ciclídeos recebem a denominação de tilápia, mas a espécie *Oreochromis niloticus* destaca-se dentre as demais por exibir características de alto interesse zootécnico (Stickney, 1997). O cultivo desta espécie ocorre a mais de três mil anos no Egito e, atualmente, espalha-se por quase todos os países do mundo, constituindo a segunda espécie de peixe de água doce mais produzida no mundo (depois da carpa), particularmente por apresentar carne de excelente qualidade e de boa aceitação pelo mercado consumidor (Torrans, 1998; FAO, 2013). Assim, a produção mundial de tilápia-nilótica tem crescido nas últimas décadas, subindo de 0,83 em 1999 para 3,5 milhões de toneladas em 2013, tornando-se a espécie mais promissora para a aquicultura do século XXI (Fitzsimmons, 2000; FAO, 2013).

Particularmente no Brasil, a tilápia-nilótica é a espécie de água doce mais cultivada, onde, de acordo com o mais recente Boletim Estatístico do MPA, para o melhorameno genético de tilápia, sua produção cresce 17% a cada ano (MPA, 2014), ultrapassando a produção de 253 mil toneladas em 2013 (Sussel, 2013). Hoje a tilápia é produzida em praticamente todas as regiões do Brasil, exceto na região Norte, onde possui produtividade pouco expressiva. A região Nordeste é a principal produtora, com destaque aos estados do Ceará, Pernambuco e Bahia. Na região Sul e Sudeste ganham destaque o

Paraná e São Paulo, nos quais grande expansão no cultivo deste teleósteo tem sido observada nos últimos dez anos (Kubitza, 2015).

Do ponto de vista biológico, a tilápia-nilótica é onívora alimentando-se de algas, fitoplâncton, zooplâncton, ovos e larvas de peixes e detritos, e esta característica faz com que esta espécie cresça rapidamente, mesmo quando alimentada com ração contendo níveis muito baixos de proteínas, além de tolerar altos níveis de carboidratos na dieta (Stickney, 2000). Apresentam também tamanho conveniente para a aquicultura em larga escala e tolerância a baixos teores de oxigênio dissolvido e a grandes variações nas condições ambientais (Beamish, 1970; Silva, 1987). Além disso, esta espécie atinge a maturidade sexual precocemente (entre 4 e 5 meses de idade), sua desova ocorre de 2 em 2 meses se a temperatura da água permanecer acima de 24ºC, e suas larvas apresentam alta viabilidade. Ainda, esta espécie se caracteriza por apresentar cuidado parental bucal desenvolvido pela fêmea (Trewavas, 1983). Portanto, a conjunção de todas estes atributos positivos faz com que a tilápia-nilótica se apresente como modelo experimental bastante atraente para se investigar a biologia reprodutiva de peixes teleósteos com alto potencial produtivo (Stickney, 2000).

1.3 *Rhamdia quelen* – jundiá

O cultivo de *Rhamdia quelen* (Quoy e Gaimard, 1824) tem distribuição neotropical, do norte do México ao sul da Argentina (Silvergrip, 1996). Esta espécie está presente na bacia do Rio São Francisco (Bockmann e Guazzelli, 2003), e seu cultivo vem crescendo no sul do Brasil, particularmente na bacia do rio Paraná. A sistemática do gênero Rhamdia é confusa desde que foi descrita. Silvergrip (1996) realizou uma ampla revisão taxonômica do gênero, baseada em caracteres da morfologia interna, e concluiu

que o gênero Rhamdia é formado de apenas 11 espécies dentre 100 anteriormente descritas. Segundo o mesmo autor, *Rhamdia quelen* pertence à seguinte divisão taxonômica:

Classe: *Osteichthyes*;

Infraclasse: *Teleostei*;

Ordem: *Siluriformes*;

Família: *Pimelodidae*;

Gênero: *Rhamdia*;

Espécie: *Rhamdia quelen*.

O jundiá é um peixe que apresenta grande aceitação pelo mercado consumidor devido à sua carne saborosa e ausência de espinhos intramusculares. Os adultos desta espécie são onívoros no ambiente natural, alimentando-se de peixes, crustáceos, insetos, restos vegetais e detritos orgânicos (Guedes, 1980; Meurer; Zaniboni Filho, 1997). Essa espécie movimenta-se à noite e sai de seus esconderijos depois das chuvas para se nutrir doa matéria orgânica que fica ao longo dos rios. Os organismos encontrados no conteúdo gastrintestinal do jundiá não são restritos ao habitat bentônico, indicando que essa espécie é generalista com relação à escolha do alimento (Gomes et al., 2000). Esta característica contribui para a adaptação deste peixe ao alimento artificial, facilitando sobremaneira a sua domesticação e condicionamento às condições de cultivo. São razoavelmente resistentes ao manejo e aceitam ração peletizada, caraterísticas estas que tornam essa espécie bastante promissora para a piscicultura.

O *Rhamdia quelen* pode atingir 50 cm ou mais de comprimento total e 3 kg de peso corporal (Ihering e Azevedo, 1936). Sua coloração varia de marrom avermelhado claro a cinza ardósia. A pigmentação da parte inferior da cabeça é variável. Os barbilhões têm crescimento alométrico negativo e esta relação é provavelmente aumentada devido à

grande possibilidade de dano dos barbilhões em exemplares de grande tamanho (Silvergrip, 1996). Segundo o mesmo autor, *R. quelen* pode ser diferenciado das outras espécies de *Rhamdia* através das seguintes características: espinho da nadadeira peitoral serrilhado em ambos os lados; nadadeira caudal com lóbulos desiguais; membrana inter-radial menor do que 2/3 do comprimento do raio do lobo superior da nadadeira caudal; com ou sem poros sensoriais múltiplos na cabeça; véu da narina posterior aberta póstero-lateralmente; barbilhões maxilares com no mínimo 29% do comprimento padrão; 5 a 16 arcos branquiais; 36 a 44 vértebras pós Weberianas (Aparelho de Weber-ossículos Werberianos); olhos de tamanho médio, com ou sem padrão de manchas; com ou sem uma marca tipo selim escuro na nuca.

Em experimentos com larvas e alevinos dessa espécie em cativeiro, observou-se uma acentuada aversão à luz e busca de locais escuros (Piaia et al., 1999). Alevinos de *R. quelen* suportam a transferência para água contendo 0% a 10% de sal (água do mar), o que indica que essa espécie é estenoalina, e suporta até 9,0g/L de sal comum (NaCl) por 96h, de modo que o tratamento de doenças com sal comum pode ser utilizado nesta espécie sem problemas (Marchioro, 1997). Essa espécie pode ser considerada euritérmica, pois os alevinos aclimatados a 31ºC suportam temperaturas de 15 a 34ºC. A aclimatação a temperaturas mais baixas proporciona maior tolerância à redução de temperatura, mas o limite superior de tolerância praticamente não se altera (Chippari-Gomes, 1998). Seu crescimento aumenta com o incremento da temperatura, sendo bastante pronunciado nos primeiros anos de vida. Porém, a taxa de crescimento dos machos é maior do que a das fêmeas até o terceiro ou quarto ano de vida, onde a situação se inverte, pois estas passam a crescer mais rapidamente. O comprimento de ambos os sexos ocorre de forma concomitante, mas as fêmeas atingem aproximadamente 67cm e os machos 52cm. Consequentemente, as fêmeas apresentam maior comprimento e idade

que os machos, e a expectativa de vida das fêmeas é de 21 anos e dos machos apenas 11 anos (Weis, 1980).

A maturidade sexual desta espécie é atingida por volta de um ano de idade nos dois sexos. Os machos iniciam o processo de maturação gonadal com 13,4cm e as fêmeas com 16,5cm. A partir de 16,5cm e 17,5cm, todos os exemplares machos e fêmeas, respectivamente, estão potencialmente aptos para reprodução (Narahara et al., 1985). Machos prontos para a espermiação liberam com facilidade o fluido espermático quando o abdome é pressionado. Além disso, o óstio genital é protraído. Fêmeas maduras apresentam o óstio genital hiperêmico, com dilatação ventral (Mardini et al., 1981). O período reprodutivo e os picos de desenvolvimento gonadal de *R. quelen* podem variar a cada ano e de um lugar para outro. Com relação ao comportamento reprodutivo de *R. quelen*, há um bom sincronismo entre machos e fêmeas na hora da desova, que ocorre logo ao amanhecer (Godinho et al., 1978). Além disso, essa espécie é ovulípara no habitat natural, e seus ovos se caracterizam por serem demersais e não aderentes. Quando prontos para a desova, grandes cardumes procuram lugares de água rasa, limpa, pouco corrente e com fundo pedregoso. Sua desova é assincrônica, uma vez que os ovos são recrutados de populações heterogêneas de oócitos em desenvolvimento e são liberados em várias ocasiões do período reprodutivo, não havendo cuidado parental. A fecundidade é baixa e apresenta relação com o comprimento total, peso total e peso da gônada (Narahara et al., 1989).

1.4 Estrutura dos testículos em teleósteos

Os testículos dos peixes estão localizados na cavidade celomática, dorsalmente ao sistema digestório, ventralmente ao mesonefro e ventro-lateralmente ao longo da bexiga

gasosa, encontrando-se presos à parede abdominal dorsal pelo mesórquio. Macroscopicamente, os testículos da maioria dos teleósteos estudados são órgãos pares, podendo estar parcial ou totalmente fundidos entre si, apresentam usualmente tamanho similar entre o direito e o esquerdo e são frequentemente alongados, embora existam outras formas como lobulados e foliáceos, como em algumas espécies de Siluriformes (Loir et al., 1989; Le Gac e Loir, 1999).

A organização estrutural básica do testículo é comum a todos os peixes e aos demais vertebrados (Schulz et al., 2010). Assim, este órgão exerce as funções de produção de gametas (espermatogênica) e endócrina (esteroidogênica), possuindo para isto dois compartimentos principais: o compartimento tubular ou seminífero, e o compartimento intertubular ou intersticial (Koulish et al., 2002). O compartimento tubular é constituído pela túnica própria, epitélio seminífero e lúmen tubular. A túnica própria reveste o túbulo externamente, sendo composta de células peritubulares mióides e membrana basal. No epitélio seminífero são encontradas dois grupos distintos de células, as células de Sertoli (células somáticas) e as células germinativas, em diferentes estágios presentes em cistos, que se diferenciarão em espermatozoides após passarem por processo bastante complexo e altamente organizado – a espermatogênese (Billard, 1990; Koulish et al., 2002). As células somáticas são os elementos chave para a função normal do testículo (Le Gac e Loir, 1999), sendo a célula de Sertoli considerada como a mais importante para o desenvolvimento das células germinativas (Russell e Griswold, 1993; Schulz et al., 2010. No compartimento intersticial estão situados vasos sanguíneos/linfáticos, células e fibras do tecido conjuntivo, além das células de Leydig que possuem função esteroidogênica. As células de Leydig encontram-se geralmente em grupos próximos ou ao redor de vasos sanguíneos (Leal et al., 2009). Em teleósteos, sob estímulo tanto do Lh quanto do Fsh, estas células secretam andrógenos que regulam as diferentes fases da espermatogênese

(Gárcia-Lopez et al., 2010; Schulz et al., 2010). Uma das principais funções das células de Leydig é a produção de testosterona (T) e 11-cetotestosterona em (11-KT), que regulam a espermatogênese, incluindo-se a diferenciação das espermatogônias tronco (SSC), principalmente via células de Sertoli (de Waal et al., 2009; Schulz et al., 2010).

De acordo com o arranjo do compartimento germinativo, os testículos de peixes ósseos, de maneira geral, podem ser classificados em duas categorias: testículo tubular anastomosado e testículo lobular (Parenti e Grier, 2004). O testículo tubular anastomosado possui compartimento germinativo formado por alças e túbulos ramificados que se interconectam da periferia até o ducto testicular principal. Esse tipo de testículo é encontrado em grupo de teleósteos filogeneticamente menos derivados ("grupos basais", filogeneticamente menos desenvolvidos, segundo Grier, 1993) como, por exemplo, os Cypriniformes, Characiformes, Siluriformes e Salmoniformes. No testículo lobular, o compartimento germinativo apresenta formato digitiforme terminando em fundo cego na periferia dorsolateral do testículo. Esse tipo de testículo é encontrado em teleósteos derivados e essa característica é, possivelmente, uma sinapomorfia dos Neoteleósteos (subdivisão de Euteleostei). Quanto à distribuição de espermatogônias, os testículos lobulares podem ainda ser classificados em restritos e irrestritos. Nos testículos lobulares restritos (distribuição espermatogonial restrita), as regiões distais do compartimento germinativo, próximas a túnica albugínea, são ocupadas por espermatogônias indiferenciadas circundadas por células de Sertoli mais imaturas. Esse tipo de testículo é característico de teleósteos mais derivados como das ordens Atheriniformes, Cyprinodonteformes e Beloniformes (Parenti e Grier, 2004). Os testículos lobulares irrestritos (distribuição espermatogonial irrestrita) apresentam espermatogônias distribuídas ao longo do compartimento germinativo por toda extensão do órgão. Esse último padrão de distribuição espermatogonial é considerado mais

primitivo, sendo encontrado em grupos taxonômicos menos relacionados (mais distantes) como em Percopsiformes, Ophidiiformes e Perciformes (Parenti e Grier, 2004; Nóbrega, 2006; Schulz et al., 2010).

Nos testículos de teleósteos a espermatogênese ocorre em estruturas denominadas "cistos", os quais são formados por células de Sertoli envolvendo células germinativas que progridem/diferenciam de maneira sincrônica. Neste sentido, atribuem-se às células de Sertoli as funções de sustentação, nutrição, secreção de fluido, de proteínas e de vários fatores de crescimento, liberação de espermátides maduras (espermiação) e intermediação hormonal da espermatogênese através de receptores para esteroides (andrógenos e estrógenos) e para Fsh (Schulz et al., 2010). Uma outra propriedade importante das células de Sertoli é sua eficiente capacidade fagocitária, retirando do epitélio seminífero produtos/corpos residuais, bem como células germinativas em apoptose e eventuais espermatozoides que permanecem no lume tubular após a espermiação (Almeida et al., 2008).

1.5 Espermatogênese

O processo espermatogênico é muito semelhante em vertebrados, sendo um evento cíclico, altamente organizado e coordenado, no qual as espermatogônias diploides se dividem e se diferenciam para formar espermatozoides maduros. Células-tronco espermatogônias (SSCs) são a base da espermatogênese e cruciais para a transmissão do informação genética para a próxima geração (Yoshida, 2012; Nagano e Yeh, 2013; Valli et al, 2014). Baseado em características morfofuncionais a espermatogênese pode ser dividida em três fases: (a) fase proliferativa ou espermatogonial, caracterizada pela auto renovação e diferenciação das espermatogônias, gerando células de reserva e células

comprometidas com a diferenciação. Estas células mais diferenciadas dão continuidade ao processo espermatogênico num intrincado processo de diferenciação e proliferação que ocorre até a última divisão mitótica originando os espermatócitos primários (pré-leptótenos); (b) fase meiótica ou espermatocitária (de Rooij e Russell, 2000), na qual ocorrem a duplicação do DNA, a recombinação gênica e a segregação dos cromossomos homólogos. Ao final desta longa fase, prófase I, subdividida em pré-leptóteno/leptóteno, zigóteno, paquíteno e diplóteno, os espermatócitos secundários serão formados. Após a segunda divisão meiótica, estes espermatócitos secundários originam células haploides denominadas espermátides (Russel et al., 1990; Hess e França, 2007). Estas espermátides constituem a fase espermiogênica (c) ou de diferenciação, na qual, através de complexo processo de diferenciação, que inclui a formação do acromossomo, a formação do flagelo e a compactação do DNA (substituição das histonas por protaminas) também ocorrem (Russell et al., 1990; Sharpe, 1994; Eddy, 1999). Após essas etapas, ocorre ainda a espermiação e a maturação espermática, na qual os espermatozoides são liberados para o lúmen do túbulo seminífero e preparados para fertilizar o oócito (Schulz e Miura, 2002; Miura e Miura, 2003; Schulz, 2003; Weltzien et al., 2004).

Biologicamente, na fase espermatogonial (mitótica), o número de divisões/gerações espermatogoniais pode variar bastante entre as diversas espécies de peixes (Schulz et al., 2010). Sendo assim, baseado em critérios morfológicos, as células espermatogoniais em peixes podem ser classificadas em dois tipos: tipo A e tipo B. Funcionalmente, as espermatogônias do tipo A podem ser subdivididas em espermatogônias indiferenciadas (A_{und}), incluindo-se aí as SSC; e espermatogônias diferenciadas (A_{diff}). As espermatogônias do tipo A_{und} originam as espermatogônias do tipo A_{diff}, que compartilham algumas características morfológicas com sua célula precursora. O comprometimento irreversível com a diferenciação/maturação e alterações

morfofuncionais, tais como menor tamanho celular e maior quantidade de heterocromatina, resulta na formação de espermatogônias do tipo B que, caracteristicamente em peixes, passam por várias e sucessivas divisões. Neste sentido, o melhor critério para discriminar espermatogônias do tipo B inicial, ou do tipo tardia, baseia-se no tamanho celular/nuclear e no número de células por cisto (Schulz et al., 2005; Leal et al., 2009; Schulz et al., 2010).

Ainda, considerando estudos em mamíferos e peixes, as SSCs são células germinativas únicas que podem ser reprogramadas em células pluripotentes, que possuem a capacidade de se diferenciar em tecido somático (Conrad et ai, 2008; Thoma et al., 2011; Oatley e Brinster, 2012). Além disso, há fortes evidências de que a regulação da atividade SSC ocorre dentro de microambiente específico nos testículos, o nicho das SSC, que pode ser influenciado por diversos elementos, tais como a célula de Sertoli, de Leydig, células peritubulares mióides, membrana basal, além de outros componentes celulares e fatores do compartimento intertubular (Chiarini-Garcia et al., 2003; Hofmann, 2008; Caires et al., 2010; Nóbrega et al., 2010; Phillips et al., 2010; Campos-Júnior et a., 2013).

1.6 Espermatogênese em teleósteos

Conforme já foi mencionado, em peixes a espermatogênese ocorre no interior de estruturas denominadas cistos espermatogênicos, que se formam quando uma espermatogônia indiferenciada ou do tipo A é completamente envolvida pelos prolongamentos das células de Sertoli (McClusky, 2012; Pudney, 1993). Em todas as espécies de vertebrados já investigados, as células resultantes da diferenciação das espermatogônias-tronco permanecem interligadas por pontes citoplasmáticas (complexos

juncionais), sincronizando o processo de desenvolvimento entre as células do mesmo clone de células germinativas (Loir et al., 1995; Pudney, 1995; Batlouni et al., 2009; Bosseboeuf et al, 2013) e delimitando física e funcionalmente um clone de células germinativas no mesmo estágio de desenvolvimento. Exceto pelo arranjo cístico, no qual as células germinativas se desenvolvem de forma clonal e apenas uma geração celular é observada neste clone (Koulish et al., 2002), o processo espermatogênico de teleósteos assemelha-se muito ao de mamíferos (Russell et al., 1990.

Num outro aspecto em particular, as células de Sertoli que delimitam os cistos espermatogênicos se apoiam na túnica própria, que é constituída pela membrana basal (camada acelular) e pelas células peritubulares mióides que possuem capacidade contrátil (Billard, 1982; Pudney, 1995; Le Gac e Loir, 1999). Portanto, diferentemente dos mamíferos, em peixes as células germinativas não estão em contato direto com a membrana basal (Billard, 1984; Schulz et al., 2010). De acordo com a literatura, o número de gerações espermatogoniais é considerado espécie-específica e filogeneticamente determinado (Schulz et al., 2010). Existe pouca informação sobre as características morfofuncionais ou moleculares das espermatogônias de teleósteos, sendo assim, as mesmas são principalmente identificadas pelo seu tamanho e características morfológicas (Nobrega et al, 2010; Schulz et al., 2010).

Particularmente, conforme já foi aventado, a disposição da cromatina nuclear e o tamanho do núcleo, aliado ao número de células germinativas por cisto, são parâmetros utilizados como referências para se distinguirem os diferentes tipos espermatogoniais (tipo A e tipo B) (Matta et al., 2002; Vilela et al., 2003; Leal et al., 2009; Lacerda et al., 2014). As espermatogônias mais indiferenciadas são as maiores células da linhagem germinativa nas espécies de teleósteos já investigadas (Schulz e Miura, 2002), apresentando um núcleo grande e claro, com pouca heterocromatina, contendo nucléolo

bastante evidente (Lacerda et al., 2013). Na tilápia-nilótica, essas células apresentam um diâmetro nuclear médio de 10µm (Vilela et al., 2003). Após sucessivas divisões, a partir da espermatogônia indiferenciada, o número de espermatogônias por cisto aumenta geometricamente, enquanto o diâmetro nuclear das mesmas sofre gradual redução (Vilela et al., 2003; Lacerda et al., 2013). A partir do número de células por cisto e do diâmetro nuclear/volume celular, foi verificada a existência de pelo menos oito gerações espermatogoniais na tilápia-nilótica (Vilela, et al., 2003; Schulz et al., 2005).

Em comparação com mamíferos, a duração total do processo espermatogênico nos peixes é normalmente mais rápida (Silva, 1987; Koulish et al., 2002; Vilela et al., 2003; Cardoso, 2007), sendo influenciada pela temperatura corporal que usualmente muito próxima da temperatura da água (Alvarenga et al, 2008). Por exemplo, na tilápia-nilótica a duração conjunta das fases meiótica e espermiogênica é de aproximadamente 5-6 dias à 35°C e de 10-11 dias à 25°C (Alvarenga et al., 2008). Em zebrafish, este processo é mais rápido, durando menos de uma semana à 25°C (Leal et al., 2009). Além disso, estudos morfométricos em testículos de tilápia-nilótica têm demonstrado que a espermatogênese dos peixes é mais eficiente que a dos mamíferos já investigados (França et al., 2015). Provavelmente, a maior eficiência da espermatogênese no arranjo cístico ocorre devido ao fato das células de Sertoli produzirem fatores para células germinativas numa mesma fase de desenvolvimento e ao menor número de apoptoses das células germinativas. Estes aspectos acima comentados, aliado ao pequeno número de células de Sertoli por cistos, resultam em enorme capacidade de suporte (eficiência) destas as células somáticas, principalmente quando se compara aos mamíferos (França et al., 2015).

Outro aspecto peculiar da função testicular em peixes é a proliferação das células de Sertoli. Nos mamíferos, é considerado que a proliferação destas células ocorre somente no período fetal e pré-púbere, sendo, portanto, o fator determinante na produção

espermática nos animais sexualmente maduros, uma vez que cada célula de Sertoli suporta um número relativamente constante e espécie-específico de células germinativas (França e Russell, 1998; França e Chiarini-Garcia, 2005). Por apresentar crescimento contínuo na fase adulta, em peixes teleósteos sexualmente maduros, a proliferação das células de Sertoli foi reportada em tilápias (*O. niloticus*) (Schulz et al., 2005), bagres africanos (*Clarias gariepinus*) (Schulz et al., 2005), zebrafish (*D. rerio*) (Leal et al., 2009) e no bacalhau (*G. morhua*) (Almeida et al., 2008). Nesses espécies citadas como exemplo, a divisão das células de Sertoli ocorre principalmente quando as mesmas estão associadas com espermatogônias, de forma a garantir número adequado destas células somáticas para suportar o desenvolvimento das células germinativas durante a espermatogênese (Assis et al., 2015; Nóbrega et al., 2009; França e Russel, 1998). Portanto, a cada período reprodutivo dos teleósteos, a proliferação das células de Sertoli é, provavelmente, o fator primário responsável pelo aumento do testículo e da produção espermática (Schulz et al., 2005), sendo a proliferação destas células também controlada por fatores ambientais como por exemplo a temperatura (Alvarenga, 2008).

1.7 Transplante de espermatogônias

O transplante de espermatogônias é uma fascinante abordagem experimental que consiste na remoção de células tronco do testículo de um animal doador e a transferência das mesmas para o testículo de um receptor infértil, onde estas células irão se desenvolver e formar espermatozoides maduros com características genéticas do doador. Esta metodologia foi desenvolvida em camundongos por Brinster e colaboradores (Brinster e Avarbock, 1994; Brinster e Zimmermann, 1994), e tem se tornado uma valiosa abordagem para se investigar a biologia da espermatogônia tronco no testículo, bem como

caracterizar os nichos espermatogoniais e verificar os efeitos da manipulação in vitro na função das espermatogônias-tronco (Orwig e Schlatt, 2005; McLean, 2005). O transplante de espermatogônias também tem proporcionado enormes avanços no estudo da espermatogênese em si, das interações entre células de Sertoli e células germinativas, além de pesquisas em potencial na área de produção animal, preservação de espécies ameaçadas de extinção, medicina reprodutiva e produção de animais transgênicos (Lacerda et al., 2014).

Um avanço muito importante propiciado por esta técnica foi o sucesso na produção de gametas a partir do transplantes interespecífico (xenogênico) de espermatogônias-tronco de rato para camundongo e de hamster para camundongo (Clouthier et al., 1996; Ogawa et al., 1999). Nesta situação de transplante interespecíficos, os camundongos receptores eram mutantes imunodeficientes como o nude mice (sem linfócitos T) ou camundongos SCID (severe combined immune deficiency - sem linfócitos B e T). A produção de espermatozoides de camundongos nos testículos de ratos também foi demonstrada após o transplante de espermatogônias (Ogawa et al. 1999; Zhang et al. 2003). Além de roedores, células germinativas de coelhos, cães, gatos, animais domésticos de grande porte (suínos, bovinos, equinos) e primatas (babuíno, macaco rhesus, e humanos) foram também transplantadas para testículos de camundongos imunodeficientes (Dobrinski, 2005). No entanto, embora diferentes graus de colonização tenham ocorrido, espermatogênese além da fase espermatogonial não foi observada (Dobrinski et al., 1999; Dobrinski et al., 2000; Reis et al., 2000; Nagano et al., 2001; Nagano et al., 2002; Kim et al. 2006). Pelo menos em mamíferos, acredita-se que o êxito do transplante esteja diretamente relacionado com o grau de proximidade filogenética das espécies durante a evolução e não com uma possível incompatibilidade imunológica, uma vez que o compartimento adluminal dos túbulos seminíferos, e mesmo

o testículo como um todo, é considerado um ambiente imunoprivilegiado (Bart et al., 2002; Nasr et al., 2005). A menor eficiência do transplante entre hamsteres e camundongos (divergem em ~16 milhões de anos), comparado com o transplante entre ratos e camundongos que são espécies consideradas mais próximas evolutivamente (10-11 milhões de anos) (Catzeflis et al., 1993; Clouthier et al., 1996; Ogawa et al., 1999), ilustra bem a influência da filogenia no sucesso do transplante. Provavelmente, esta limitação ocorra devido à falhas ou incompatibilidade na associação estrutural e interações funcionais entre as células de Sertoli do animal receptor e as células germinativas transplantadas (Khaira et al., 2005).

Num outro aspecto, o sucesso do transplante de células germinativas criopreservadas (Avarbock et al., 1996; Kanatsu-Shinohara et al., 2003; Wang et al., 2008) e cultivadas (Brinster e Nagano, 1998; Nagano et al., 1998) foram também avanços bastante importantes conseguidos através desta técnica. Pois, diferentemente do congelamento de sêmen onde uma quantidade finita de células é preservada, o congelamento e o armazenamento de células germinativas permitem preservar indefinidamente as linhagens de células espermatogênicas de espécies em extinção, animais experimentais valiosos e animais de alto valor zootécnico. Outro aspecto muito importante a ser considerado é que a criopreservação das espermatogônias preserva todo o potencial genético do macho, ao possibilitar que, após o transplante, essas células continuem replicando e sofrendo recombinação gênica (crossing-over) durante a meiose, garantindo assim variabilidade genética (Dobrinski, 2005).

1.8 Transplante de espermatogônias em peixes

Na mesma linha dos estudos desenvolvidos em mamíferos por Brinster e colaboradores, o transplante de espermatogônias foi também feito com sucesso em peixes teleósteos no Laboratório de Biologia Celular do ICB/UFMG, a partir de estudos desenvolvidos por Lacerda e colaboradores (2006, 2010). Resumidamente, todos os procedimentos necessários para o transplante neste grupo de vertebrados foram padronizados utilizando-se a tilápia-nilótica como modelo experimental. Desta forma, foi demonstrado que as espermatogônias transplantadas diretamente nos testículos de tilápias adultas tratadas com a droga quimioterápica busulfan são capazes de sobreviver, colonizar e proliferar nos túbulos seminíferos dos receptores, gerando espermatozoides funcionais e uma prole normal apresentando o genótipo dos animais doadores (Lacerda, 2006; Lacerda et al., 2010 e 2012).

Em outros estudos, utilizando diferentes procedimentos, Takeuchi e colaboradores (2003) descreveram as técnicas necessárias para o transplante de células germinativas primordiais (PGCs), que são células precursoras dos gonócitos que originam oogônias e espermatogônias, em trutas arco-íris (*O. mykiss*). Assim, nesta investigação, PGCs de trutas GFP (Green Fluorescent Protein) transgênicas foram isoladas e transplantadas na cavidade celomática de larvas recém eclodidas da mesma espécie. A verificação posterior de células GFP positivas nas gônadas dos receptores indicou que as PGCs dos doadores migraram para as gônadas dos peixes receptores, proliferaram e se diferenciaram em espermatozoides e ovócitos, dependendo do sexo genético do indivíduo receptor. Além disso, os ovos de trutas foram inseminados com o ejaculado de machos transplantados, formando embriões com genótipo do doador (carreando o gene *gfp*). Mais recentemente, a produção de ovócitos a partir do transplante alogênico de espermatogônias de trutas arco-íris adultas foi observada. Demonstrando, assim, a grande

plasticidade e bipotencialidade das espermatogônias de peixes, mesmo após os machos terem atingido a maturidade sexual (Okutsu et al., 2006; Lacerda et al, 2013).

Dando continuidade a esta linha de pesquisa, trutas foram geradas a partir do transplante xenogênico de espermatogônias na cavidade do celoma de larvas de salmões triploides, que usualmente são inférteis por não apresentarem células germinativas endógenas em suas gônadas, permitindo assim a produção de espermatozoides somente dos doadores (Okutsu et al., 2007). Ainda neste contexto, utilizando procedimento cirúrgico para transplantar espermatogônias de peixe-rei (pejerey, *Odontesthes bonariensis*) diretamente nos testículos de peixe-rei patagônicos (Patagonian pejerey; *O. hatcheri*), Majhi e colaboradores (2009) verificaram a produção de gametas xenogênicos, vários meses após o transplante. Mais recentemente (2014), os mesmos autores realizaram o transplante xenogênico de espermatogônias e oogônias, através da papila urogenital, entre as mesmas espécies citadas, tendo obtido gametas viáveis que geraram prole saudável. Em outra vertente, Saito e colaboradores (2008) mostraram que PGCs de zebrafish (*D. rerio*) micro injetadas em blástulas de Danio pérola (*D. albolineatus*) foram capazes de se incorporar ao embrião, dando origem a quimeras que produzem espermatozoides funcionais com características genéticas do doador. Estendendo este estudo, os mesmos pesquisadores demonstraram que PGCs isoladas de goldfish (*Carassius auratus*) e dojô (*Misgurnus anguillicaudatus*) são também capazes de se diferenciar em espermatozoides, quando transplantadas em zebrafish no estágio de blástula (Lacerda et al., 2013).

1.9 Referências bibliográficas

ALMEIDA, F. F., KRISTOFFERSEN C., TARANGER G. L., SCHULZ R. W. (2008) Spermatogenesis in Atlantic cod (*Gadus morhua*, L): a novel model of cystic germ cell development. Biol Reprod. 78, 27-34.

ALVARENGA, E. R. (2008). Efeitos de diferentes temperaturas sobre as células germinativas e somáticas do testículo de tilápias (*Oreochromis niloticus*) adultas. Dissertação de Mestrado, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG. p.112.

ASSIS, L. H., Crespo, D., Morais, R. D., França, L. R., Bogerd, J., Schulz, R. W. (2015) Cell Tissue Res.INSL3 stimulates spermatogonial differentiation in testis of adult zebrafish (Danio rerio). Jun 16, Epub ahead of print.

AVARBOCK, M. R., BRINSTER, C. J., BRINSTER RL. (1996). Reconstitution of spermatogenesis from frozen spermatogonial stem cells. Nature Med. 2: 693-696.

BART, J., GROEN, H. J., VAN DER GRAAF, W. T., HOLLEMA, H., HENDRIKSE, N. H., VAALBURG, W., SLEIJFER, D. T., DE VRIES, E. G. (2002). An oncological view on the blood-testis barrier. Lancet Oncol.3: 357-363.

BATLOUNI, S. R., NÓBREGA, R. H., FRANÇA, L. R. (2008). Cell junctions in fish seminiferous epithelium. Fish Physiol Biochem. 35: 207-17.

BEAMISH, F. W. (1970). Influence of temperature and salinity acclimation on temperature preference of the euryhaline fish Tilapia nilotica. J Fish. Res. Board Can. 27:1209-1214.

BILLARD, R., FOSTIER, A., WEIL, C., BRETON, B. (1982). Endocrine control of spermatogenesis in teleost fish. Can. J. Aquat. 39: 65-79

BILLARD, R. (1984). Ultrastructural changes in the spermatogonia and spermatocytes of Oecilia reticulata during spermatogenesis. Cell Tiss. Res.237: 219-226.

BILLARD, R. (1990). Spermatogenesis in teleost fish. In: Lamming Ge. (eds). Reproduction in the male. Churchill Livingstone. p 183-212.

BOCKMANN, F., GUAZZELLI, A. Y .G. M. (2003). Heptapteridae. In Check list of the freshwater fishes of South and Central America. (R.E. Reis, S.O. Kullander & C.J Ferraris Jr., orgs). EDIPUCRS, Porto Alegre, p. 406-431.

BOUSSEBOEUF, A., GAUTIER, A., AUVRAY, P., MAZAN, S., SOURDAINE, P., (2013). Characterization of spermatogonial markers in the mature testis of the dogfish (*Scyliorhinus canicula*L). Reproduction 147, 125–139.

BRINSTER, R.L., AVARBOCK, M.R., (1994). Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci, USA. 91, 11303–11307.

BRINSTER, R. L., ZIMMERMANN, J. W. (1994). Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci USA. 91:11298-302.

BRINSTER, R. L., NAGANO, M. (1998). Spermatogonial stem cell transplantation, cryopreservation and culture. Cell Dev Biol. 9:401-409.

CAIRES, K., BROADY, J., McLean, D. (2010). Maintaining the male germline: regulation ofspermatogonial stem cells. J. Endocrinol. 205, 133–145.

CAMPOS-JÚNIOR, P. H., COSTA, G. M., AVELAR, G. F., SEGATELLI, T. M., LACERDA, S. M., APONTE, P. M., FRANÇA, L. R. (2013). Morphometric evaluation of the spermatogonial stem cell distribution and niche in vertebrates.Methods Mol Biol. 2013;1035:35-42.

CARDOSO, E. (2007). Cinética espermatogonial, duração da espermatogênese e eficiência das células de Sertoli em zebrafish (Danio rerio) adultos. Dissertação de Mestrado, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG. p 81.

CATZEFLIS, F., DICKERMAN, A., MICHAUX, J., KIRSCH, J. (1993). DNA Hybridization and rodent phylogeny. In: Szalay F, Novacek M, Mckenna M (eds). Mammal Phylogeny. New York, NY: Springer Verlag. p 159-172.

CHIARINI-GARCIA, H., RAYMER, A. M., RUSSELL, L. D. (2003). Non-random distribution of spermatogonia in rats: evidence of niches in the seminiferous tubules. Reproduction. 126: 669-80.

CHIPPARI-GOMES, A. R. Temperaturas letais de larvas e alevinos de jundiá, *Rhamdia quelen* (QUOY & GAIMARD, 1824 – PISCES, PIMELODIDAE). Santa Maria – RS, 1998. 70 p. Dissertação (Mestrado em Zootecnia) – Curso de Pósgraduação em Zootecnia, Universidade Federal de Santa Maria, 1998.

CLOUTHIER, D. E., AVARBOCK, M. R., MAIKA, S. D., HAMMER, R. E., BRINSTER, R. L. (1996). Rat spermatogenesis in mouse testis. Nature. 381: 418-21.

CONRAD, S., RENNINGER, M., HENNENLOTTER, J., WIESNER, T., JUST, L., BONIN, M., AICHER, W., BUHRIG, H.J., MATTHEUS, U., MACK, A., WAGNER, H.J., MINGER, S., MATZKIES, M., REPPEL, M., HESCHELER, J., SIEVERT, K.D., STENZL, A., SKUTELLA, T., (2008). Generation of pluripotent stem cells from adult human testis. Nature 456, 344–349.

CUPP, A. S., SKINNER, M. K. (2005). Embryonic Sertoli Cell Differentiation. In: Sertoli Cell Biology (eds). MD Griswold and MK Skinner, Elsevier -Academic Press, San Diego, CA, p. 43-70.

DE ROOIJ DG, RUSSELL LD. (2000). All you wanted to know about spermatogonia but were afraid to ask. J Androl. 21:776-98.

DE WALL, P. P., LEAL, M. C., GARCÍA-LÓPEZ, A., LIARTE, S., DE JONGE, H., HINFRAY, N., BRION, F., SCHULZ, R. W., BOGERD, J. (2009). J Endocrinol. Oestrogen-induced androgen insufficiency results in a reduction of proliferation and differentiation of spermatogonia in the zebrafish testis. 2:287-97.

DOBRINSKI, I., AVARBOCK, M. R., BRINSTER, R. L. (1999). Transplantation of germ cells from rabbits and dogs into mouse testes. Biol Reprod .61:1331-39.

DOBRINSKI, I., AVARBOCK, M. R., BRINSTER, R. L. (2000). Germ cell transplantation from large domestic animals into mouse testes. Mol Reprod Dev. 57:270-279.

DOBRINSKI, I. (2005). Germ cell transplantation and testis tissue xenografting in domestic animals. Anim Reprod Sci., 89: 137-45.

EDDY, E. M. (1999). Role of heat shock protein HSP70-2 in spermatogenesis. Rev. Reprod. 4: 23-30.

FAO. (2013). Cultured Aquatic Species Information Programme. Fisheries and Aquaculture Department. http://www.fao.org/fishery/culturedspecies/Oreochromis_niloticus/en. Acesso em 2 de Agosto de 2015.

FEITSMA, H., Leal, M. C., MOENS, P. B., CUPPEN, E., SCHULZ, R. W. (2007). Mlh1 deficiency in zebrafish results in male sterility and aneuploid as well as triploid progeny in females. Genetics. 4:1561-9.

FITZSIMMONS, K. (2000). Tilapia aquaculture in the Americas. In: Costa Pierce Ba, Rackoy (eds.). Baton Rouge: World Aquaculture Society. p.100-118.

FRANÇA, L. R., NÓBREGA, R.H., MORAIS, R. D. V. S., ASSIS, L. H. C., SCHULZ, R. W., (2015). Sertoli cell structure and function in anamniote vertebrates In: Griswold MD (ed) Sertoli cell biology, Second edition. Elsevier Inc. p385-407.

FRANÇA, L. R., CHIARINI-GARCIA, H. (2005). Célula de Sertoli. In: CARVALHO HF, COLLARES-BUZATO, CB (eds.). Células: uma abordagem multidisciplinar. Barueri, SP: Manole. p302-324.

FRANÇA, L. R., RUSSELL, L. D. (1998). The testis of domestic animals. In: Male Reproduction. Martínez-García F, Regadera J (eds). Churchill Livingstone, Madrid, Spain. 197-219.

GÁRCIA-LOPEZ, A., DE JONGE, H., NÓBREGA, R. H., DE WALL, P. P., VAN DJIK, W., HEMRIKA, W., TARANGER, G. L., BOGERD, J., SCHULZ, R. W. (2010) Studies in zebrafish reveal unusual cellular expression patterns of gonadotropin receptor messenger ribonucleic acids in the testis and unexpected functional differentiation of the gonadotropins. Endocrinology. 5:2349-60.

GODINHO, H. M., FENERICH, N. A., NARAHARA, M. Y. (1978). Developing of embryos and larvae of Rhamdia hilarii (Valenciennes, 1840) (Siluriformes, Pimelodidae). Rev. Bras. Biol.38:151–156.

GOMES, L. C., GOLOMBIESKI, J. I., CHIPPARI, A. R., BALDISSEROTTO, B. (2000). Biologia do jundiá - *Rhamdia quelen* (Teleostei, Pimelodidae) Ciência Rural, Santa Maria. 30:179-185.

GRIER HJ. 1993. Comparative organization of Sertoli cells including the Sertoli cell barrier. In: The Sertoli Cell. Russell LD, Griswold MD (eds.). Clearwater: Cache River Press. 703-739.

GUEDES, D.S. (1980). Contribuição ao estudo da sistemática e alimentação de jundiás (Rhamdia spp) na região central do Rio Grande do Sul (Pisces, Pimelodidae). Santa Maria – RS, 1980. 99p. Dissertação (Mestrado em Zootecnia) - Curso de Pós-graduação em Zootecnia, Universidade Federal de Santa Maria.

HESS, R. A., FRANÇA, L. R. (2007). Spermatogenesis and cycle of the seminiferous

histopathological evaluation of the testis. Clearwater: Cache River Press. 1990.

HOFMANN, M. C. (2008). GDNF signaling pathways within the mammalian spermatogonial stem cell niche. Mol Cell Endocrinol. 25: 95-103.

IHERING, R. Von; AZEVEDO, P. (1936). A desova e a hipofisação dos peixes. Evolução de dois Nematognathas. Arq. Inst. Biol., São Paulo. 7:107-117.

KANATSU-SHINOHARA, M., KATO, M., TAKEHASHI, M., MORIMOTO, H., TAKASHIMA, S., CHUMA, S., NAKATSUJI, N., HIRABAYASHI, M., SHINOHARA, T. (2008). Production of transgenic rats via lentiviral transduction and xenogeneic transplantation of spermatogonial stem cells. Biol Reprod. 79:1121-8.

KHAIRA, H., MCLEAN, D., OHL, D. A., SMITH, G. D. (2005). Spermatogonial stem cell isolation, storage, and transplantation. J Androl. 26:442-50.

KIM, Y., SELVARAJ, V., DOBRINSKI, I., LEE, H., McENTEE, M. C., TRAVIS, A. J. (2006). Recipient preparation and mixed germ cell isolation for spermatogonial stem cell transplantation in domestic cats. J Androl. 27:248-256.

KOULISH, S., KRAMER, C. R., GRIER, H. J. (2002). Organization of the male gonad in a protogynous fish, Thalassoma bifasciatum (Teleostei: Labridae). J Morphol. 254:292-311.

KUBITZA, F. (2015). HTTP://WWW.MATSUDA.COM.BR/MATSUDA/WEB/ENTREVISTAS/DETALHE.ASPX?IDNOT=H12101114130328ELANG=PT-BR . ACESSO EM 02 DE AGOSTO DE 2015.

LACERDA, S. M. S. N., BATLOUNI, S. R., SILVA, S. B. G., HOMEM, C. S. P., FRANÇA, L. R. (2006). Germ cells transplantation in fish: the Nile tilapia model. Animal Reproduction. 2:146–159.

LACERDA, S. M. S. N., BATLOUNI, S. R., COSTA, G. M. J., SEGATELLI, T. M., QUIRINO, B. R., FRANÇA, L. R. (2010). A new and fast technique to generate offspring after germ cells transplantation in adult fish: the Nile tilapia (*Oreochromis niloticus*) model. PLoSONE. 5:e10740.

LACERDA, S. M., APONTE, P. M., COSTA, G. M., CAMPOS-JUNIOR, P. H., SEGATELLI, T. M., SILVA, M. A., FRANÇA, L. R. (2012). An overview of spermatogonial stem cell physiology, niche and transplantation in fish. Anim. Reprod. 9, 798–808.

LACERDA, S. M. S. N., COSTA, G. M. J., SILVA, M. A., CAMPOS-JUNIOR, P. H. A., SEGATELLI, T. M., PEIXOTO, M. T. D., RESENDE, R. R., FRANÇA, L. R. (2013). Phenotypic characterization andin vitro propagation and transplantation of the Nile tilapia (Oreochromis niloticus) spermatogonial stem cells. General and Comparative Endocrinology. 192:95–106.

LACERDA, S. M., Costa, G. M., FRANÇA, L. R. (2014). Biology and identity of fish spermatogonial stem cell. Gen Comp Endocrinol. 207:56-65.

LACERDA, S. M. S. N.; COSTA, G. M. J.; TONELLI, F. M. P.; AVELAR, G. F.; FRANÇA, L. R. (2015). Transplante de espermatogônias-tronco como abordagem biotecnológica para a produção de animais transgênicos. Capítulo 28, Biotecnologia Aplicada à Saúde. Ed. 1 Blucher.

LE GAC, F., LOIR, M. (1999). Male reproductive system, fish. In: Knobil E, Neill Jd (eds). Encyclopedia of Reproduction. San Diego: Academic Press. 3:20-30.

LEAL, M. C., CARDOSO, E. R., NÓBREGA, R. H., BATLOUNI, S. R., BOGERD, J., FRANÇA, L. R., SCHULZ, R. W. (2009). Histological and stereological evaluation of zebrafish (Danio rerio) spermatogenesis with an emphasis on spermatogonial generations. Biol Reprod. 81:177-87.

LOIR, M.; CAUTY, C.; PLANQUETTE, P.; LE BAIL, P. Y. (1989). Comparative study of the male reproductive tract in seven families of South-American catfishes. Aquatic Living Resources, Montrouge, 2:45-56.

LOIR, M., SOURDAINE, P., MENDIS-HANDAGAMA, S.M., JÉGOU, B. (1995). Cellcellinteractions in the testis of teleosts and elasmobranchs. Microsc. Res. Tech.32, 533–552.

MAJHI, S. K., HATTORI, R. S., YOKOTA, M., WATANABE, S., STRÜSSMANN, C.A. (2009). Germ cell transplantation using sexually competent fish: an approach for rapid propagation of endangered and valuable germlines. Plos One. 4:e6132.

MAJHI, S. K., HATTO, R. S., RAHMAN, S. M., STRUSSMANN, C. A. (2014). Surrogate Production of Eggs and Sperm by Intrapapillary Transplantation of Germ Cells in Cytoablated Adult Fish. Plos One. 18:e95294.

MARCHIORO, M. I. (1997). Sobrevivência de alevinos de jundiá (Rhamdia quelen) à variação de pH e salinidade da água de cultivo. 1997. 87p. Dissertação (Mestrado) - Universidade Federal de Santa Maria, Santa Maria.

MARDINI, C. V., SILVEIRA, M. A., BARENHO D. H. L. (1981). Técnica de indução da desova em jundiá (*Rhamdia quelen*) empregada na estação experimental de piscicultura da Lagoa dos Quadros. Porto Alegre: Secretaria da Agricultura, p.14. Documento ocasional n.4.

MATTA, S. L., VILELA, D. A., GODINHO, H. P., FRANÇA, L. R. (2002). The goitrogen 6-n-propyl-2-thiouracil (PTU) given during testis development increases Sertoli and germcell numbers per cyst in fish: the tilapia (*Oreochromis niloticus*) model.Endocrinology 143, 970–978.

McCLUSKY, L. M. (2012) Coordination of spermatogenic processes in the testis: lessons from cystic spermatogenesis. Cell Tissue Res. 3:703-15.

McLean, D. J. (2005). Spermatogonial stem cell transplantation and testicular function. (2005) Cell Tissue Res. 1:21-31.

MIURA, T., MIURA, C. I. (2003). Molecular control mechanisms of fish spermatogenesis. Fish Physiol Biochem. 28:181-186.

MOYLE, P. B.; CECH JR., J. J. (2003). Fishes: An Introduction to Ichthyology. 5th Edition, Hardcover.

MPA. (2014). Melhoramento Genético da Tilápia. Acesso em 2 de Agosto de 2015.

NAGANO, M.; AVARBOCK, M, R.; LEONIDA, E. B..; BRINSTER, C. J. ; BRINSTER, R. L. (1998) Culture of mouse spermatogonial stem cells. Tissue and Cell. 30:389–397.

NAGANO, M.; BRINSTER, C. J.; ORWIG, K. E.; RYU, B. Y.; AVARBOCK, M. R.; BRINSTER, R. L. (2001). Transgenic mice produced by retroviral transduction of male germ-line stem cells. Proc Natl Acad Sci *USA*. 98:13090-13095.

NAGANO, M.; PATRIZIO, P.; BRINSTER, R.L. (2002). Long-term survival of human spermatogonial stem cells in mouse testes. Fertility and Sterility. 78:1225-1233.

NAGANO, M. C.; YEH, J. R.; (2013). The identity and fate decision control of spermatogonial stem cells: where is the point of no return? Curr. Top. Dev. Biol. 102:61–95.

NAKATANI, K.; A.A. AGOSTINHO; G. BAUMGARTNER; A. BIALETZKI; P.V. SANCHES; M.C. MAKRAKIS; C.S. PAVANELLI.

(2001).Ovos e larvas de peixes de água doce: Desenvolvimento e manual de identificaçã o.Maringá, EDUEM, 378p.

NARAHARA, M. Y.; GODINHO, H. M.; ROMAGOSA, E. (1985). Estrutura da população de Rhamdia hilarii (Valenciennes, 1840) (Osteichthyes, Siluriformes, Pimelodidae). Boletim do Instituto de Pesca. 12:123-137.

NARAHARA, M. Y.; GODINHO, H. M.; ROMAGOSA, E. (1989). Tipo de desova e fecundidade do bagre, Rhamdia hilarii (Valenciennes, 1840) (Siluriformes, Pimelodidae). Boletim do Instituto de Pesca. 16:37-45.

NASR IW, WANG Y, GAO G, DENG S, DIGGS L, ROTHSTEIN DM, TELLIDES G, LAKKIS FG, DAI Z. (2005). Testicular immune privilege promotes transplantation tolerance by altering the balance between memory and regulatory T cells.J Immunol. 10:6161-6168.

NELSON, J. S. (2006). Fishes of the World, 4.ed., New York, John Wiley & Sons. 523.

NÓBREGA, R. H.; BATLOUNI, S. R.; FRANÇA, L. R. (2009). An overview of functional and stereological evaluation of spermatogenesis and germ cell transplantation in fish. Fish Physiol Biochem. 35:197-206.

NÓBREGA, R. H. (2006). Alterações do epitelio germinativo masculino, celulas endocrinas testic ulares e celulas gonadotropicas durante o ciclo reprodutivo de Serrasalmus spilopleura (Kner, 1859) e Pimelodus maculatus (Lacepede, 1803). Dissertação de Mestrado. Instituto de Biologia, Universidade Federal de Campinas. Campinas SP. p 185.

NÓBREGA, R. H.; GREEBE, C. D.; VAN DE KANT, H.; BOGERD, J.; FRANÇA, L. R.;SCHULZ, R. W. (2010). Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. Plos One 5:e12808.

OATLEY, J. M.; BRINSTER, R. L. (2012). The germline stem cell niche unit in mammalian testes. Physiol Rev. 2:577-95.

OKUTSU, T.; SHIKINA, S.; KANNO, M.; TAKEUCHI, Y.; YOSHIZAKI, G. (2007). Production of trout offspring from triploid salmon parents. Science. 317, 1517.

OKUTSU, T.; SUZUKI, K.; TAKEUCHI, Y.; TAKEUCHI, T.; YOSHIZAKI, G. (2006). Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. Proc Natl Acad Sci USA. 103:2725–2729.

ORWIG, K. E.; SCHLATT, S. (2005). Cryopreservation and transplantation of spermatogonia and testicular tissue preservation of male fertility. J National Cancer Institute Monographs. 51-56.

PARENTI, L.R.; GRIER, H. J. (2004). Evolution and phylogeny of gonad morphology in bony fishes. Integr. Comp. Biol. 44:333-348

PHILLIPS, B. T.; GASSEI, K.; ORWIG, K. E. (2010). Spermatogonial stem cell regulation and spermatogenesis. Philos Trans R Soc Lond B Biol Sci. 1546:1663-78.

PIAIA, R.; TOWNSEND, C. R.; BALDISSEROTTO, B. (1999). Growth and survival of fingerlings of *Rhamdia quelen* exposed to different light regimes. Aquaculture International. 7:201-205.

POUGH, F.H.; HEISER, J. B.; JANIS, C.M. (2008). A vida dos vertebrados. 4ºEdição. São Paulo: Editora Atheneu, 750.

PUDNEY, J. (1993). Comparative cytology of the non-mammalian vertebrate Sertoli cell. In: RUSSELL LD & GRISWOLD MD (eds). The Sertoli cell. Clearwater, FL: Cache River Press. 611-657.

PUDNEY, J. (1995). Spermatogenesis in nonmammalian vertebrates. 1995. Microsc. Res. Tech. 6:459-497.

REIS, M. M.; TSAI, M. C.; SCHLEGEL, P. N.; FELICIANO, M.; RAFFAELLI, R.; ROZENWAKS, Z; PALERMO, G. D. (2000). Xenogeneic transplantation of human spermatogonia. Zigote. 8:97-105.

RUSSELL, L. D.; ETTLIN, R. A.; SINHA-HIKIM, A. P.; CLEGG, E. D. (1990). Tissue preparation for evaluation of the testis. In: Russell LD, Ettlin RA, Sinha-Hikim AP & Clegg ED (Editors), Histological and Histopathological Evaluation of the Testis. Cache River Press, Clearwater, FL, USA.

RUSSELL, L. D.; GRISWOLD, M. D. (1993). The Sertoli Cell. Clearwater: Cache River Press; 801.

SAITO, T.; GOTO-KAZETO, R.; ARAI, K.; YAMAHA, E. (2008). Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation. Biol. Reprod.78:159-166.

SCHULZ, R. W.; MIURA, T. (2002). Spermatogenesis and its endocrine regulation. Fish Physiol Biochem 26:43-56.

SCHULZ, R. W. (2003). Endocrine regulation of spermatogenesis in teleost fish. ARBS Annu Rev Biomed Sci. 5:57-68.

SCHULZ, R. W.; MENTING, S.; BOGERD, J.; FRANCA, L. R.; VILELA, D. A.; GODINHO, H. P. (2005). Sertoli cell proliferation in the adult testis--evidence from two fish species belonging to different orders.Biol Reprod.73:891-8.

SCHULZ, R. W.; FRANÇA, L. R.; LAREYRE, J. J.; LEGAC, F.; CHIARINI-GARCIA, H.; NÓBREGA, R.H.; MIURA, T. (2010). Spermatogenesis in fish. Gen Comp Endocrinol. 165: 390–411.

SHARPE, R. M. (1994). Regulation of spermatogenesis. In: KNOBIL E, NEILL JD (eds). The Physiology of Reproduction. New York: Raven Press. 1363-1434.

SILVA, M. (1987). Morfologia ultra-estrutural do testículo, cinética da espermatogênese e barreira hemotesticular da tilápia do nilo, Oreochromis niloticus. Tese de Doutorado. Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais. Belo Horizonte, MG. 164.

SILFVERGRIP, A. M. C. (1996). A sistematic revision of the neotropical catfish genus *Rhamdia* (Teleostei, Pimelodidae). Stockholm, Sweden (PhD Thesis) - Department of Zoology, Stockholm University and Department of Vertebrate Zoology, Swedish Museum of Natural History. 156.

STICKNEY, R. R. (1997). Tilapia update 1996. World Aquaculture. 28: 20-25.

STICKNEY, R. R. (2000). Tilapia Culture. In: Stickney, R.R. (ed.). Encyclopedia of Aquaculture, New York: John Wiley & Sons. 934-941.

SUSSEL, F. R. (2013). ftp://ftp.sp.gov.br/ftppesca/TilapiculturaEntraves2013.pdf. Acesso em 02 de Agosto de 2015.

TAKEUCHI, Y.; YOSHIZAKI, G.; TAKEUCHI, T. (2003) Generation of live fry from intra-peritoneally transplanted primordial germ cells in rainbow trout. Biol Reprod 69:1142–1149.

THOMA, E. C.; WAGNER, T. U.; WEBER, I. P.; HERPIN, A.; FISCHER, A.; SCHARTL, M. (2011) Ectopic expression of single transcription factors directs differentiation of a medaka spermatogonial cell line. Stem Cells Dev. 20:1425-1438.

TORRANS, L. (1998). Blue tilapia culture in arkansas. Pine Bluff: Cooperative Extension Service Program Publication University of Arkansas. EC560.

TREWAVAS, E. (1983). Tilapiine: Fishes of The Genera Sarotherodon, Oreochromis and Danakilia. British Museum of Natural History, London.

VALLI, H., PHILLIPS, B.T., SHETTY, G., BYRNE, J.A., CLARK, A.T., MEISTRICH, M.L., ORWIG, K.E. (2014). Germline stem cells: toward the regeneration of spermatogenesis. Fertil.Steril. 101:3–13.

VILELA, D. A. R.; SILVA, S. G. B.; PEIXOTO, M. T. D.; GODINHO, H. P.; FRANÇA, L. R. (2003). Spermatogenesis in teleost: insights from the nile tilapia (*Oreochromis niloticus*) model. Fish Physiol Biochem. 28:187-190.

WANG, Y. B.; CHEN, B; WANG, Z. (2008). Transplantation of spermatogonial stem cells and prospects of its application. Zhonghua Nan Ke Xue. 14:635-639.

WEIS, M. L. C. (1980). Interpretação da idade e cálculo da curva de crescimento do jundiá, Rhamdia quelen (QUOY & GAIMARD, 1824) do banhado de Santa Catarina - RS. Santa Maria – RS. Dissertação (Mestrado em Zootecnia) - Curso de Pós-graduação em Zootecnia, Universidade Federal de Santa Maria, 1980. 93.

WELTZIEN, F. A.; ANDERSSON, E.; ANDERSEN, O.; SHALCHIAN-TABRIZI, K.; NORBERG, B. (2004). The brain–pituitary–gonad axis in male teleosts, with special emphasis on flatfish (Pleuronectiformes). Comparative Biochemistry And Physiology - part a: Molecular &Integrative Physiology. 137: 447-477.

YOSHIDA, S. (2012). Elucidating the identity and behavior of spermatogenic stem cells in the mouse testis. Reproduction. 3:293-302.

ZANIBONI FILHO, E. (1997). O desenvolvimento da piscicultura brasileira sem a deterioração da qualidade de água. Rev. Bras. Biol. 57:3-9

ZHANG, Z.; RENFREE, M. B.; SHORT, R. V. (2003). Successful intra- and interspecific male germ cell transplantation in the rat. Biol Reprod. 68: 961-967.

2. JUSTIFICATIVA

2. JUSTIFICATIVA

Até o presente momento, o transplante de espermatogônias é a única abordagem experimental funcional disponível para se investigar a biologia das espermatogônias-tronco. Em peixes teleósteos, esta técnica foi desenvolvida recentemente, utilizando-se a tilápia nilótica como modelo experimental (Lacerda, 2006). Assim, todas as abordagens necessárias para o transplante de espermatogônias em machos sexualmente maduros foram padronizadas. A utilização dessas abordagens demonstrou que o transplante alogênico (tilápia-tilápia) de células germinativas a fresco ou criopreservadas resulta na formação de espermatozoides viáveis (cerca de dois meses após o transplante), com prole possuindo as características genéticas do animal doador (células a fresco) (Lacerda et al, 2006 e 2010). Estes resultados, inéditos e promissores, demonstraram que células germinativas podem ser transplantadas com sucesso diretamente nos testículos de peixes adultos. Num contexto ainda mais amplo, a investigação envolvendo o transplante xenogênico em peixes poderia trazer inúmeros benefícios para pesquisas no campo da agropecuária, preservação de espécies ameaçadas de extinção e de animais valiosos do ponto de vista zootécnico, medicina reprodutiva e produção de animais transgênicos (Lacerda et al., 2014). Neste sentido, utilizamos a tilápias-nilóticas (*Oreochromis niloticus*) sexualmente maduras como receptor para o transplante xenogênico de espermatogônias-tronco, com a finalidade de se avaliar o sucesso da técnica utilizando-se células germinativas de jundiá (*Rhamdia quelen*), espécie pertencente à ordem Siluriforme, com a finalidade de se produzir gametas desta espécie. Espécie esta apresenta algumas características interessantes, como por exemplo a facilidade de se manter em cativeiro e, principalmente, por ser filogeneticamente próxima do jaú (*Zungaro jahu)* que se encontra atualmente ameaçada de extinção. Ainda, buscando a melhor compreensão das características reprodutivas do jundiá, realizamos experimentos para descrever a morfologia e morfometria de suas células germinativas, e estimar a duração da espermatogênese do jundiá.

3. OBJETIVOS

3.OBJETIVOS

3.1 Objetivo Geral

Investigar parâmetros morfofuncionais da espermatogêse do jundiá (ordem Siluriforme; *Rhamdia quelen*) e a viabilidade do transplante xenogêncio de células germinativas desta espécie para o receptor tilápia-nilótica (ordem Perciforme; *Oreochromis niloticus*).

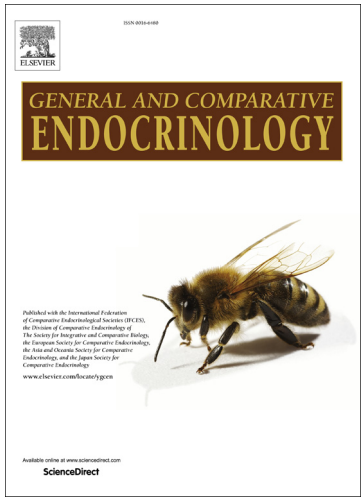
3.2 ObjetivosEspecíficos

• Investigar a presença de eventuais células germinativas marcadas com PKH26 e oriundas do doador nos testículos de tilápias receptoras, através de microscopia de fluorescência, em diferentes tempos após o transplante;

• Confirmar geneticamente a presença de células germinativas derivadas de jundiá no testículo das tilápias receptoras através de análises de microssatélites do DNA mitocondrial.

• Caracterizar a espermatogênese do jundiá, através de avaliações morfológica e morfométrica de suas células germinativas, bem como estimar sua duração após injeção de BrdU.

4. CAPÍTULO I



Successful Xenogeneic Germ Cell Transplantation from Jundia Catfish (*Rhamdia quelen*) into Adult Nile tilapia (*Oreochromis niloticus*) Testes

M.A. Silva, G.M.J. Costa, S.M.S.N. Lacerda, P.F.P. Brandão-Dias, E. Kalapothakis, A.F. Silva Júnior, E.R. Alvarenga, L.R. França

PII: S0016-6480(16)30056-9
DOI: http://dx.doi.org/10.1016/j.ygcen.2016.03.012
Reference: YGCEN 12338

To appear in: *General and Comparative Endocrinology*

Received Date: 21 July 2015
Revised Date: 8 March 2016
Accepted Date: 9 March 2016

Please cite this article as: Silva, M.A., Costa, G.M.J., Lacerda, S.M.S.N., Brandão-Dias, P.F.P., Kalapothakis, E., Silva Júnior, A.F., Alvarenga, E.R., França, L.R., Successful Xenogeneic Germ Cell Transplantation from Jundia Catfish (*Rhamdia quelen*) into Adult Nile tilapia (*Oreochromis niloticus*) Testes, *General and Comparative Endocrinology* (2016), doi: http://dx.doi.org/10.1016/j.ygcen.2016.03.012

Successful Xenogeneic Germ Cell Transplantation from Jundia Catfish (*Rhamdia quelen*) into Adult Nile tilapia (*Oreochromis niloticus*) Testes

Silva, M.A.[1], Costa G.M.J.[1], Lacerda S.M.S.N.[1], Brandão-Dias, P.F.P.[2], Kalapothakis, E.[2], Silva Júnior., A. F.[3]; Alvarenga, E. R.[3]; França L.R.[1,4]

[1]Laboratory of Cellular Biology (Dept. of Morphology), [2]Laboratory of Biotechnology and Molecular Markers (Dept. of General Biology), [3]Laboratory of Aquaculture (Veterinary School). Federal University of Minas Gerais, MG, Brazil - 31270-901. [4]National Institute for Amazonian Research, Manaus, AM, Brazil, 69067-375.

CORRESPONDING AUTHOR:

Dr. Luiz Renato de França

Phones: +55 (31) 96181992; +55 (92) 99605-5655

e-mails: lrfranca@icb.ufmg.br and lrfranca@inpa.gov.br

ABSTRACT

Fish germ cell transplantation presents several important potential applications for aquaculture, including the preservation of germplasm from endangered fish species with high genetic and commercial values. Using this technique in studies developed in our laboratory with adult male Nile tilapias (*Oreochromis niloticus*), all the necessary procedures were successfully established, allowing the production of functional sperm and healthy progeny approximately 2 months after allogeneic transplantation. In the present study, we evaluated the viability of the adult Nile tilapia testis to generate sperm after xenogeneic transplant of germ cells from sexually mature Jundia catfish (*Rhamdia quelen*) that belong to a different taxonomic order. Therefore, in order to investigate at different time-periods post-transplantation, the presence and development of donor PKH26 labeled catfish germ cells were followed in the tilapia seminiferous tubules. From 7 to 20 days post-transplantation, only PKH26 labeled spermatogonia were observed, whereas spermatocytes at different stages of development were found at 70 days. Germ cell transplantation success and progression of spermatogenesis were indicated by the presence of labeled PKH26 spermatids and sperm on days 90 and 120 post-transplantation, respectively. Confirming the presence of the catfish genetic material in the tilapia testis, all recipient tilapias evaluated (n=8) showed the genetic markers evaluated. Therefore, we demonstrated for the first time that the adult Nile tilapia testis offers the functional conditions for development of spermatogenesis with sperm transplanted from a fish species belonging to a different order, which provides an important new venue for aquaculture advancement.

KEYWORDS: Jundia catfish (*Rhamdia quelen*), Nile-tilapia (*Oreochromis niloticus*), xenogeneic germ cells transplantation, spermatogonial stem cells, spermatogenesis.

INTRODUCTION

For fish, it has been assumed that preservation of the genetic resources is limited to the rearing of live individuals (Zhang et al., 2007). However, although the cryopreservation of fish milt is feasible, several other alternative reproductive technologies have been developed to efficiently produce functional gametes and offspring from endangered and commercially important species, which are normally difficult to breed in captivity (Majhi et al., 2014). Among these approaches, the induction of ovulation, *in vitro* gametogenesis and germ cell transplantation are the most popular (Kawasaki et al., 2015; Lacerda et al., 2013a; Mylonas et al., 2010). The latter is an important technique that involves the isolation of spermatogonial stem cells (SSCs) from a donor animal and the transplantation of these cells into a recipient testis (Brinster et al., 1994), where they will be able to develop and form mature fertile sperm carrying the donor's genetic characteristics (Brinster, 2002; Shinohara et al., 2006).

In several fish species, intraspecific allogeneic germ cell transplantation has been performed by microinjecting primordial germ cells and/or spermatogonial cells into blastula-stage fish embryos, as well as in the coelomic cavity of newly hatched fish larvae (Ciruna et al., 2002; Kise et al, 2012; Kobayashi et al., 2007; Okutsu, 2006; Takeuchi et. al, 2009; Takeuchi, 2003; Wong et al., 2011). Although these methodologies allow the production of fertile donor sperm in the recipient gonads, a long time-interval is required to generate functional gametes, mainly because recipient animals need firstly to reach puberty (reviewed by Lacerda et al., 2013a; Yoshizaki et al., 2012). Studies developed in our laboratory by Lacerda and colleagues (2013b, 2010, 2006) standardized all the necessary methodologies to perform successful SSCs transplantation using, as recipient, the adult Nile tilapia (*Oreochromis niloticus*). Donor SSCs were injected through the common spermatic duct located in the urogenital papilla

and this procedure provided a faster (approximately two months) generation of donor-derived spermatozoa and the production of normal offspring carrying the donor genetics characteristics. Additionally, in association with the Utrecht University (The Netherlands), we performed intraspecific germ cells transplantation in zebrafish (Nobrega et al., 2010). Although the transplantation efficiency was very low, the recipient male zebrafish were able to develop donor-derived gametogenesis (Nobrega et al., 2010).

The feasibility of interspecific (xenogeneic) spermatogonial transplantation was also determined in several fish species using embryos, newly hatched larvae, and adult fish as recipient (reviewed by Lacerda et. al, 2013a). In this regard, the key studies related to xenogeneic germ cell transplantation, using embryos and newly hatched larvae, were performed respectively in zebrafish (Ciruna et al., 2002; Wong et al., 2011) and rainbow trout (Kise et al., 2012; Kobayashi et al., 2007; Okutsu et al., 2006; Takeuchi et al., 2003). From the obtained results, the authors concluded that the spermatogonial transplantation technique is valid for interspecies applications, resulting in donor-derived sperm and offspring (reviewed by Lacerda et al., 2012), even for some fish species (donor and recipients) that are phylogenetically distant. In the first study where xenogeneic germ cell transplantation was successfully developed in adult fish, using *Odontesthes hatcheri* as recipients and *Odontesthes bonariensis* as donors, a surgical procedure was used (Majhi et al., 2009). More recently, using the same species, Majhi and colleagues (2014) described a successful production of eggs and sperm by intrapapillary transplantation of germ cells in cytoablated adult fish.

Based on the results obtained for xenogeneic germ cell transplantation in adult fish, a very promising scenario has emerged, as this approach can be used to assist the preservation of the genetic resources of endangered species or valuable genetic strains.

Therefore, in the present study, we evaluated the feasibility of xenogeneic germ cell transplantation using two species belonging to different taxonomic orders, using the adult Nile tilapia (*Oreochromis niloticus* from the Perciformes order) as a recipient and sexually mature Jundia catfish (*Rhamdia quelen* from the Siluriformes order) as a germ cell donor.

MATERIALS AND METHODS

Animals

Eighteen sexually mature male Nile tilapia (*Oreochromis niloticus*; mean body weight = 208g) and ten Jundia catfish (*Rhamdia quelen*; mean body weight = 289g) were used, respectively, as recipient and donor for germ cells transplantation. These fish were obtained from the Aquaculture Laboratory (School of Veterinary Sciences or Veterinary School, Federal University of Minas Gerais, Belo Horizonte, Brazil) and from commercial farmers located nearby Belo Horizonte city (state of Minas Gerais, Southeastern Brazil). The experimental design used in the present study is shown in Figure 1. All experimental procedures were performed in accordance with the guidelines approved by the local ethics committee on animal experimentation – CEUA, UFMG (protocol # 89/2012).

Recipient preparation

The adequate preparation of the recipient fish, in which endogenous spermatogenesis is naturally absent or experimentally deprived, is crucial for the success and efficiency of germ cells transplantation. In this condition, niches for germ cells colonization and development are available. In the present study, the endogenous

spermatogenesis was depleted according to the procedures already described by Lacerda and colleagues (2006). Eighteen tilapias were kept at temperatures of 35°C (Figure 1A) for at least two weeks before receiving intraperitoneally two injections of busulfan (Sigma, St. Louis, MO, USA) (18 mg/kg body weight and 15 mg/kg body weight), with a two-week interval between the injections (Figure 1B-C). The germ cell transplantation was performed one week after the second injection (Figure 1D).

Donor germ cell isolation

Donor spermatogonial cells were harvest from the testes of eight adult Jundia catfish (mean testis weight = 8g; Figure 2B) through enzymatic digestion according to standardized protocols (Lacerda et al., 2010). Briefly, testes were dissociated with 2% collagenase (Sigma, St. Louis, MO) in Dulbecco Modified Eagle medium/Ham F-12 medium (DMEM/F12- Gibco, Grand Island, NY) for four hours at 28°C. The dispersed testicular tissue was then incubated with 0.25% trypsin/1mM EDTA (Sigma, St. Louis, MO) and 0.03% DNase I (Sigma, St. Louis, MO) under constant shaking at 28°C for 30 minutes. After this step, an equal volume of DMEM containing 10% of fetal bovine serum (FBS, Gibco) was added to the cell suspension in order to inactivate the trypsin. The testicular cell suspension was sequentially filtered using 70 and 40µm mesh and then centrifuged at 400g for 10 minutes and re-suspended in DMEM/F12 (Figure 2C). An enriched type A spermatogonia cell suspension, characterized according to the germ cell morphology in Jundia (Figure 2A) was obtained after centrifugation, at 800g for 30 minutes, using a 40% percoll gradient, followed by another centrifugation using 35% percoll. The pellet was discarded and the supernatant (Figure 2D) was subjected to differential plating (Figure 2F-H), in order to deplete testicular somatic cells that adhere to the culture bottle (Figure 2G) (Lacerda et al., 2010). A total of $22x10^7$ cells was

obtained, and $7.3x10^7$ cells per culture bottle (75cm², TPP, Switzerland) were cultured in FSM (fish spermatogonial medium – DMEM/F12 supplemented) for 18 hours, at 28°C in an atmosphere of 5% CO_2. These methodologies allowed an efficient enrichment of germ cell for transplantation (Figure 2H). Additionally, in order to morphologically characterize Jundia germ cells along spermatogenesis, testes samples from donors were fixed in 4% glutaraldehyde and embedded in plastic for routine histological analysis (Alvarenga and França, 2009; Leal et al., 2009).

Donor germ cell labeling and transplantation

Before germ cell transplantation into recipient Nile tilapia testes, donor germ cells were incubated with the fluorescent membrane dye PKH26-GL (Sigma, St. Louis, MO), an adequate molecular tracer for the investigation of transplanted donor germ cell fate and location into the recipient seminiferous tubules (Lacerda et al., 2013b, 2010, 2006; Majid et al. 2014) (Figure 3A-B). The cell labeling was performed following the manufacture's guidelines with the final optimal concentration of 9mM PKH26. The collected cells were then suspended in DMEM/F12 at a concentration of 10^7 cells/mL and approximately 1 mL of cell suspension was injected in each recipient. The cells were transplanted using a non-surgical access, via the common spermatic duct that opens in the urogenital papilla through the urogenital pore (Figure 3C). For this purpose, the recipient Nile tilapias were anesthetized with Quinaldin solution (1:5000, added in the aquarium water; Merck & Co.) and the donor germ cells were transplanted using a glass micropipette (outside diameter 70µm) under a stereomicroscope (Olympus SZX-ILLB2-100). As Nile tilapia usually reproduce at a temperature of 26-28°C, the water temperature was gradually decreased 1–2°C per day after the transplantation, until 28°C was reached.

Microscopic evaluation of donor-derived spermatogenesis in the recipient Nile tilapia testis

Testes were collected from ten recipient tilapia for analysis of PKH26 positive donor spermatogonia behavior in the Nile tilapia testis (Figure 1E-I). In order to evaluate the progression of spermatogenesis and the eventual spermatozoa formation after germ cell transplantation, the recipient fish testes were collected at 7, 20, 70, 90 and 120 days post germ cell transplantation. The testes were immediately embedded in Jung Tissue Freezing Medium (Leica Instruments, Nussloch, Germany), frozen in liquid nitrogen and stored at -80°C freezer. These testis samples were serially cryosectioned (7µm of thickness), stained with DAPI (targeting DNA in the cell nucleus) and then analyzed under the fluorescence microscope (Olympus IX-70).

Genetic analysis

A PCR approach was used to detect donor germ cell DNA in eight recipient Nile tilapia testes (Table 1 and Figure 1J). The primers consisted of three (outer, middle and inner) pairs amplifying the Rhq7 microsatellite sequence for *R. quelen* (GenBank access number KC117544.1); primer specificity and sensitivity was tested both *in silico* and *in vitro*. *In silico* tests consisted of a primer-BLAST specificity tool (Ye et al., 2012) against GenBank *O. niloticus* database and no evidence of a cross amplification was observed. *In vitro* tests comprised serial dilutions of small quantities of total *R. quelen* genomic DNA (from 0.000089 to 2.23 $ng.\mu L^{-1}$) into large amounts of total *O. niloticus* genomic DNA (from 2.53 to 7.04 $ng.\mu L^{-1}$), and no evidence of an unspecific amplification was detected. Total DNA was extracted from the controls (Nile tilapia and Jundia), and from the recipient Nile tilapia testes following a standard proteinase k,

phenol/chloroform extraction (Sambrook and Russell, 2001), and PCR reactions were run in a Veriti 96-Well thermal cycler (Applied Biosystems). The best reaction cycle was an initial outer primer pair reaction consisting of a 95°C denaturation step (5 minutes), followed by 5 cycles of 92°C denaturation (20 seconds), 64°C annealing (30 seconds) and 72°C of extension (30 seconds). One µL of first amplification product was then used as template DNA for a second reaction with middle primers, which differed from the first one by adding 20 additional cycles (total 25 cycles) of denaturation, annealing and extension steps. Immediately after that, a $1{:}10^{-3}$ dilution of the second PCR product was applied as a template for an inner primer reaction, using again 25 cycles.

RESULTS

Jundia germ cell morphology and Jundia PKH26 labeled germ cells in the recipient testis

As depicted in Figure 2A, type A spermatogonial cells show a large ovoid nucleus with a prominent nucleolus and these cells are noticeably larger than early and late type B spermatogonia.

At one week post-transplantation (Figure 4A-C), labeled donor germ cells were observed in the recipient tilapia seminiferous tubules epithelium. Based on established morphological criteria (Figure 2A), these cells were considered spermatogonia. Twenty days post-transplantation, a higher number of labeled germ cells, similar to spermatogonial cells, were also identified in the seminiferous tubules epithelium (Figure 4D-N).

Demonstrating the progression and differentiation of donor-derived germ cells through spermatogenesis, seventy days after transplantation, PKH26 labeled germ cells

cysts at different stages of development were observed in the recipient seminiferous tubules (Figure 5A-G). The number and size of the germ cells present in these cysts indicated that they were spermatocytes (meiotic cells). At 90 (Figure 5H-J) and 120 days (Figure 6) post-transplantation, labeled germ cells presented morphological characteristics of spermatids and sperm, respectively. These cells were arranged in cystic structures (90 days) inside the seminiferous tubules (Figure 5J) and at 120 days in the tubular lumen (Figure 6). All recipient tilapia testes presented PKH26 positive donor germ cells. Particularly in the later time-periods, cysts containing germ cells in different developmental stages (spermatogonia, spermatocytes and spermatids) were noted. Some germ cells at later stages may have been present among the transplanted cells, but it seems unlikely that these more differentiated germ cells contributed to the long-term spermatogenesis in the recipient testis, for which usually stem cell properties would be required.

Genetic analysis of the Nile tilapia recipient testes

All recipient fish (n=8; Figure 1J) showed the presence of Jundia catfish genetic material in their testis (Figure 7E-L). The evaluated testis samples displayed different intensities of the amplicon bands, and although no qPCR was performed in the present study, these results suggest different transplantation/colonization efficiencies. In all cases, however, the data strongly suggest a successful donor germ cell colonization and spermatogonial progression in the Nile tilapia testis. Importantly, the primers, which were designed for Jundia specific sequences (Figure 7B), did not recognize any tilapia sequences (Figure 7C).

DISCUSSION

Previous studies developed in our laboratory using Nile tilapias demonstrated that this species represents an excellent recipient for fish SSCs transplantation (Lacerda et al., 2013b, 2010, 2006). This finding motivated us to investigate if the Nile tilapia testis environment could support the development of germ cells from phylogenetically distant fish species. In the present investigation we evaluated the success of xenogeneic germ cell transplantation using *Rhamdia quelen* (a species belonging to the Siluriformes order) as a donor and the Nile tilapia (species belonging to the Perciformes order) as a recipient. The formation of *Rhamdia quelen* sperm in the tilapia seminiferous tubules strongly suggests that the Nile tilapia has high plasticity as a recipient, providing an excellent possibility for use in aquaculture to preserve the germplasm of endangered fish species and the production of gametes in species with high commercial and genetic values.

Although Majhi and colleagues (2014 and 2009) have already carried out a xenogeneic transplantation in adult fish, the species used (donor and recipient) belonged to the same genus. In their study, spermatogonia and oogonia from *Odontesthes bonariensis* were transplanted, respectively, in male and female *Odontesthes hatcheri*. After germ cell transplantation, the establishment of spermatogenesis and oogenesis was observed in the recipient species with the donor sperm and oocytes production. The gametes were functionally tested, leading to healthy offspring generation. In our study, although we have demonstrated the presence of *Rhamdia quelen* sperm in the Nile tilapia testis, their viability was not evaluated due to the lack of information about the induction of ovulation and/or artificial insemination in the *Rhamdia quelen*. Therefore, in order to demonstrate the fertilization capacity and generation of healthy embryos derived from these gametes produced after germ cell transplantation, further studies are being conducted.

As reviewed by Lacerda and colleagues (2012), the successful production of xenogeneic sperm was also described from germ cell transplantation using zebrafish embryos (Saito et al., 2010, 2008) and salmon larvae (Takeuchi et al., 2004) as recipients. Based on the literature for higher vertebrates (Honaramooz and Yang, 2011), it is possible that xenotransplantation in mammals is less efficient than that performed in fishes, which could be based on represent a higher plasticity provided by the testicular somatic cells. In fact, oogonia can differentiate into spermatogonia in the fish testis, resulting in viable gamete production (Wong et al., 2011; Yoshizaki et al., 2011, 2010) and the opposite is also true (Okutsu et al., 2007; Nobrega et al., 2010). From these results, it can be concluded that the somatic cells in the fish testis are crucial for gametogenesis development when different cell lineages (male or female) or germ cells from distinct fish species are transplanted.

The duration of spermatogenesis is considered species-specific and controlled by the germ cell genotype (França et al., 1998). Although the time required for sperm formation, from preleptotene primary spermatocytes, takes about 11 days in the Nile tilapia (Vilela et al., 2003), in this species sperm production after allogeneic germ cell transplantation is observed approximately 9 weeks post-transplantation (Lacerda et al., 2010). Studies developed in mammals have shown that the commitment of SSCs to differentiation occurs at approximately two weeks after germ cell transplantation due to the time-period required for colonization of the seminiferous epithelium by these cells (Ishii et al., 2013; Nagano et al., 1999; Ohta et al., 2000; Parreira et al., 1998). In the present investigation, the required time to form *Rhamdia quelen* sperm in the Nile tilapia testis was approximately 17 weeks, which is a much longer time than that described for intraspecific germ cell transplantation in tilapias. A long time-period (24-34 weeks) for sperm production was also observed when germ cells from *O.*

bonariensis were transplanted to the *O. hatcheri* seminiferous tubules (Majhi et al., 2014). Although this time could be considered as rather long, the production of spermatozoa using adult fish as recipients is much faster in comparison to germ cell transplantation using embryos or larvae as recipients (Kise et al., 2012; Okutsu et al., 2007; Saito et al., 2011, 2010, 2008; Takeuchi et al., 2004; Yazawa et al., 2010; Yoshizaki et al., 2010). In order to evaluate whether the duration of spermatogenesis in Jundia *Rhamdia quelen* is longer than in Nile tilapias, or if their spermatogonial SSCs need a longer time-period to colonize and differentiate in the tilapia testis, further studies related to these aspects are being developed in our laboratory.

The species *Rhamdia quelen* is an important source of protein for human consumption (Sampaio and Sato, 2006), and therefore has been used as germ cell donor in the present study. The phylogenetic distance between this species and the Nile tilapia is approximately 110 million years (Wittbrodt et al., 2002) and a similar distance separates man from mouse (Nagano et al., 2002). However, while in the present investigation we could observe *Rhamdia quelen* sperm formation in the Nile tilapia testis, human spermatogonia were only able to colonize the mouse testis without any further progression of spermatogenesis (Nagano et al., 2002). These xenogeneic germ cell transplantation studies suggest that, different from mammals (Dobrinski and Hill, 2007; González and Dobrinski, 2015), fish apparently have no tight phylogenetic barrier that would limit the success of this very important biotechnology.

In the present study, we applied the technique of spermatogonial transplantation using Jundia as the donor and Nile tilapia as the recipient species. We demonstrated that the somatic microenvironment of the Nile tilapia gonad can support colonization, survival, proliferation and the differentiation of transplanted progenitor spermatogonial cells. The novelty of our report lies in the phylogenetic distance between donor and

recipient, which are species from different taxonomic orders. These findings indicate a remarkable plasticity in the capacity of the somatic cells in the teleost testis to support xenogenic germ cells. A functional test of catfish sperm matured in tilapia testis must await the availability of catfish eggs and the development of an in vitro fertilization protocol.

ACKNOWLEDGEMENTS

Technical help from Mara Lívia Santos is highly appreciated. The assistance from the local farmers, José Geraldo da Silva and Marco Túlio Diniz Peixoto, was of great value. The scholarships awarded to M.A.S. and S.M.S.N.L. and the financial support from the FAPEMIG and CNPq are fully appreciated.

REFERENCES

Alvarenga, E.R., França, L.R., 2009. Effects of different temperatures on testis structure and function, with emphasis on somatic cells, in sexually mature Nile tilapias (Oreochromis niloticus). Biol Reprod. 80: 537-544.

Brinster, R.L., 2002. Germ cell transplantation. Science. 296, 2174–2176.

Brinster, R.L., Avarbock, M.R., 1994. Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci, USA. 91, 11303–11307.

Ciruna, B., Weidinger, G., Knaut, H., Thisse, B., Thisse, C., Raz, E., Schier, A.F., 2002. Production of maternal-zygotic mutant zebrafish by germ-line replacement. Proc Natl Acad Sci USA. 23, 14919-14924.

Dobrinski, I., Hill, J.R., 2007. Germ cell transplantation - A novel approach to 'designer' animals? In: Swain D, CharmleySteel, E.J, Coffey S., (Eds). Redesigning Animal Agriculture: The challenge of the 21st century. Oxford, UK; Cambridge, MA: CAB International. pp. 81–93.

França, L.R., Ogawa, L., Avarbock, M.R., Brinster, R.L., Russell, L.D., 1998. Germ Cell Genotype Controls Cell Cycle during Spermatogenesis in the Rat. Biology of Reproduction. 23, 1371-1377.

González, R., Dobrinski, I., 2015. Beyond the mouse monopoly: studying the male germ line in domestic animal models. ILARJ. 56, 83-98.

Honaramooz, A., Yang, Y., 2011. Recent advances in application of male germ cell transplantation in farm animals. Vet Med Int. 4, pii–657860.

Ishii, K., Kanatsu-Shinohara, M., Shinohara, T., 2014. Cell-cycle-dependent colonization of mouse spermatogonial stem cells after transplantation into seminiferous tubules. J Reprod Dev. 60, 37-46.

Kawasaki T., Siegfried K.R., Sakai N., 2015. Differentiation of zebrafish spermatogonial stem cells to functional sperm in culture. Development. 30:129643.

Kise, K., Yoshikawa, H., Sato, M., Tashiro, M., Yazawa, R., Nagasaka, Y., Takeuchi, Y., Yoshizaki, G., 2012. Flow-cytometric isolation and enrichment of teleost type A spermatogonia based on light-scattering properties. Biol Reprod. 86, 107.

Kobayashi, T., Takeuchi, Y., Takeuchi, T., Yoshizaki, G., 2007. Generation of viable fish from cryopreserved primordial germ cells. Mol Reprod Dev. 74, 207–213.

Lacerda, S.M., Aponte, P.M., Costa, G.M., Campos-Junior, P.H., Segatelli, T.M., Silva, M.A., França, L.R., 2012. An overview of spermatogonial stem cell physiology, niche and transplantation in fish. Anim. Reprod. 9, 798–808.

Lacerda, S.M., Costa, G.M., Campos-Junior, P.H., Segatelli, T.M., Yazawa, R., Takeuchi, Y., Morita, T., Yoshizaki, G., França, L.R., 2013a. Germ cell transplantation as a potential biotechnological approach to fish reproduction. Fish Physiol Biochem. 39, 3-11.

Lacerda, S.M.S.N., Batlouni, S.R., Costa, G.M.J., Segatelli, T.M., Quirino, B.R., França, L.R, 2010. A new and fast technique to generate offspring after germ cells transplantation in adult fish: the Nile tilapia (*Oreochromis niloticus*) model. Plos One. 20, e10740.

Lacerda, S.M.S.N., Batlouni, S.R., Silva, S.B.G., Homem, C.S.P., França, L.R., 2006. Germ cells transplantation in fish: the Nile tilapia model. Animal Reproduction. 2, 146–159.

Lacerda, S.M.S.N., Costa, G.M.J., Silva, M.A., Campos-Junior, P.H.A., Segatelli, T.M., Peixoto, M.T.D., Resende, R.R., França, L.R., 2013b. Phenotypic characterization and in vitro propagation and transplantation of the Nile tilapia (Oreochromis niloticus) spermatogonial stem cells. General and Comparative Endocrinology. 192, 95–106.

Leal, M.C., Cardoso, E.R., Nóbrega, R.H., Batlouni, S.R., Bogerd, J., França, L.R., Schulz, R.W., 2009. Histological and stereological evaluation of zebrafish (Danio rerio) spermatogenesis with an emphasis on spermatogonial generations. Biol Reprod. 81: 177-187.

Majhi, S.K., Hattori, R.S., Rahman, S.M., Strüssmann, C.A., 2014. Surrogate Production of Eggs and Sperm by Intrapapillary Transplantation of Germ Cells in Cytoablated Adult Fish. Plos One. 18, e95294.

Majhi, S.K., Hattori, R.S., Yokota, M., Watanabe, S., Strüssmann, C.A., 2009. Germ cell transplantation using sexually competent fish: an approach for rapid propagation of endangered and valuable germlines. Plos One. 4, e6132.

Mylonas, C.C., Fostier, A., Zanuy, S., 2010. Broodstock management and hormonal manipulations of fish reproduction. Gen Comp Endocrinol. 165: 516-534.

Nagano, M., Avarbock, M.R., Brinster, L.R., 1999. Pattern and Kinetics of Mouse Donor Spermatogonial Stem Cell Colonization in Recipient Testes. Biology of Reproduction. 60, 1429–1436.

Nagano, M., Patrizio, P., Brinster, R.L., 2002. Long-term survival of human spermatogonial stem cells in mouse testes. Fertility and Sterility. 78, 1225-1233.

Nóbrega, R.H., Greebe, C.D., van de Kant, H., Bogerd, J., de França, L.R., Schulz, R.W., 2010. Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. Plos One 5, e12808.

Ohta, H., Yomojida, K., Yahamada, S., Okabe, M., Nishimune, Y., 2000. Real-time observation of transplanted 'green germ cell': Proliferation and differentiation of stem cells. Develop. Growth Differ. 42, 105-112.

Okutsu, T., Shikina, S., Kanno, M., Takeuchi, Y., Yoshizaki, G., 2007. Production of trout offspring from triploid salmon parents. Science 317, 1517.

Okutsu, T., Suzuki, K., Takeuchi, Y., Takeuchi, T., Yoshizaki, G., 2006. Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. Proc Natl Acad Sci USA. 103, 2725–2729.

Parreira, G.G., Ogawa, T., Avarbock, M.R., França, L.R., Brinster, R.L., Russell, L.D., 1998. Development of germ cell transplants in mice. Biol Reprod. 59, 1360-1370.

Saito, T., Goto-Kazeto, R., Arai, K., Yamaha, E., 2008. Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation. Biol. Reprod.78, 159-166.

Saito, T., Goto-Kazeto, R., Fujimoto, T., Kawakami, Y., Arai, K., Yamaha, E., 2010. Inter-species transplantation and migration of primordial germ cells in cyprinid fish. Int Journal Dev. Biol. 54, 1481-1486.

Saito, T., Goto-Kazeto, R., Kawakami, Y., Nomura, K., Tanaka, H., Adachi, S., Arai, K., Yamaha, E., 2011. The mechanism for primordial germ-cell migration is conserved between Japanese eel and zebrafish. Plos One. 6, e24460.

Sambrook, J., Russell, D., 2001. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory. p. 1885.

Sampaio, E.V., Sato, Y., 2006. Biologia reprodutiva e desova induzida de duas espécies de bagres (Osteichthyes: Siluriformes) da bacia do (Osteichthyes: Siluriformes) da bacia do rio São Francisco São Francisco. Acta Sci. Biol. Sci. 28, 263-268.

Shinohara, T., Kato, M., Takehashi M., Lee J., Chuma, S., Nakatsuji, N., Kanatsu-Shinohara, M., Hirabayashi, M., 2006. Rats produced by interspecies spermatogonial transplantation in mice and in vitro microinsemination. Proc Natl Acad Sci, USA. 12, 13624-13628.

Schulz, R.W., França, L.R., Lareyre, J.J., LeGac, F., Chiarini-Garcia, H., Nóbrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen Comp Endocrinol. 165: 390–411.

Takeuchi, Y., Higuchi, K., Yatabe, T., Miwa, M., Yoshizaki, G., 2009. Development of spermatogonial cell transplantation in Nibe croaker (*Nibea mitsukurii*). Biol Reprod 81, 1055–1063.

Takeuchi, Y., Yoshizaki, G., Takeuchi, T., 2003. Generation of live fry from intraperitoneally transplanted primordial germ cells in rainbow trout. Biol Reprod. 69, 1142–1149.

Takeuchi, Y., Yoshizaki, G., Takeuchi, T., 2004. Biotechnology: surrogate broodstock produces salmonids. Nature. 5, 629-30.

Vilela, D.A.R., Silva, S.G.B., Peixoto, M.T.D., Godinho, H.P., França, L.R., 2003. Spermatogenesis in teleost: insights from the nile tilapia (*Oreochromis niloticus*) model. Fish Physiol Biochem, 28, 187-190.

Wittbrodt, J., Shima, A., Schart, M., 2002. Medaka —A model organism from the far east. Nature Reviews Genetics. 3, 53-54.

Wong, T., Saito, T., Crodian, J., Collodi, P., 2011. Zebrafish germline chimeras produced by transplantation of ovarian germ cells into sterile host larvae. Biology of Reproduction. 84, 1190–1197.

Yazawa, R., Takeuchi, Y., Higuchi, K., Yatabe, T., Kabeya, N., Yoshizaki, G., 2010. Chub mackerel gonads support colonization, survival, and proliferation of intraperitoneally transplanted xenogenic germ cells. Biol Reprod. 82, 896-904.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T.L., 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. 13:134.

Yoshizaki, G., Fujinuma, K., Iwasaki, Y., Okutsu, T., Shikina, S., Yazawa, R., Takeuchi, Y., 2011. Spermatogonial transplantation in fish: A novel method for the preservation of genetic resources. Comparative Biochemistry and Physiology. 6, 55–61.

Yoshizaki, G., Ichikawa, M., Hayashi, M., Iwasaki, Y., Miwa, M., Shikina, S., Okutsu, T., 2010. Sexual plasticity of ovarian germ cells in rainbow trout. Development. 137, 1227-1230.

Yoshizaki, G., Okutsu, T., Morita, T., Terasawa, M., Yazawa, R., Takeuchi, Y., 2012. Biological characteristics of fish germ cells and their application to developmental biotechnology. Reprod. Domest. Anim. 47, 187–192.

Zhang, T., Rawson, D.M., Pekarsky, I., Blais, I., Lubzens, E., 2007. Low-temperature preservation offish gonad cells and oocytes. In: Babin, P.J., Cerda, J., Lubzens, E. (Eds.), The Fish Oocyte, from Basic Studies to Biotechnological Applications. Springer, pp. 412–432.

FIGURE LEGENDS

Figure 1 - Experimental design timeline. To deplete the endogenous spermatogenesis of recipient tilapia, the fish (n = 18) were kept at temperatures of 35°C for at least two weeks (A) before receiving intraperitoneally two busulfan injections (B; 18 mg/kg body weight and C; 15 mg/kg body weight), with a two-week interval between injections. Three weeks after the first busulfan injection, recipient tilapia received PKH26-labeled donor germ cells through the common spermatic duct (D). To evaluate the development (proliferation and differentiation) of donor germ cells in the recipient gonads, the testes were collected at specific time periods (E-I; from 7 to 120 days). In order to demonstrate the presence of Jundia catfish DNA in the recipient testis 140 days post germ cell transplantation, genetic analysis was also performed (J).

Figure 2 - Germ cell morphology and preparation of enriched spermatogonia cell suspension from Jundia catfish for transplantation. (A) Morphological illustration of germ cell cysts along spermatogenesis in adult Jundia: type A undifferentiated spermatogonia (Aund), type A differentiated spermatogonia (Adiff), type B early spermatogonia (Bearly), type B late spermatogonia (Blate), primary spermatocyte (SpcI), meiotic divisions (M); secondary spermatocyte (SpcII), spermatid (Sptd) and spermatozoa (Sptz) located in the tubular lumen. The nuclei of type A spermatogonia are much larger than the nuclei of all other germ cell types. For transplantation, donor germ cells were harvest from the testes of adult males (B) through enzymatic digestion. The cell suspension (C) was filtered and submitted to percoll gradient centrifugation. After 40% percoll, the pellet contained only erythrocytes and sperm (not shown), whereas the supernatant (D) contained type A spermatogonia (SpgA), spermatocytes (Spc), spermatids (Sptd) and sperm (Sptz). This cell suspension (D) was then subjected to the 35% percoll and the supernatant (E) showed a high concentration of type A

spermatogonia (SpgA); however, sperm (Sptz) and somatic cells (SC) were also observed. A further enrichment of type A spermatogonia was obtained through differential plating (F) for 12 hours: testicular somatic cells adhered firmly to the culture dish and hence were drastically reduced (G), whereas spermatogonia remained in suspension (H). Inset photos in G and H show the morphology of adherent (G`; somatic cells) and non-adherent (H`; type A spermatogonia) cells, after differential plating. Scale bars: A, C, D, and E = 10µm; F, G, and H = 15µm; B = 2cm; G` and H` (inserts) = 10µm.

Figure 3 - Jundia catfish spermatogonial cell labeling and transplantation. (A and B) Isolated donor spermatogonia were labeled with 9 µM red fluorescent dye (PKH26) and all cells in the brightfield (A) are strongly stained as shown in the fluorescent image (B). The inset photos in A and B at higher magnification show the typical PKH26 membrane-associated labeling. (C) Transplantation of PKH26-labeled Jundia spermatogonial cells into the adult Nile tilapia testis. Before transplantation, the cells were mixed with trypan blue and then injected using a glass micropipette (GM) into the common spermatic duct, located in the urogenital papilla (UGP). The dotted circle delimits the urogenital pore opening. Scale bars: A and B = 15µm; inserts = 7µM; and C = 3mm.

Figure 4 - Microscopic evaluation of recipient tilapia testes following one week and twenty days after Jundia catfish spermatogonial cells transplantation. At one-week (A-C) post-transplantation, labeled spermatogonial cells (white arrowheads) were observed in the tilapia seminiferous tubules epithelium. Twenty days (D-N) after transplantation, labeled spermatogonial cells were also identified in the seminiferous tubules epithelium

(white arrowheads). PKH26 labeled spermatogonial cells are observed in red (B, C, E, F, H, I, L, and M). Cell nuclei labeled in blue with DAPI are also visualized (A, C, D, F, G, I, K, and M). The brightfield images (J and N) correspond to the fluorescent images shown in I and M (merge images). The dashed lines delimit the tubular lumen (*). Scale bar = 50 µm in all images.

Figure 5 - Microscopic evaluation of recipient tilapia testis following seventy and ninety days after Jundia catfish spermatogonial cells transplantation. At seventy days (A-G) post-transplantation, labeled spermatocytes (white arrowheads) were observed in the tilapia seminiferous tubules epithelium. Ninety days (H-J) after transplantation, evident labeled spermatids were also present in the seminiferous epithelium (white arrowheads). PKH26 labeled spermatocytes (B-C and F-G) and spermatids (I-J) are observed in red. Cell nuclei labeled in blue with DAPI are also visualized (A, C, E, G, H, and J). The brightfield image (D) corresponds to the fluorescent image shown in C (merge image). The dashed lines delimit the tubular lumen (*). The insert (J´) shows PKH26 negative Sertoli cells (SC - white arrowhead) enveloping PKH26 positive spermatids. Scale bar = 50 µm in all images, except for the insert (5 µm).

Figure 6 - Microscopic evaluation of recipient tilapia testis following 120 days after Jundia catfish spermatogonial cells transplantation. In all images, labeled spermatozoa (white arrowheads) are observed in the tilapia seminiferous tubules lumen. PKH26 labeled spermatozoa (B, C, E, F, I, and J) are observed in red. Cell nuclei labeled in blue with DAPI are also visualized (A, C, D, F, H, and J). The brightfield images (G and K) correspond to the fluorescent images shown in F and J (merge images). The dashed lines delimit the tubular lumen (*). The inset photos (C´, F´, G´, J´, and K´) show at

higher magnification labeled Jundia spermatozoa. Scale bar = 50 µm in all images, except for the inserts (5 µm).

Figure 7 - Electrophoresis in 2% agarose gel for amplicons of the region corresponding to Rhq7 microsatellite sequence for *Rhamdia quelen* (Jundia) by nested PCR. Ladder molecular weight of 100 pb (lanes A and M); positive control with Jundia DNA (lane B); negative control with tilapia DNA (lane C); negative control with H_2O (lane D); and DNA from recipient Nile tilapia testis (lanes E-L). All recipient testes (lanes E-L) show the presence of Jundia genetic material (bands), but the amplicon bands display different intensities, suggesting different degrees of germ cell transplantation colonization/efficiency.

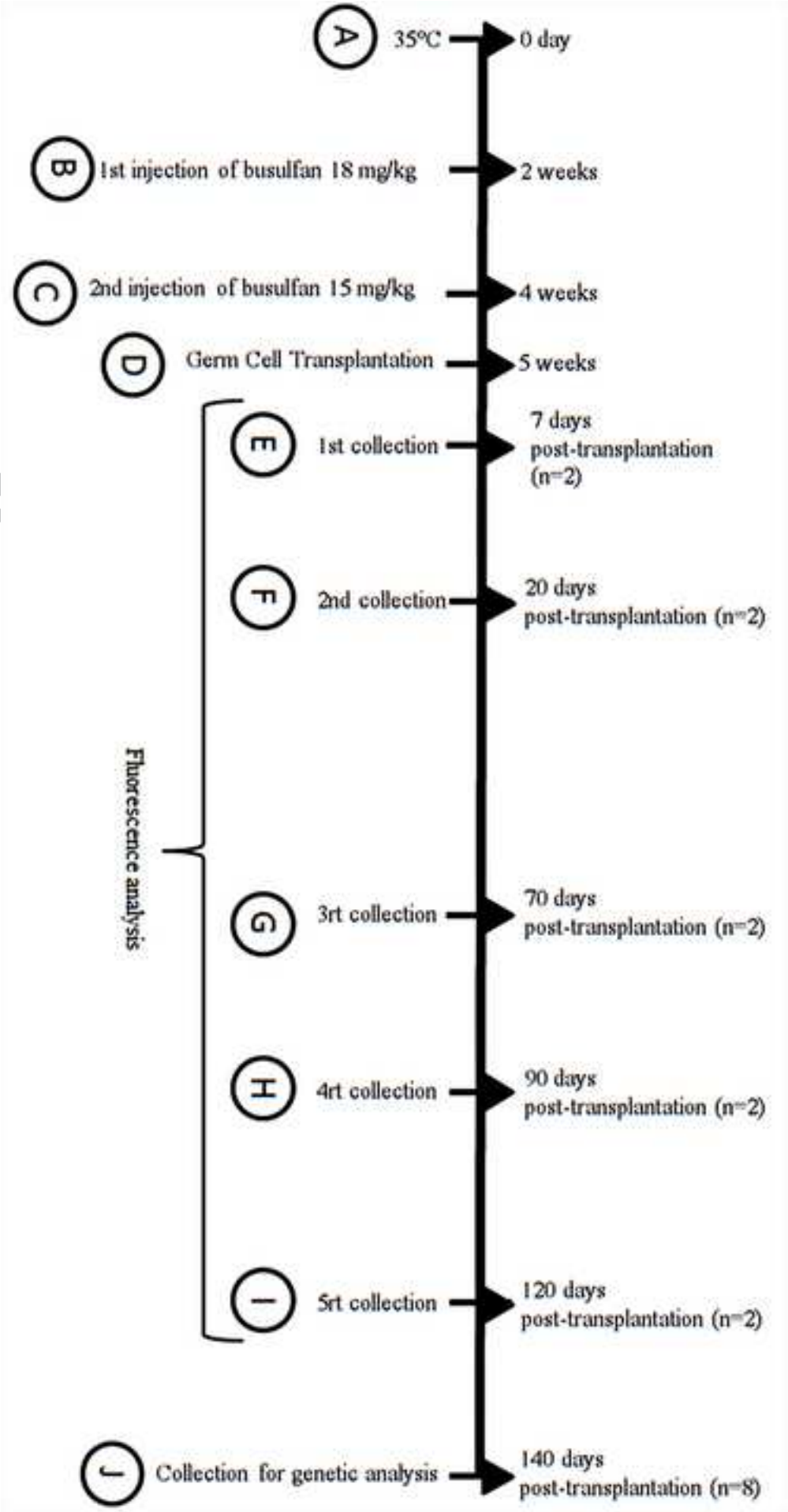


Figure 2

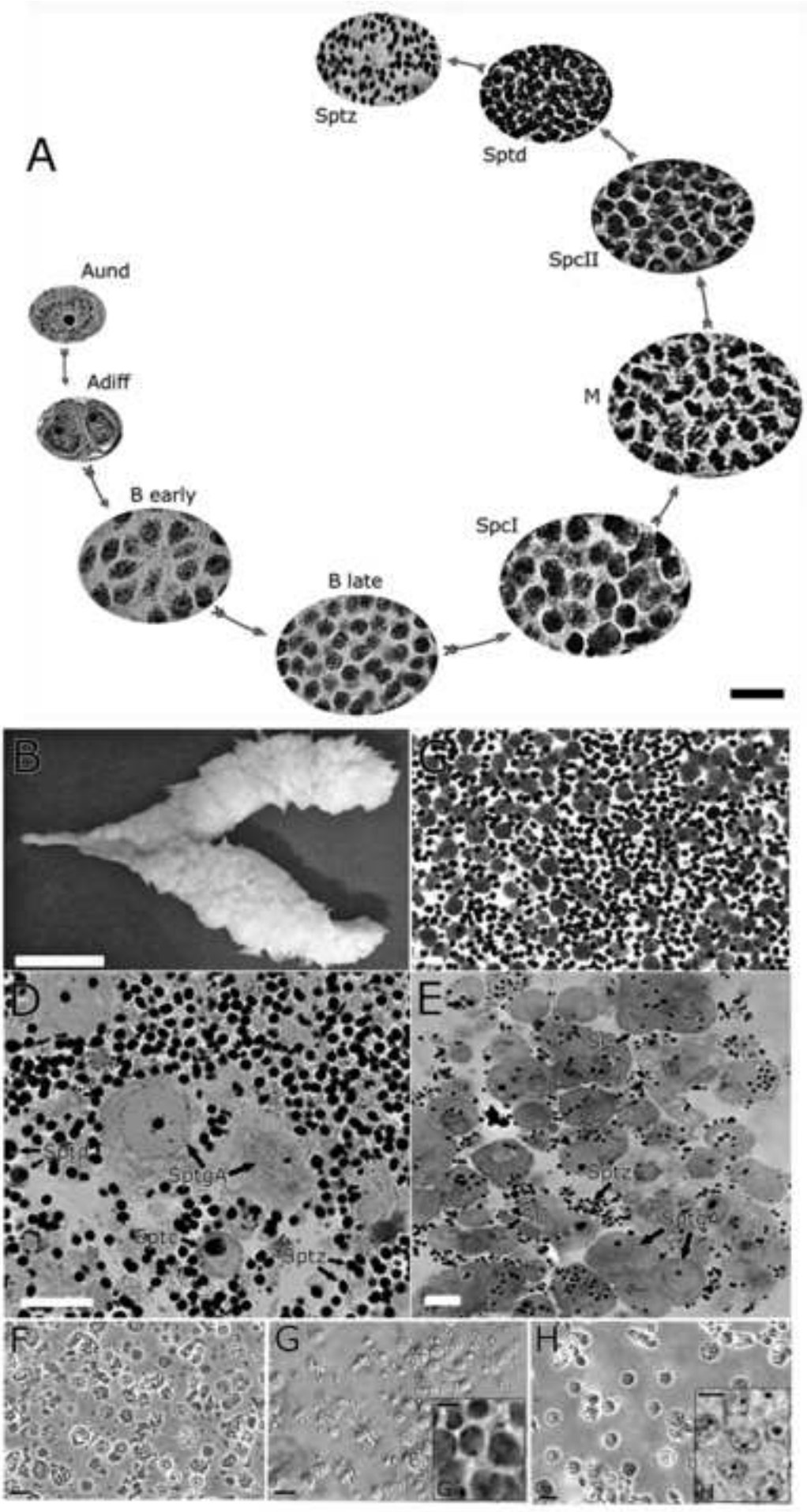


Figure 3

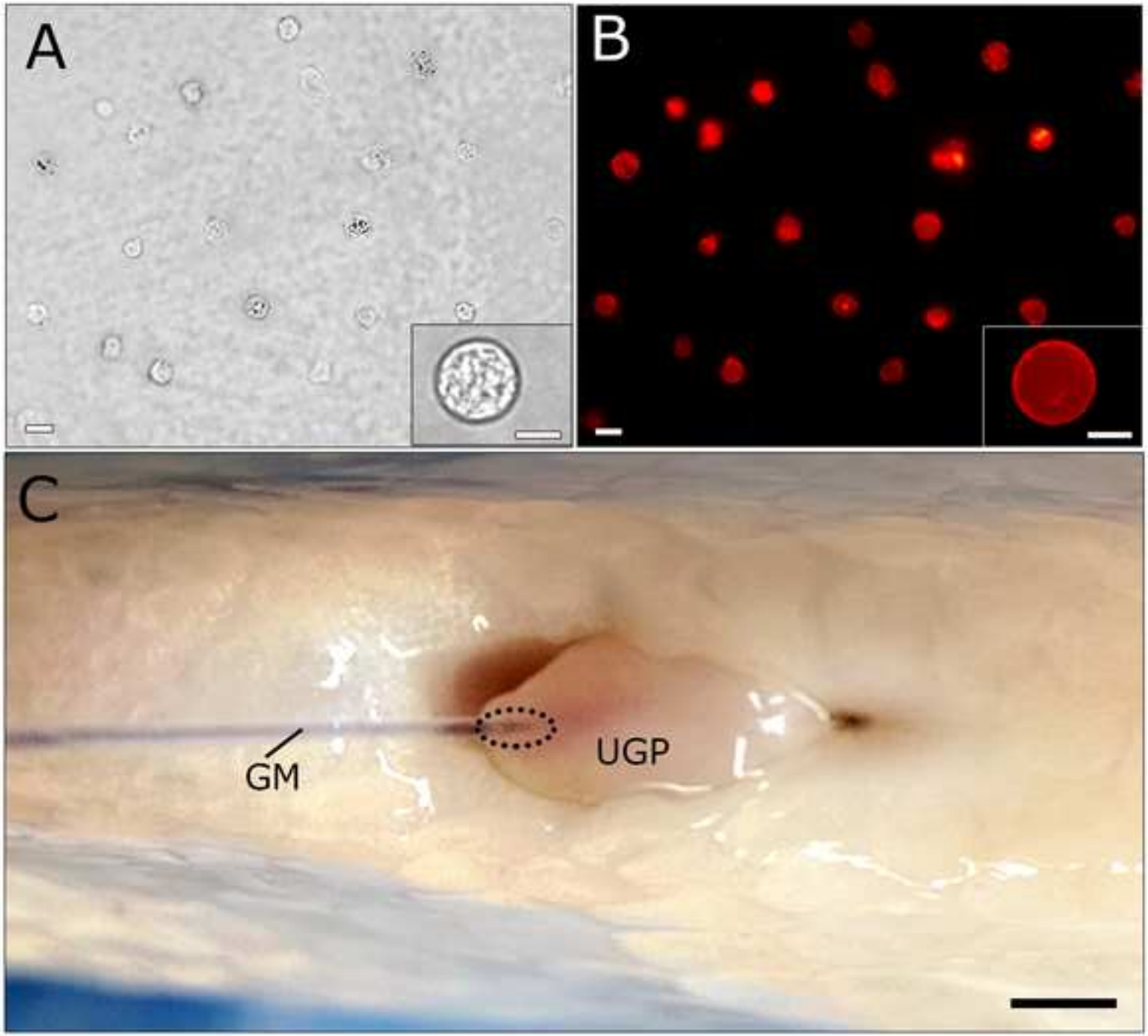


Figure 4

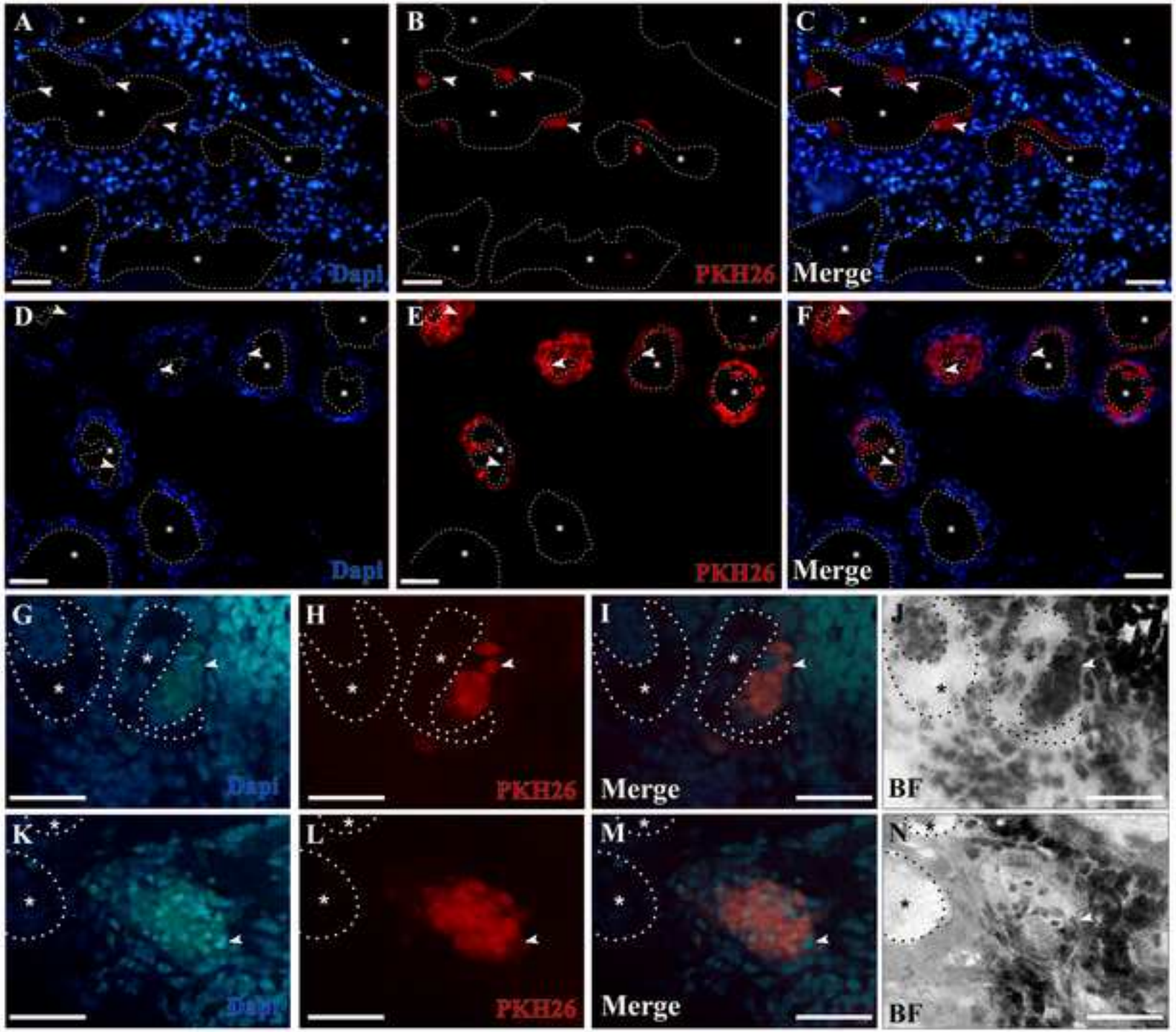


Figure 5

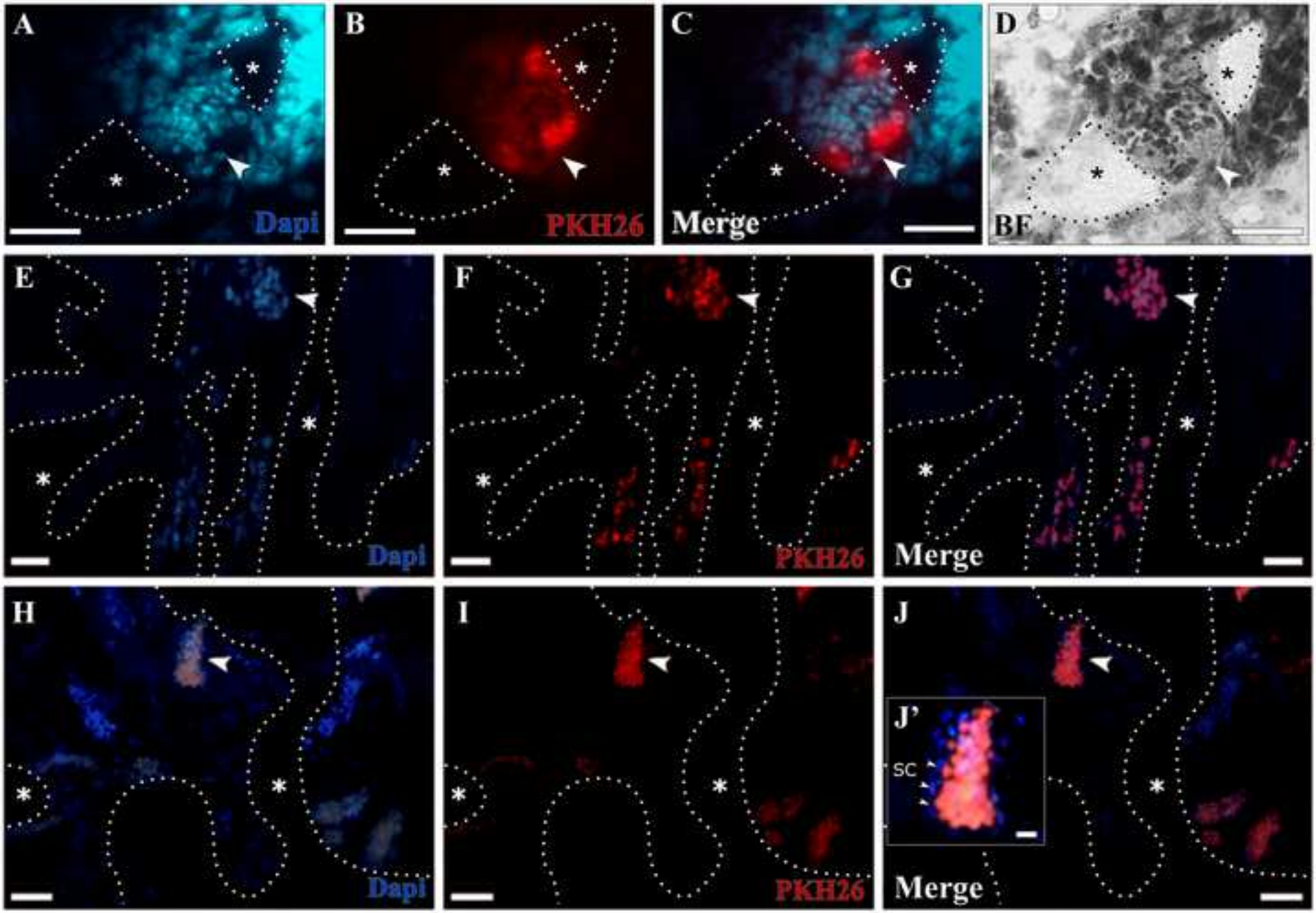


Figure 6

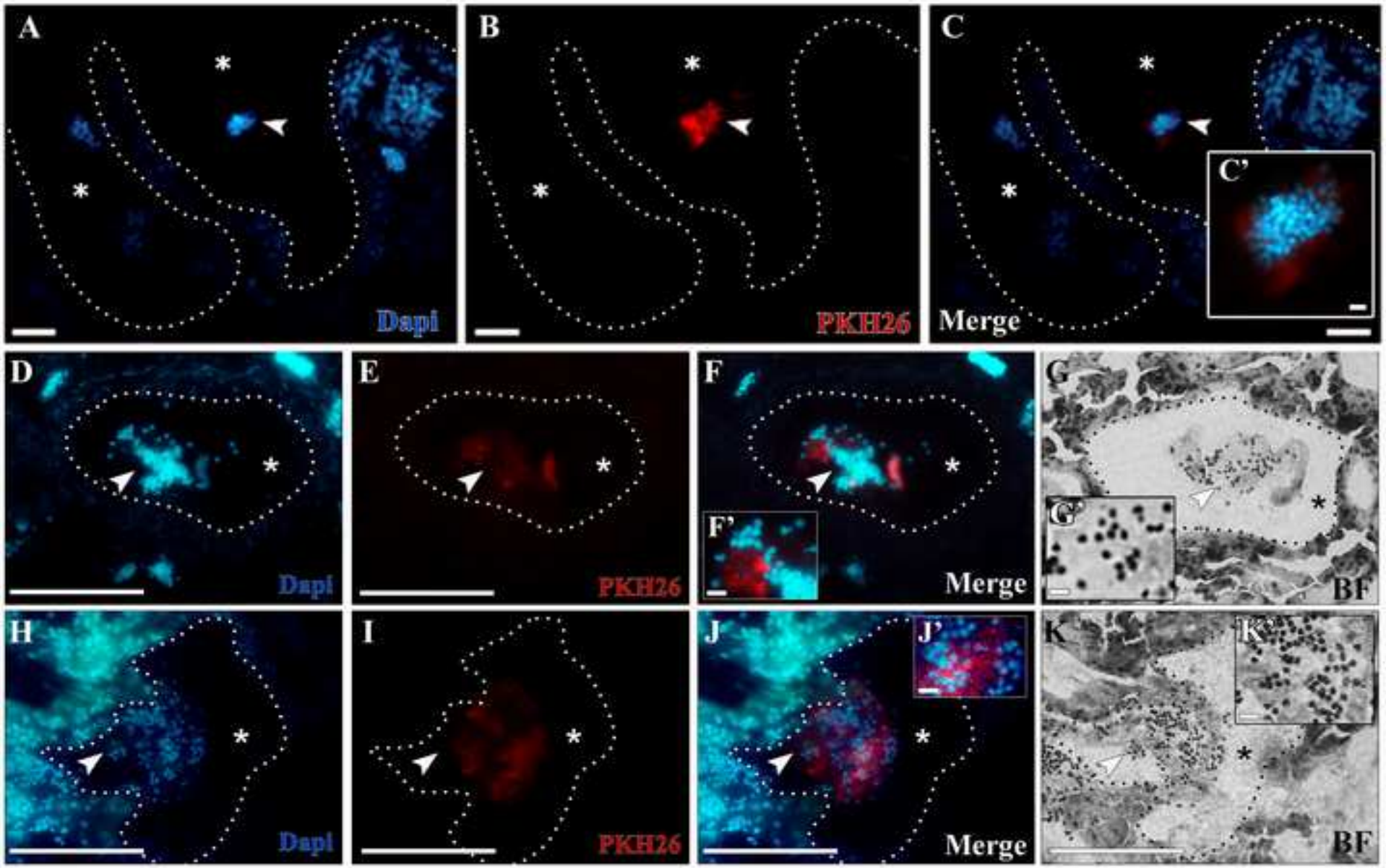


Figure 7

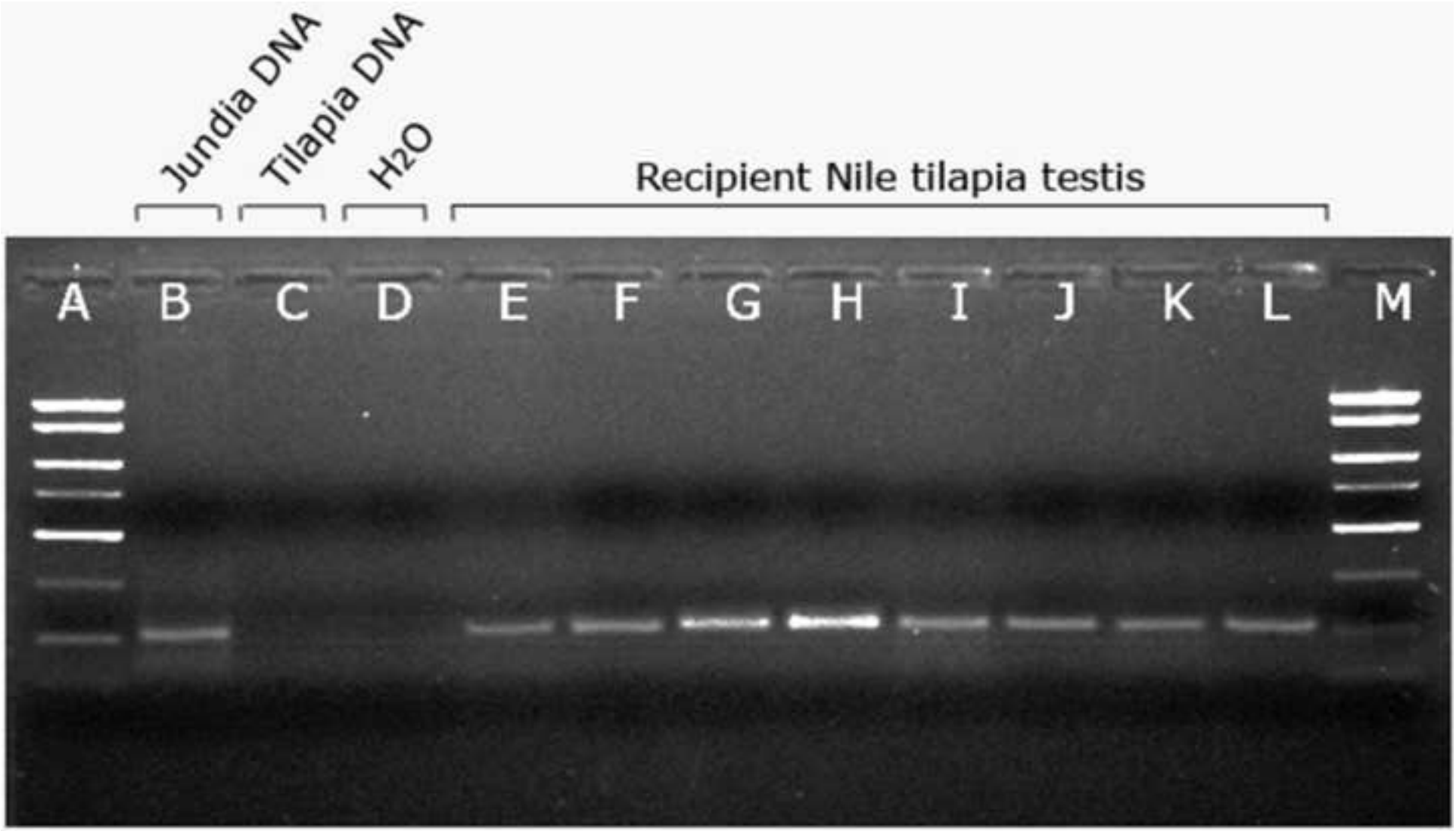


Table 1 - Primer pairs used in the genetic analysis to detect donor jundia catfish germ cells DNA in the Nile tilapia recipient testis.

Primer		Sequence
Outer	Forward	5'- GAC GCC TTT CTG GGG TCT AC -3'
	Reverse	5'- CTC TGC AGC CTC AGT CCT TC -3'
Middle	Forward	5'- GCC TTT CTG GGG TCT ACC TG -3'
	Reverse	5'- AGC CTC AGT CCT TCC AGA GA -3'
Inner	Forward	5'- GGA AGG TCT CTG TCC AAC AGG -3'
	Reverse	5'- TAA ACA CCC CTC GTA GCC CA -3'

Highlights:

- Tilapia testis offers proper environment for catfish germ cell transplantation.
- Jundia catfish spermatogonia are able to fully develop in the tilapia testis.
- Successful spermatogonial transplantation is observed using two different fish orders.

5. CAPÍTULO II

Fish Physiology and Biochemistry

Duration of spermatogenesis in Jundiá Catfish (Rhamdia quelen)

--Manuscript Draft--

Manuscript Number:	
Full Title:	Duration of spermatogenesis in Jundiá Catfish (Rhamdia quelen)
Article Type:	Short article
Keywords:	Jundiá catfish (Rhamdia quelen), germ cell morphology, duration of spermatogenesis, testis.
Corresponding Author:	Luiz Renato França, PhD, DVM Federal University Belo Horizonte, MG BRAZIL
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Federal University
Corresponding Author's Secondary Institution:	
First Author:	Mariana A Silva
First Author Secondary Information:	
Order of Authors:	Mariana A Silva
	Guilherme MJ Costa, PHD
	Samyra MSN Lacerda
	Luiz R França
Order of Authors Secondary Information:	
Funding Information:	CNPq — Dr Luiz R França
	FAPEMIG — Dr Luiz R França
Abstract:	In mammals the spermatogenic process lasts from 30 to 75 days, is under the control of the germ cell genotype, and has been generally considered to be constant for a given species. In teleosts, the duration of this process is usually much faster and is influenced by the temperature. Due to its fast growth, good adaptability to different environmental conditions, and economical importance, the jundiá catfish (Rhamdia quelen) emerges as an appropriate model to investigate testis structure and function in teleost. The optimal sperm production in this fish species occurs when they are kept at temperatures around 30°C. Here, we morphologically characterized germ cells along spermatogenesis and estimated the duration of this process through the injection of bromodeoxyuridine (BrdU), in jundiá kept at 30°C. Similar to other fish species, we observed a dramatic decrease of germ cell nuclear volume during spermatogenesis, particularly from type A undifferentiated to late type B spermatogonia and from diplotene to late spermatids. The most advanced germ cells observed at 1h, 3 and 5d after BrdU injection were respectively preleptotene, pachytene and secondary spermatocytes. Initial spermatids were found after 6d of BrdU exposure, whilst spermatozoa were observed in the seminiferous tubules lumen after 7d. Based on these observations, the combined duration of meiotic and spermiogenic phases in this species was very short and lasted approximately 7 days. Besides contributing to a better understanding of reproductive biology, we expect that the results herein obtained will help to improve the production and management of jundiá catfish.

Duration of spermatogenesis in Jundiá Catfish (*Rhamdia quelen*)

[1]Silva, M.A., [1]Costa G.M.J., [1]Lacerda S.M.S.N., [1,2,*]França L.R.

[1]Laboratory of Cellular Biology, Department of Morphology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil. [2]National Institute for Amazonian Research, Manaus, AM, Brazil.

*CORRESPONDING AUTHOR:
Dr. Luiz Renato de França
Phones: +55 (31) 96181992; +55 (92) 99605-5655
e-mails: lrfranca@icb.ufmg.br and lrfranca@inpa.gov.br

ABSTRACT

In mammals the spermatogenic process lasts from 30 to 75 days, is under the control of the germ cell genotype, and has been generally considered to be constant for a given species. In teleosts, the duration of this process is usually much faster and is influenced by the temperature. Due to its fast growth, good adaptability to different environmental conditions, and economical importance, the jundiá catfish (*Rhamdia quelen*) emerges as an appropriate model to investigate testis structure and function in teleost. The optimal sperm production in this fish species occurs when they are kept at temperatures around 30°C. Here, we morphologically characterized germ cells along spermatogenesis and estimated the duration of this process through the injection of bromodeoxyuridine (BrdU), in jundiá kept at 30°C. Similar to other fish species, we observed a dramatic decrease of germ cell nuclear volume during spermatogenesis, particularly from type A undifferentiated to late type B spermatogonia and from diplotene to late spermatids. The most advanced germ cells observed at 1h, 3 and 5d after BrdU injection were respectively preleptotene, pachytene and secondary spermatocytes. Initial spermatids were found after 6d of BrdU exposure, whilst spermatozoa were observed in the seminiferous tubules lumen after 7d. Based on these observations, the combined duration of meiotic and spermiogenic phases in this species was very short and lasted approximately 7 days. Besides contributing to a better understanding of reproductive biology, we expect that the results herein obtained will help to improve the production and management of jundiá catfish.

KEYWORDS: Jundiá catfish (Rhamdia quelen), germ cell morphology, duration of spermatogenesis, testis.

INTRODUCTION

Spermatogonial stem cells are the foundation of spermatogenesis and male fertility in eukaryotes and their development is functionally supported by somatic cells (Oatley and Brinster 2012; Lacerda et al. 2014). In vertebrates, fish differ from amniotes (mammals, birds, and reptiles) by having a cystic type arrangement of spermatogenesis where germ cells are nurtured by mitotically active Sertoli cells (Schulz et al. 2010; França et al. 2014, 2016). Therefore, cyst formation is initiated when the cytoplasmic extensions of Sertoli cells surround a spermatogonium (Uribe et al. 2015; França et al. 2016) and a group of germ cells comprise a single syncytium that synchronously divides (incompletely) and differentiate into the male gamete (Yoshida, 2016). Also in this vertebrate group, the number of spermatogonial mitotic divisions is quite high, considered species-specific, and phylogenetically determined (Nóbrega et al. 2009; Schulz et al. 2010). Thus, in association with the loss of a fairly low number of germ cells during progression of spermatogenesis and low of Sertoli cells enveloping germ cells, hundreds of meiotic and post meiotic germ cells are usually present in a cyst (Vilela et al. 2003; Leal et al. 2009; Schulz et al. 2010; França et al. 2015). These morphofunctional aspects result in a rather high Sertoli cell efficiency or support capacity for germ cells. Thus, a very high number of sperm are produced from a single spermatogonium (Schulz et al. 2005; Schulz et al. 2010; França et al. 2014, 2016).

It is well established in the literature that the duration of spermatogenesis is very important in determining the speed of sperm formation and hence spermatogenic efficiency and reproductive strategies (Hess and França, 2007). According to studies developed in mammals using xenogenic (rats to mice) germ cells transplantation, it was found that the duration of spermatogenesis is determined by the germ cell genotype (França et al. 1998). In most mammalian species already investigated, the total duration of spermatogenesis, that is species-specific, takes from 40 to 60 days (Hess and França 2007). In fish, however, data from the literature show that the duration of this process is usually much faster (Vilela et al. 2003; Lacerda et al. 2006; Leal et al. 2009; Nóbrega et al. 2009). Moreover, although it is classically considered that the duration of spermatogenesis is not influenced by any factor or agent, in fish and amphibian there is strong evidence that the pace of spermatogenesis is altered by temperature (Morgan 1979; Lacerda et al. 2006).

The *Rhamdia quelen*, also known as jundiá, is a catfish species that is an important source of protein for human consumption in some regions of Brazil (Sampaio and Sato

2006). This catfish species reaches 600 to 800 g of body mass in 8 months, growing faster in the warm summer months (Barcelos et al. 2002). The literature is deficient in data regarding reproduction of *Rhamdia quelen*, particularly those related to testis function and spermatogenesis. Therefore, in order to better understand the reproductive biology and to help with future studies of production and management in this species, we performed histological and stereological investigations of the germ cells, focused mainly on an estimate of the duration of spermatogenesis.

MATERIALS AND METHODS

Animals

Ten sexually mature jundiá catfish (*Rhamdia quelen*; mean body weight = 200g) were used to estimate the duration of spermatogenesis and to perform germ cell histomorphometric analysis during the spawning season (September to March). These fish were obtained from commercial farmers located nearby Belo Horizonte city (state of Minas Gerais, Southeastern Brazil). All procedures used followed approved guidelines for the ethical treatment of animals and national laws (CEUA; #89/2012).

Experimental design and tissue preparation

After 1 week of acclimation, the fishes were kept at a water temperature of 30°C, which is considered the optimal temperature for this fish species to reproduce in captivity conditions (Gomes et al. 2000). After that, they received a single intracelomic BrdU (Sigma) injection as a marker of cells that were synthesizing DNA at the time of injection. The fish were weighed and killed at 1 hour and 3, 5, 6 and 7 days after the injection (Figure 1). After euthanasia, the testis samples were fragmented (2–3 mm in thickness), fixed and embedded according to the analysis to be performed. Therefore, for germ cell histological and morphometric evaluation, testis fragments were fixed by immersion in 5% buffered glutaraldehyde and embedded in glycol methacrylate (Leica, Wetzlar, Germany). To estimate the duration of spermatogenesis, testis fragments were fixed in Bouin's solution (Merk, Kenilworth, New Jersey, United States) and embedded in Paraplast® Tissue Embedding Media (Sigma-Aldrich, St. Louis, Missouri, United States).

Histological and morphometric analysis

In order to evaluate the germ cell nuclear volume, fifty nuclei were measured for each germ cell type investigated per fish. Because the nucleus of all germ cells investigated in Jundiá catfish is spherical or nearly round, their nucleus volume was estimated using the mean nuclear diameter. Individual nuclear volume was expressed (in cubic micrometers) and obtained using the formula $4/3\ \pi\ R3$, where R = nuclear diameter/2. The morphometric data obtained allowed us to estimate the changes in nucleus volume that occur during the spermatogenic process, from undifferentiated spermatogonia to mature spermatids. Images were captured through Cell F software (Olympus) using 40X and 100X oil immersion objectives in an Olympus BX60 Microscope.

BrdU incorporation detection

Five-micrometer-thick sections were subjected to antigen retrieval (1% [v/v] periodic acid in water at 60°C for 30 min and peroxidase blocking (1% [v/v] H_2O_2 in PBS for 10 min). Subsequently, slides were incubated at room temperature for 1 h with mouse anti-BrdU (1:100 [BD Biosciences, San Jose, CA] diluted in PBS containing 1% [w/v] bovine serum albumin [BSA; Sigma-Aldrich, St. Louis, MO]), and then for an additional hour with biotinylated horse anti-mouse (1:100 [Vector Laboratories, Burlingame, CA] diluted in PBS containing 1% [w/v] BSA). Detection of immunostaining was done using avidin-biotin complex incubation for 1 h (Vector Laboratories) followed by diaminobenzidine (Dako, Glostrup, Denmark) substrate development for 20 sec. Nuclei were counterstained with hematoxylin Gill no. 3 (Sigma-Aldrich) for 30 sec. For negative control, the primary antibody (mouse anti-BrdU) was omitted. After these procedures, the most advanced BrdU labeled germ cells at each time period considered was evaluated.

RESULTS

Germ Cell Characterization

As shown in Figure 2 and 3, type A undifferentiated spermatogonia (Aund) are single cells involved by Sertoli cells and are the largest germ cell types observed in jundiá catfish testis. These cells also present a large nucleus containing low condensed chromatin and one compact nucleolus. Aund spermatogonia mitotically divide and give rise to type A differentiated spermatogonia (Adiff) that still share some morphological characteristics with Aund. However, they exist in groups inside the cyst and are smaller than the type

Aund spermatogonia. Type B spermatogonia are drastically smaller that type A and present an elliptical/round nucleus with one or two small nucleoli and an evident larger amount of heterochromatin compared to both type Aund and Adiff spermatogonia. Type B spermatogonia are present in several generations, therefore their number per cyst increases markedly, while their size decreases continually. For illustrative purpose only early (Bearly), intermediate (Bint) and late (Blate) type B spermatogonia are here in shown.

All spermatogonial cells together, from Aund/Adiff to typeB, compose what is morphofunctionally defined as spermatogonial phase of spermatogenesis. After the large expansion of the number of spermatogonial cells, the last type B spermatogonial generation mitotically divides to form preleptotene spermatocytes (Pl), whose size and morphology resemble their predecessor cells and constitute the beginning of the meiotic phase of spermatogenesis. After that, these cells progress through meiosis and grow continually forming leptotene/zygotene (L/Z), pachytene, (P), diplotene (D) spermatocytes. Because the morphological characteristics of these cells are very similar in vertebrates, we will not describe them here. Also, due to their usually long duration in vertebrates, pachytene spermatocytes are the most frequent meiotic germ cells observed in the seminiferous tubules. At the end of meiosis I, diplotene spermatocytes divide to form secondary spermatocytes (S), which quickly go through meiosis II and divide forming the haploid spermatids. Due to their usually very short duration, the presence of secondary spermatocytes cysts are quite rare.

As it occurs in fish in general, in the last phase of spermatogenesis (spermiogenic) a remarkable reduction in the cellular volume is observed. Based on the increasing nuclear compaction, round spermatids in jundiá catfish could be didactically classified in three different types called initial (E1), intermediate (E2) and final (E3) spermatids.

BrdU labeling

The most advanced labeled germ cells type following BrdU injection, after different periods, are shown in Figure 4. As it can be noted in this figure, these labeled cells were preleptotene spermatocytes (at one hour; Fig. 4A), pachytene spermatocytes (at 3 days; Fig. 4B), diplotene spermatocytes (at 5 days; Fig. 4C), spermatids (at 6 days; Fig. 4D), and spermatozoa in the tubular lumen (at 7 days; Fig. 4E). Figure 4F summarizes these findings. Based on these observations and in the temperature used (30°C), the

combined duration of meiotic and spermiogenic phases (from preleptotene to spermatozoa) in jundiá catfish could be estimated as lasting approximately 7 days.

DISCUSSION

Knowledge of the germ cell differentiation steps and the duration of spermatogenesis are very useful for understanding reproductive biology, in particular the functional and regulatory mechanisms of spermatogenesis and reproductive strategies (Nóbrega et al. 2009). Jundiá catfish cultivation have been steadily increasing in South America, particularly in southern Brazil (Baldisserotto and Radünz Neto 2004; Carneiro and Mikos 2005; Canton et al. 2007). However because several biological parameters for this species are not available, including those related to reproductive physiology, jundiá production is still far below its potential (Carneiro and Mikos 2005). Here, for the first time, we describe germ cell morphology and the duration of spermatogenesis for this species during the spawning season. As for most fish species, our results show that the combined duration of meiosis and spermiogenesis in jundiá catfish is very fast and lasts approximately one week.

Although some specific molecular markers for fish germ cells have been described (reviewed by Lacerda et al. 2014; Ozaki et al. 2011; Bellaiche et al. 2014), these cells are mainly characterized by morphological criteria (Nóbrega et al. 2010; Uribe et al. 2015). Overall, the morphological characteristics of the different jundiá germ cell types are very similar to those observed for other teleost species already investigated (Grier 1981; Selman and Wallace 1986; Billard 1990; Le Gac and Loir 1999; Miura 1999; Grier and Neidig 2000; Leal et al. 2009; Schulz et al. 2010). Also, in jundiá catfish undifferentiated spermatogonia are the largest germ cells observed and a remarkable decrease in germ cell nuclear diameter was observed from type Aund spermatogonia to late type B spermatogonia, as well as from pachytene/diplotene to final spermatids. This trend is quite similar to other fish species investigated in this aspect (Almeida et al. 2008; Melo et al. 2014), including data obtained in our laboratory for tilapia (Schulz et al. 2005) and zebrafish (Leal et al. 2009).

The literature shows that the jundiá is capable of adjusting to a wide range of environmental temperatures (Lermen et al. 2004). However, it has been found that in captivity conditions, this species presents a better development and higher growth rate in a temperature around 30°C (Andrews et al. 1972; Andrews and Stickney 1972; Gomes et al. 2000). In this condition, in the present study we found that the combined duration of

meiotic and spermiogenic phases of spermatogenesis was rather fast and lasted approximately one week. This value was similar to those found for other fish species investigated in our laboratory, such as the Nile tilapia kept in temperatures between 27°C and 30°C (Silva and Godinho 1983; Vilela et al. 2003) and zebrafish kept at 20°C (Nóbrega et al. 2010).

Several environmental factors are known to influence fish sperm production and spermatogenesis (Alvarenga and França 2009; Schulz et al. 2010). However, temperature is one of the most prevalent environmental factors capable of affecting reproductive physiology of aquatic wildlife (Pankhurst and Munday 2011). For comparative purpose, a data compilation related to the duration of spermatogenesis (meiotic and spermiogenic phases) of several fish species kept at different temperatures is shown in Table 1. It is noteworthy that in cold-water fish exposed to lower temperatures (<10°C) (group A), the duration of spermatogenesis is much longer, lasting more than 4 weeks. On the other hand, in fish kept in temperatures ranging from 20 to 25°C (group B), the time period from the appearance of early spermatocytes to the formation of sperm is about 2 to 3 weeks. At higher temperatures (27 to 30°C; group C), the time required to form spermatozoa, including the jundiá catfish investigated in the present study, is in the range of 1 to 2 weeks. Whereas, in temperatures around 35°C (ex: Nile tilapia; group D), the time required to form sperm is less than 1 week. Therefore, independent of the fish species considered, water temperature exerts a high influence on the spermatogenesis, either an acceleration or a delay in the process. A very clear illustration of this aspect are the data shown for zebrafish and Nile tilapia for which the duration of spermatogenesis changed according to the experimental temperature used. Taking advantage of this knowledge and showing a practical application of these findings, in order to deplete endogenous spermatogenesis before germ cells transplantation, in association with busulfan treatment, we experimentally manipulated (increased) the water temperature where tilapias and zebrafish were kept (Lacerda et al. 2006; Nóbrega et al. 2010). In these conditions, spermatogenesis was accelerated and the germ cells became more vulnerable to the antineoplastic drug used (Lacerda et al. 2006; Alvarenga et al. 2009). These approaches were also used in other studies (Majhi et al. 2009; Majhi et al. 2014).

Using Nile tilapia as an experimental fish model we also demonstrated that higher temperatures (30°C and 35°C) resulted in germ cell differentiation, as well as faster and larger sperm production (Alvarenga and França 2009). In contrast, lower temperature (20°C) triggered self-renewal of type A spermatogonia, Leydig cell and Sertoli cell

proliferation, apoptosis of more advanced germ cells, and arrest of spermatogenesis (Vilela et al. 2003; Alvarenga and França 2009). These findings again illustrate the effects of higher temperatures on the acceleration of spermatogenesis and provide some clues for reproductive strategies. However, it is interesting that there is a physiological limit for this acceleration. Because, in comparison to a water temperature of 30°C, in which only two days is enough to form sperm in zebrafish, the temperature of 35°C promotes spermatogenic arrest (Table 1) (Nóbrega et al. 2010), whereas such arrest in Nile tilapia occurs at a low temperature (20°C; Vilela et al. 2003). In mammals, that are homoeothermic, the duration of each phase of spermatogenesis (spermatogonial, meiotic and spermiogenic) is similar (reviewed by Bittman 2016), whereas in fish and amphibian the spermiogenic phase is much faster (Segatelli et al. 2009). Surely, in order to better understand the molecular mechanism involved in the determination of the germ cell pace of development during spermatogenesis, as well the role of temperature in this very important reproductive parameter in fish, more studies are necessary.

ACKNOWLEDGEMENTS

Technical help from Mara Lívia Santos is highly appreciated. The assistance from the local farmers, José Geraldo da Silva and Marco Túlio Diniz Peixoto, was of great value. The scholarships awarded to M.A.S. and S.M.S.N.L. and the financial support from the FAPEMIG and CNPq are fully appreciated.

REFERENCES

1. Almeida FFL, Kristoffersen C, Taranger GL, Schulz RW (2008). Spermatogenesis in Atlantic Cod (Gadus morhua): A Novel Model of Cystic Germ Cell Development. Biol Reprod. 78:27-34

2. Alvarenga ER, França LR (2009) Effects of different temperatures on testis structure and function, with emphasis on somatic cells, in sexually mature nile tilapias (Oreochromis niloticus). Biol Reprod. 80:537–544

3. Andrews JW, Knight LH, Murai T (1972). Temperature Requirements for High Density Rearing of Channel Catfish from Fingerling to Market Size. The Progressive Fish-Culturist. 34: 240-241

4. Andrews JW, Stickney RR (1972) Interactions of feeding rates and environmental temperature on growth, food conversion, and body composition of channel catfish. Trans Am Fish Soc. 101:94-99.

5. Baldisserotto B, Radünz Neto J (2004). Criação de Jundiá. UFSM, Santa Maria

6. Barcelos LJG, Wassermann GF, Scott AP, Woehl VM, Quevedo RM, Ittizés I, Krieger MH, Lulhier F (2002). Plasma esteroid concentrations in relation to the reproductive cycle of cultured male Rhamdia quelen. J Fish Biol.. 61:751-763

7. Bellaiche J, Lareyre JJ, Cauty C, Yano A, Allemand I, Le Gac F (2014) Spermatogonial stem cell quest: nanos2, marker of a subpopulation of undifferentiated A spermatogonia in trouttestis. Biol Reprod. 79:90-94

8. Billard R (1990). Spermatogenesis in teleost fish. In: Lamming Ge. (ed) Reproduction in Males. Churchill Livingstone, Edinburgh, pp 183:212

9. Bittman EL (2016). Timing in the Testis. Journal of Biological Rhythms. doi:10.1177/0748730415618297

10. Canton R, Weingartner M, Fracalossi DM, Zaniboni Filho E (2007). Influência da freqüência alimentar no desempenho de juvenis de jundiá. R. Bras. Zootec. 36:749-753

11. Carneiro PCF, Mikos JD (2005). Freqüência alimentar e crescimento de alevinos de jundiá, Rhamdia quelen. Cienc. Rural. 35:187-191

12. França LR, Hess RA, Dufour JM, Hofmann MC, Griswold MD (2016). The Sertoli cell: one hundred fifty years of beauty and plasticity. Andrology. doi: 10.1111/andr.12165

13. França LR, Nóbrega RH, Morais RDVS, Assis LHC, Schulz RW (2015) Sertoli cell structure and function in anamniote vertebrates In: Griswold MD (ed) Sertoli cell biology, 2nd edn. Elsevier Academic Press, San Diego, pp 385-407

14. França LR, Russel LD (1998). The testis of domestic animals. In: Male Reproduction. Martínez-García F, Regadera J (ed) Churchill Livingstone, Madrid, pp 197-219

15. Gomes LC, Golombiesk JI, Chippari AR, Baldisserotto B (2000). Biologia do jundiá - Rhamdia quelen (Teleostei, Pimelodidae) Cienc. Rural. 30:179-185

16. Grier H, Neiding C (2000). Gonads and gametes of fishes. In: Tiersch TR, Mazik PM (ed) Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, pp. 1-12

17. Grier HJ (1981). Cellular organization of testis and spermatogenesis in fishes. Am Zool. 21: 345-357.

18. Hess RA, França LR (2007). Spermatogenesis and cycle of the seminiferous histopathological evaluation of the testis. In: Molecular Mechanisms in Spermatogenesis, Clearwater: Cache River Press, Texas, pp 1-15

19. Lacerda SMSN, Batlouni SR, Silva SBG, Homem CSP, França LR (2006). Germ cells transplantation in fish: the Nile tilapia model. Anim. Reprod. 2:146–159

20. Lacerda SMSN, Costa GM, França LR (2014). Biology and identity of fish spermatogonial stem cell. Gen Comp Endocrinology. 207:56-65

21. Le Gac F, Loir M (1999). Male reproductive system, fish. In: Knobil E, Neill Jd (eds). Encyclopedia of Reproduction. Academic Press, San Diego, pp 20-30

22. Leal MC, Cardoso ER, Nóbrega RH, Batlouni SR, Bogerd J, França LR, Schulz RW (2009). Histological and stereological evaluation of zebrafish (Danio rerio) spermatogenesis with an emphasis on spermatogonial generations. Biol Reprod. 81:177-187.

23. Lermen CL, Lappe R, Crestani M, Vieira VP, Gioda CR, Schetinger MRC, Baldisserotto B, Moraes G, Morsch VM (2004). Effect of different temperature regimes on metabolic and blood parameters of silver catfish Rhamdia quelen. Aquaculture. 239:497–507

24. Majhi SK, Hatto RS, Rahman, SM, Strüsmann CA (2014). Surrogate Production of Eggs and Sperm by Intrapapillary Transplantation of Germ Cells in Cytoablated Adult Fish. PLoS One. 18:e95294

25. Majhi SK, Hattori RS, Yokota M, Watanabe S, Strüsmann CA (2009). Germ cell transplantation using sexually competent fish: an approach for rapid propagation of endangered and valuable germlines. PLoS One. 4:e6132.

26. Melo MC, Andersson E, Fjelldal PG, Bogerd J, França LR, Taranger GL, Schulz RW (2014). Salinity and photoperiod modulate pubertal development in Atlantic salmon (Salmo salar). J Endocrinol. 220:319-332

27. Miura T, Miura C, Ohta T, Nader MR, Todo T, Yamauchi (1999). Estradiol-17β Stimulates the Renewal of Spermatogonial Stem Cells in Males. Biochem Bioph Res Co. 264:230-234

28. Morgan GT (1979). The time course of male meiosis in the red-backed salamander, Plethodon cinereus. J Cell Sci. 38:345-356

29. Nóbrega RH, Batlouni SR, França LR (2009). An overview of functional and stereological evaluation of spermatogenesis and germ cell transplantation in fish. Fish Physiol Biochem. 35:197-206

30. Nóbrega RH, Greebe CD, Van De Kant H, Bogerd J, França, LR, Schulz, RW (2010). Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. PLoS One 5:e12808

31. Oatley JM, Brinster RL (2012). The germline stem cell niche unit in mammalian testes. Physiol Rev. 2:577-595

32. Ozaki Y, Saito K, Shinya M, Kawasaki T, Sakai N (2011). Evaluation of Sycp3, Plzf and Cyclin B3 expression and suitability as spermatogonia and spermatocyte markers in zebrafish. Gene Expression Patterns. 11:309-315

33. Pankhurst AC, Philip L, Munday B (2011). Effects of climate change on fish reproduction and early life history stages Ned W. Marine and Freshwater Research. 62:1015-1026

34. Sampaio EV, Sato Y (2006). Biologia reprodutiva e desova induzida de duas espécies de bagres (Osteichthyes: Siluriformes) da bacia do rio São Francisco. Acta Sci. Biol. Sci. 28: 263-268

35. Schulz RW, Franca, LR, Lareyre JJ, Le Gac F, Chiarini-Garcia H, Nóbrega RH, Miura T (2010). Spermatogenesis in fish. Gen Comp Endocrinol. 165:390-411

36. Schulz RW, Menting S, Bogerd J, França LR, Vilela DA, Godinho HP (2005). Sertoli cell proliferation in the adult testis evidence from two fish species belonging to different orders. Biol Reprod. 73:891-898

37. Segatelli TM, Batlouni SR, França LR (2009). Duration of spermatogenesis in the bullfrog (Lithobates catesbeianus). Theriogenology. 72:894-901

38. Selman K, Wallace RA (1986). Gametogenesis in Fundulus heteroclitus. Amer. Zool. 26:173-192

39. Silva M, Godinho HP (1983). Timing of some events of the gametogenesis in the male nile tilapia, Sarotherodon niloticus. Archives d'anatomie microscopique 72:23l-277

40. Uribe MC, Grier HJ, Mejía-Roa V (2015). Comparative testicular structure and spermatogenesis in bony fishes. Spermatogenesis. 23:e983400.

41. Vilela DAR, Silva SGB, Peixoto MTD, Godinho HP, França LR (2003). Spermatogenesis in teleost: insights from the nile tilapia (Oreochromis niloticus) model. Fish Physiol Biochem. 28:187-190

42. Yoshida S (2016). From cyst to tubule: innovations in vertebrate spermatogenesis. Wiley Interdiscip Rev Dev Biol. 5:119-131

FIGURE LEGENDS

Figure 1. Diagram showing the experimental timeline for BrdU injection in jundia Rhamdia quelen. The animals were sacrificed at 1 hour and at 3, 5, 6, and 7 days after BrdU injection.

Figure 2. Different germ cell cysts observed during the progression of spermatogenesis in jundia Rhamdia quelen from a type A undifferentiated spermatogonium to late spermatids. Undifferentiated type A spermatogonium (Aund; pressumably stem cell) (A); type A differentiated spermatogonia (Adiff) (B); early type B spermatogonia (Bearly) (C); inetrmediate type B spermatogonia (Bint) (D); late type B spermatogonia (Blate) (E); primary spermatocytes (F-I) in preleptotene (Pl), in the transition from leptotene to zygotene (L/Z), pachytene (P), diplotene (D), and meiotic figures from the first (MI) and second (MII) meiotic division; secondary spermatocytes (SS) (J); spermatids (K–M) at the initial (E1), intermediate (E2), and final (E3) steps of development. Bars = 10 μm.

Figure 3. Germ cell nuclear volume in jundia Rhamdia quelen. As it can be noted, type A undifferentiated spermatogonial cells (Aund) are the largest cell. During the progression of spermatogenesis, the germ cell nuclear volume shows a dramatic reduction. Aund, type A undifferentiated spermatogonia; Adif, type A differentiated spermatogonia; Bearly, early type B spermatogonia; Blate, late type B spermatogonia; Pl, preleptotene spermatocyte; L/Z, transition from leptotene to zygotene spermatocyte; P, pachytene spermatocyte; D, diplotene spermatocyte; S, secondary spermatocyte; E1, initial spermatids; E2, intermediate spermatids; E3, final spermatids.

Figure 4. Most advanced labelled germ cell type observed at different time periods after BrdU injection Rhamdia quelen jundia kept at 30°C. The following labeled cells (arrowheads) were observed: (A) at 1 h, preleptotene spermatocytes; (B) at 3 days, pachytene spermatocyte; (C) at 5 days, secondary spermatocyte; (D) at 6 days, initial spermatids; (E) at 7 days, spermatozoa in the lumen of the seminiferous tubules. The inset in (F) shows the kinetics of labelled germ cell progression during spermatogenesis. Black bars = 30 μm and White bars = 5 μm.

TABLE

Table 1. Comparative duration of spermatogenesis in various fish species kept at different temperatures.

Figure 1

Click here to download Figure Figure 1.tif

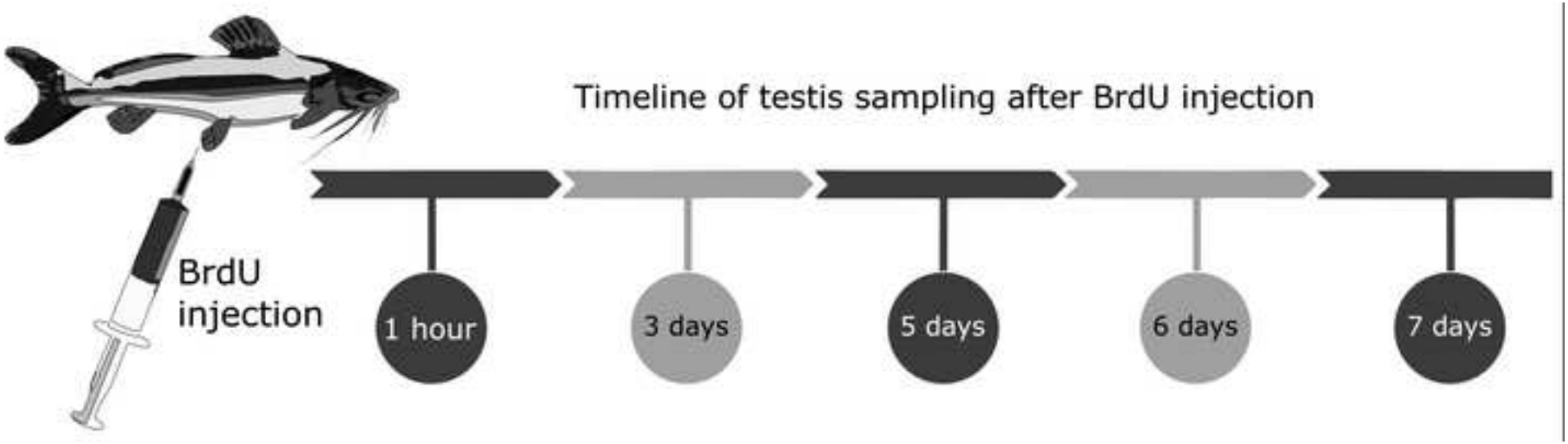


Figure 2

Click here to download Figure Figure 2.tif

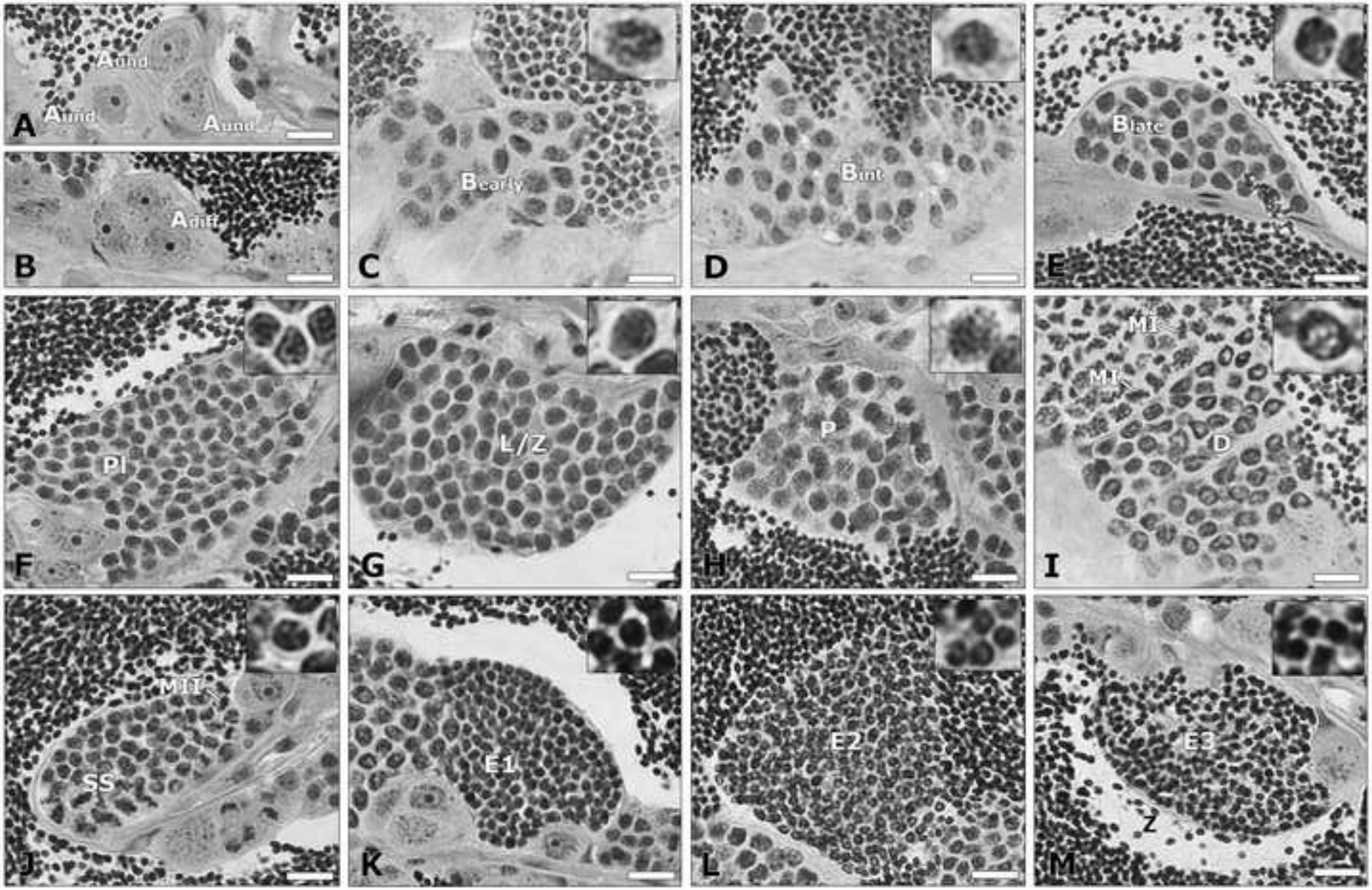
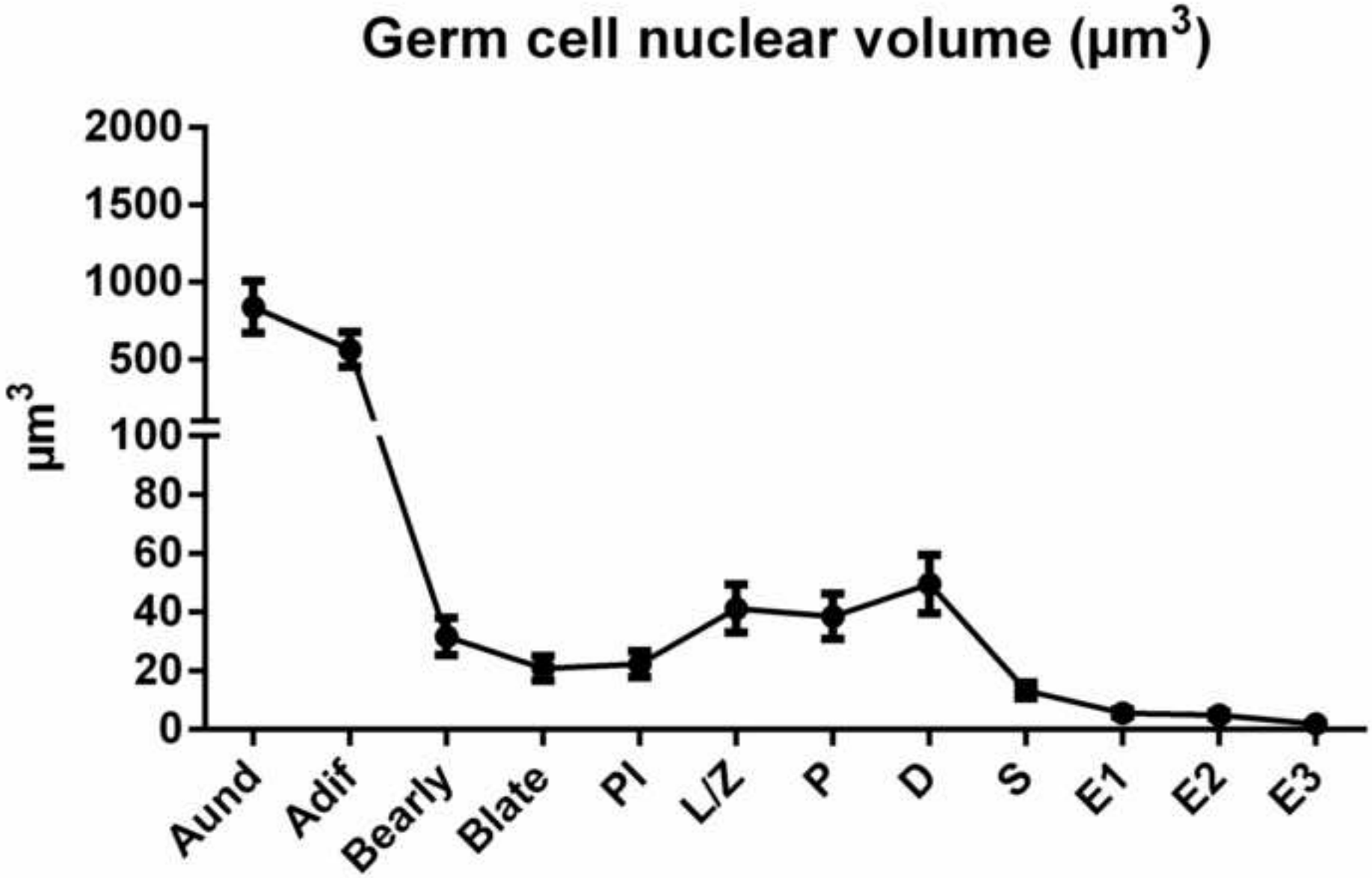


Figure 3

Click here to download Figure Figure 3.tif



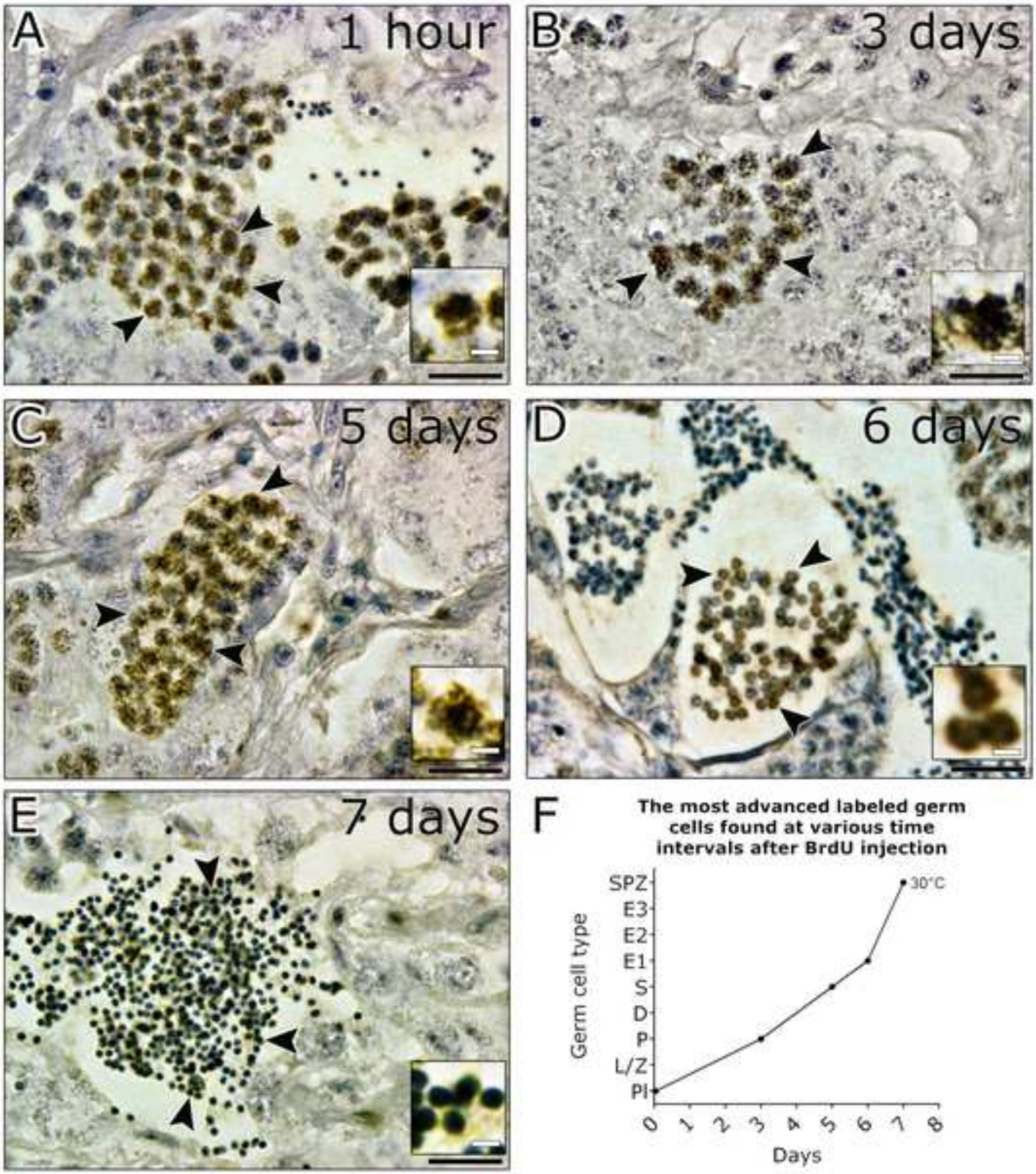


Table 1. Comparative duration of spermatogenesis in various fish species kept at different temperatures.

Group	Species	Water temperature	Spermatogenic duration*	References
A	Rainbow trout (*Oncorhynchus mykiss*)	09°C	6-12 weeks	Whitehead et al., 1978
	Roach (*Rutilus rutilus*)	10°C	30 days	Billard, 1986
B	Nile tilapia (*Orechromis niloticus*)	20°C	spermatogenic arrest	Vilela et al., 2003
	Zebrafish (*Danio rerio*)	20°C	6 days	Nobrega et al., 2010
	Black molly (*Poecilia sphenops*)	21-25°C	21days	Felice et al., 1969
	Climbing perch (*Anabas testudineus*)	25°C ∆	21 days	Sinha et al., 1983
	Guppy (*Poecilia reticulata*)	25°C	14 days	Billard, 1969
	Nile tilapia (*Orechromis niloticus*)	25°C	14 days	Lacerda et al., 2006
C	Zebrafish (*Danio rerio*)	27°C	4 days	Nobrega et al., 2010
	Medaka (*Oryza latipes*)	27°C	12 days	Kuwahara et al., 2003
	Colisa fasciata (*Trichogaster fasciata*)	27°C ∆	9,7 days	Sinha et al., 1979
	Nile tilapia (*Sarotherodon niloticus*)	27°C	7 days	Silva and Godinho, 1983
	Nile tilapia (*Orechromis niloticus*)	30°C	7 days	Vilela et al., 2003
	Jundia Catfish (*Rhamdia quelen*)	**30°C**	**7 days**	**Present study**
	Zebrafish (*Danio rerio*)	30°C	2 days	Nobrega et al., 2010
D	Nile tilapia (*Orechromis niloticus*)	35°C	5-6 days	Lacerda et al., 2006
	Zebrafish (*Danio rerio*)	35°C	spermatogenic arrest	Nobrega et al., 2010

*spermatocytes untill spermatozoa formation

∆ assumed temperature

6. PERSPECTIVA

Perspectivas

O presente estudo teve como focos principais de investigação o transplante xenogênico de espermatogônias-tronco de jundiá adultos para tilápias sexualmente maduras e a estimativa da duração da espermatogênese em jundiás. Os resultados obtidos nos estudos envolvendo o transplante xenogênico demonstrou que que, diferentemente de mamíferos, os testículos de peixes apresentam grande plasticidade filogenética em relação aos fatores necessários para o suportar o desenvolvimento de espermatogênese exógena. Desta forma, estes achados nos permitem esperar que os espermatozoides de jundiá formados nos testículos de tilápia-nilótica sejam viáveis. Neste sentido, são necessários estudos sobre a biologia reprodutiva de fêmeas de jundiá, com o objetivo de se estabelecerem protocolos de indução da ovulação e, assim, otimizar os testes de viabilidade e fertilidade dos gametas obtidos após o transplante xenogênico.

Ainda, o fato do jundiá ser uma espécie filogeneticamente muito próxima do jaú (*Zungaro jahu*) que está ameaçada de extinção e que não se reproduz em cativeiro (Alves, 2006), também nos permitirá desenvolver futuros estudos usando o transplante xenogênico de espermatogônias-tronco como ferramenta. Neste sentido, como ambas espécies pertencem a família *Pimelodidae*, esperamos que o transplante de espermatogônias-tronco de jaú para o testículo de tilápia-nilótica também possa resultar na formação de espermatozoides eventualmente férteis, o que propiciaria a preservação desta importante espécie nativa.

Quanto a duração da espermatogênese, a partir de estudos desenvolvidos em mamíferos usando a técnica do transplante de espermatogônias ficou demonstrado que a duração da espermatogênese, que é um evento espécie-específica, é controlada pelo genótipo das células germinativas. No entanto, em peixes, como a temperatura corporal é próxima da temperatura da água, a duração da espermatogênese é modulada por este

importante parâmetro abiótico. O segundo artigo de nosso estudo mostrou que a duração conjunta das fases meiótica e espermiogênica da espermatogênese no jundiá é muito similar àquele observada na tilápia-nilótica mantida à 30°C. De modo interessante, apesar de estudos previamente desenvolvidos em nosso laboratório terem demonstrado que cerca de dois meses (9 semanas) são necessários para que ocorra a produção de espermatozoides após o transplante singênico em tilápias-nilótica, o tempo requerido para este evento após o transplante de células germinativas de jundiá para os testículos de tilápia-nilótica foi praticamente o dobro (17 semanas). Neste sentido, seria muito importante investigar os aspectos fisiológicos (fatores) envolvidos na adaptação e colonização de espermatogônias-tronco de jundiás no ambiente testicular (nicho espermatogonial) após o transplante. Com foco em particular nos fatores referentes a auto-renovação/diferenciação das espermatogônias-tronco que estejam conservados ou não entre diferentes espécies. No nosso entendimento, se melhor compreendidos estes importantes aspectos poderão contribuir para maior eficiência do transplante xenogênico de espermatogônias-tronco em peixes.

7. CONCLUSÕES

Conclusões

- A partir da padronização da técnica de transplante de espermatogônias-tronco utilizando o jundiá como doador e a tilápia-nilótica como receptora, demonstramos que o microambiente somático da gônada da tilápia-nilótica é capaz de permitir a colonização, proliferação e diferenciação de células-tronco espermatogoniais transplantadas.

- Também mostramos o sucesso desta abordagem com a produção de espermatozoides após o transplante xenogênico entre espécies pertencentes à diferentes ordens taxonômicas. Este resultado sugere que em peixes as que células germinativas apresentam grande plasticidade para se adaptarem a um diferente ambiente somático.

- Após a caracterização morfológica das células germinativas de jundiá, foi demonstrado que, à semelhança de outras espécies de peixes, drástica diminuição do volume nuclear das células germinativas durante a espermatogênese ocorre. Esta redução é observada principalmente entre as espermatogônias indiferenciadas e espermatogônias do tipo B, bem como entre diplótenos e espermátides finais.

- As análises imunohistoquímicas do testículo de jundiá, feitas após a injeção de BrdU em diferentes intervalos de tempos, mostrou que a duração conjunta das fases meiótica e espermiogênica nesta espécie foi muito curta, requerendo cerca de 7 dias para a diferenciação de espermatócitos primários em espermatozoides.

8. ANEXO I

General and Comparative Endocrinology 192 (2013) 95–106

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Phenotypic characterization and *in vitro* propagation and transplantation of the Nile tilapia (*Oreochromis niloticus*) spermatogonial stem cells

Samyra Maria Santos Nassif Lacerda [a], Guilherme Mattos Jardim Costa [a], Mariana de Araújo da Silva [a], Paulo Henrique Almeida Campos-Junior [a], Tânia Mara Segatelli [a], Marco Túlio Diniz Peixoto [a], Rodrigo Ribeiro Resende [b], Luiz Renato de França [a,*]

[a] *Laboratory of Cellular Biology, Department of Morphology, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais 31270-901, Brazil*
[b] *Laboratory of Cell Signaling and Nanobiotechnology, Department of Biochemistry and Immunology, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais 31270-901, Brazil*

ARTICLE INFO

Article history:
Available online 20 June 2013

Keywords:
Nile tilapia (*Oreochromis niloticus*)
Spermatogonial stem cells
Stem cell markers
Gfra1
Nanos2
Spermatogonial transplantation

ABSTRACT

In association with *in vitro* culture and transplantation, isolation of spermatogonial stem cells (SSCs) is an excellent approach for investigating spermatogonial physiology in vertebrates. However, in fish, the lack of SSC molecular markers represents a great limitation to identify/purify these cells, rendering it difficult to apply several valuable biotechnologies in fish-farming. Herein, we describe potential molecular markers, which served to phenotypically characterize, cultivate and transplant Nile tilapia SSCs. Immunolocalization revealed that Gfra1 is expressed exclusively in single type A undifferentiated spermatogonia (A_{und}, presumptive SSCs). Likewise, the expression of Nanos2 protein was observed in A_{und} cells. However, Nanos2-positive spermatogonia have also been identified in cysts with two to eight germ cells that encompass type A differentiated spermatogonia (A_{diff}). Moreover, we also established effective primary culture conditions that allowed the Nile tilapia spermatogonia to expand their population for at least one month while conserving their original undifferentiated (stemness) characteristics. The maintenance of A_{und} spermatogonial phenotype was demonstrated by the expression of early germ cell specific markers and, more convincingly, by their ability to colonize and develop in the busulfan-treated adult Nile tilapia recipient testes after germ cell transplantation. In addition to advancing our knowledge on the identity and physiology of fish SSCs, these findings provide the first step in establishing a system that will allow fish SSCs expansion *in vitro*, representing an important progress towards the development of new biotechnologies in aquaculture, including the possibility of producing transgenic fish.

1. Introduction

Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis and crucial for transmitting the genetic information to the next generation. In addition, SSCs are unique stem cells that can also be reprogrammed into pluripotent cells with the ability to differentiate into somatic tissues (Conrad et al., 2008; Oatley and Brinster, 2012; Thoma et al., 2011), rendering SSCs into a potential substitute for embryonic stem cells (ES). Moreover, in combination with transplantation techniques, SSCs could be powerful vectors for modified genes (Honaramooz and Yang, 2010).

In mammals, several molecular markers (cell surface markers, RNA-binding proteins, zinc finger proteins, cytokines, cell cycle proteins and others) have been described in order to identify SSCs and their early progeny (Reviewed by Kolasa et al., 2012 and Phillips et al., 2010). Although most molecular mechanisms controlling the onset of spermatogenesis have remained elusive, the roles of some components of these pathways are now being elucidated. For instance, in mice the growth factor GDNF (glial cell-line derived neurotrophic factor) signal emanated from Sertoli cells and the germ cell-intrinsic factor Nanos2 represent key regulators for the maintenance and modulation of SSCs self-renewal (Hofmann 2008; Sada et al., 2009; Suzuki et al., 2009). GDNF acts in the testis through a membrane receptor complex formed by the GDNF family receptor alpha 1 (GFRA1) and the receptor tyrosine kinase RET, both expressed in SSCs of different mammalian species (Gassei et al., 2009, 2010; He et al., 2010; Hofmann et al., 2005), including peccaries (Campos-Junior et al., 2012) and equids (Costa et al., 2012) in studies developed in our laboratory. GFRA1 and RET mutant mice have shown a similar spermatogenic phenotype to that

* Corresponding author. Tel.: +55 31 34092816; fax: +55 31 34092780.
E-mail address: lrfranca@icb.ufmg.br (L.R. de França).

0016-6480/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ygcen.2013.06.013

of the GDNF-*null* mice, in which SSCs cannot maintain an undifferentiated state that results in germ cell depletion (Buageaw et al., 2005; Jijiwa et al., 2008; Naughton et al., 2006; Tokuda et al., 2007). *Nanos* genes encode evolutionarily conserved zinc-finger RNA-binding proteins that play important roles for germline stem cell function (Draper et al., 2007; Jaruzelska et al., 2003; Mochizuki et al., 2000; Wang and Lin 2004). In adult mouse testes, a heterogeneity is clearly observed in the undifferentiated spermatogonia population and it has been proposed that GFRA1^{+}Nanos2^{+} spermatogonia represents the stem cell population (Sada et al., 2012; Suzuki et al., 2009). Recently, it was demonstrated that GDNF signaling is essential to maintain Nanos2 expression in murine undifferentiated spermatogonia and that overexpression of *Nanos2* can alleviate the stem cell loss phenotype caused by the depletion of the *Gfra1* gene. Therefore, Nanos2 is a possible target of GDNF signaling to suppress differentiation of mice SSCs (Sada et al., 2012).

Except for some investigations in which early spermatogonial cells have been more accurately characterized by morphological criteria (Lacerda et al., 2013; Nóbrega et al., 2010; Schulz et al., 2010), in teleosts few studies have addressed a more comprehensive phenotypic characterization of undifferentiated spermatogonia. More specifically, microarray analysis of enriched rainbow trout (*Oncorhynchus mykiss*) type A spermatogonia showed that the receptor notch1 would be a useful molecular marker for this cells (Yano et al., 2009). Also, the lymphocyte antigen 75 (Ly75/CD205) was identified as a potential cell surface marker on spermatogonia in the Pacific bluefin tuna (*Thunnus orientalis*) and rainbow trout (Nagasawa et al., 2010, 2012). Within this context, recently, the transcripts showing preferential expression in rainbow trout type A spermatogonia were isolated by microarray analysis (Hayashi et al., 2012).

As in mammals, to date germ cell transplantation represents the only functional bioassay to retrospectively test the stemness of putative SSCs in fish (Lacerda et al., 2012, 2013; Okutsu et al., 2006; Takeuchi et al., 2004; Yoshizaki et al., 2012). Therefore, the appropriate identification and isolation of fish SSCs is essential not only for a high efficiency of spermatogonial transplantation techniques, but also for the establishment of effective SSC culture systems that would offer a better understanding of the proliferation, self-renewal and differentiation behavior of these very important cells, as well as to develop valuable reproductive biotechnologies in aquaculture. In this regard, aiming to develop long-term culture conditions for propagating SSCs *in vitro*, as well as to test their stemness using germ cell transplantation as a functional approach, we investigated in the present study the expression of Gfra1 and Nanos2 in the testis of adult Nile tilapia.

2. Material and methods

2.1. Animals

Twenty-five sexually mature male Nile tilapia (*Oreochromis niloticus*) were used in this study. The fish were obtained from the commercial aquaculture station 3D Aqua Ltda (Morada Nova de Minas, MG) located in the Southeast region of Brazil. All experimental procedures used were conducted in accordance with the guidelines approved by the local ethics committee on animal experimentation – CETEA, UFMG (protocol # 89/2012).

2.2. SDS–PAGE and Western blotting

To qualitatively evaluate the presence of specific markers for SSCs (Gfra1 and Nanos2) immunoblots were performed using total protein lysates from Nile tilapia testes ($n = 4$). For this evaluation, 300 mg of testis parenchyma were immersed in PBS containing protease inhibitors (SIGMA*FAST*™ Protease Inhibitor Tablets; Sigma Aldrich's Corp., St. Louis, MO, USA), and the tissues were submitted to ultrasonic homogenization. After sonication, the lysates were centrifuged at 14,000*g* for 30 min. Supernatants were collected, and then frozen at −80 °C. Protein samples were diluted to 1:2 in a solution of 10% sodium dodecyl sulfate (SDS, Sigma Aldrich), glycerol, 10% bromophenol blue in 0.5 M Tris buffer, pH 6.8, and the samples were boiled for 5 min.

Denatured 12% SDS polyacrylamide mini-gels were prepared and 20 μL samples were loaded into the wells. Protein molecular weight markers (Thermo Fisher Scientific Inc.) were run parallel to the samples. Electrophoresis was carried out at 100 Volts constant voltage and separated proteins were then transferred onto a 0.45 μm PVDF membrane for 60 min. The membranes were blocked with 1% bovine serum albumin (BSA, Sigma Aldrich) in PBS for 1 h at room temperature and then blotted with anti-GDNF receptor alpha 1 antibody (Abcam, ab84106, 1:500), anti-Nanos2 antibody (Abcam, ab76568, 1:200) overnight at 4 °C. After incubation, the membranes were washed three times with PBS 0.05% Tween-20 (PBS-T) solution and then incubated in biotinylated goat anti-rabbit IgG antibody (Abcam, ab97049, 1:500) for 60 min. The membranes were washed three times in PBS-T and incubated in streptavidin-peroxidase solution (Thermo Scientific, TS-125-HR) for 15 min. After washing, proteins were revealed with peroxidase substrate 3, 3′-diaminobenzidine (DAB, Sigma Aldrich), 4-cloronapthol and hydrogen peroxide (Sigma Aldrich) for 5 min and washed in water. Finally, the membranes were scanned in Epson Perfection 4990 photo scanner. Molecular weights of Nile tilapia proteins were determined using SignalP 4.1 Server (www.cbs.dtu.dk/services/SignalP) and ExPASy/ProtParam tool (www.expasy.org/tools/protparam.html).

2.3. Immunostaining analyses

In order to evaluate the *in situ* expression of proteins analyzed by Western blotting, we performed immunostaining using the immunoperoxidase and immunofluorescence methods. Slides were analyzed by light (BX-60 Olympus) and confocal (510 META Laser Scanning Confocal, Zeiss) microscopy. The testicular samples ($n = 6$) were fixed in 4% paraformaldehyde in PBS and embedded in paraplast (Sigma Aldrich). Five micrometer thick serial sections were obtained and used for the immunoperoxidase and immunofluorescence reactions.

Tissue sections were immunostained using protocols specifically developed for each antigen and antibody dilutions previously tested. After dewaxing and rehydration, antigen retrieval was performed in 500 ml of citrate buffer (pH 6.0) for 5 min after boiling in a microwave oven on high power (∼1000 watts). Endogenous peroxidase was quenched for 30 min with 3% H_2O_2 (Vetec) in TBS. Non-specific binding was blocked with 10% normal goat serum (Sigma Aldrich) in TBS 1% BSA. The primary antibodies anti-GDNF receptor alpha 1 (Abcam, ab84106, 1:200) and anti-Nanos2 (Abcam, ab76568, 1:40) were applied to slides and incubated at 4 °C overnight. Biotinylated anti-rabbit IgG antibody (Abcam, ab97049, 1:200) was applied and incubated for 60 min. Detection of the signal was performed by incubating the section in streptavidin-HRP for 15 min, followed by the reaction with DAB and counterstaining with hematoxylin (Merck). After dehydration, sections were mounted and analyzed. For immunofluorescence, antigens were detected by incubation with Alexa Fluor® 488 Donkey Anti-Rabbit IgG (Invitrogen, 1:500) for 1 h at room temperature. After nuclear counterstaining using propidium iodide (Sigma Aldrich), sections were mounted with Mowiol 4–88 solution (Merck).

In order to quantify the percentage of A_{und} spermatogonia that expressed Gfra1 receptor and Nanos2, 100 A_{und} cells were counted

per animal ($n = 4$). Type A undifferentiated spermatogonia were identified according to the criteria proposed by Schulz et al., (2010). The two-parameter analysis was tested for student's T test. The analyzes were performed using the GraphPad Prism (version 5) software. All data were expressed as mean ± SD and the significance level considered was $p < 0.05$.

2.4. Spermatogonia culture

Testes were removed from adult Nile tilapia ($n = 5$) and dissociated with collagenase, hyaluronidase and DNAse (Sigma Aldrich) in Dulbecco Modified Eagle medium/Ham F-12 medium (DMEM/F12 medium; Gibco, Grand Island, NY, USA), and an enriched type A spermatogonia cell suspension was obtained by percoll gradient centrifugation and differential plating ($\sim 2 \times 10^6$ cells/testis) according to methods previously established for this species (Lacerda et al., 2006, 2010). The cells were then plated on 1% gelatin-coated dishes (Sigma Aldrich) and incubated at 28 °C in an atmosphere of 5% CO_2. For evaluation of the characteristics of testis cells in culture, multi-well tissue culture plates were employed in order to provide multiple samples of the same preparation for examination at several times after initiation of culture. Culture cells were analyzed and documented in phase contrast and differential interference contrast (DIC) microscopy. The culture medium was DMEM/F12 containing 4.5 g/L glucose buffered with 10 mM Na_2-HCO_3 (pH 7.3) supplemented with 10,000 U/L penicillin, 10 mg/L streptomycin, glutamine (2 mM), Na-pyruvate (1 mM), Na-selenite (2 nM), nonessential amino acids solution (1%), 2-mercaptoethanol (100 mM), insulin (25 μg/ml), apo-Transferrin bovine (100 μg/ml), human recombinant basic fibroblast growth factor (10 ng/ml), human recombinant epidermal growth factor (100 ng/ml), fetal bovine serum (10%, Gibco), KnockOut Serum Replacement (1%, Life Technologies, Invitrogen), fish serum (1%) from adult Nile tilapia (Hong and Schartl,1996), and tilapia embryo extract (extract from one embryo/ml; Hong and Schartl,1996; Westerfield, 1995). Supplements were purchased from Sigma Aldrich unless otherwise indicated. The cells were replated using StemPro Accutase (Life Technologies, Invitrogen).

For immunocytochemistry, cells were directly fixed with 4% paraformaldehyde in culture dishes for 20 min. Subsequently, they were incubated for 1 h in a blocking solution containing PBS, 5% BSA, and 0.05% Triton-X-100 and then incubated overnight at 4 °C with primary antibodies: anti-DDX4/Vasa (Abcam, ab13840, 1:400) and anti-Oct4 (Abcam, ab18976, 1:100). Secondary Alexa Fluor488 donkey anti-Rabbit IgG (Invitrogen, 1:500 was used for fluorescent detection. Cell nuclei were counterstained with propidium iodide.

In order to observe spermatogonia proliferation *in vitro*, after 10 days in culture, the cells were incubated for 30 h with 100 nM of 5-bromo-2′-deoxyuridine (BrdU, Sigma Aldrich) in culture medium. Cells were detached, fixed by 70% methanol, and the pellets obtained were embedded in plastic glycol methacrilate (Leica Historesin, Heidelberg, Germany). Immunocytochemical detection of BrdU was performed with an anti-BrdU monoclonal antibody (1:200, BD Biosciences, San Jose, CA), biotinylated anti-mouse IgG secondary antibody and ABC Elite kit (1:200, Vector Laboratories, Foster City, CA). Labeled spermatogonia were observed under a light microscope.

For DNA ploidy analysis cells were collected after 30 days in culture. Attached cells were harvested by trypsinization, and testicular cells dissociated from mature testes were used as reference. Cells were fixed in cold 100% ethanol, incubated at 37 °C for 30 min in PBS containing RNase A (100 μg/ml), sodium citrate (0,1%), Triton-X (0,05%) and propidium iodide (50 μg/ml) for DNA staining. Data were acquired on flow cytometer FACScan (BD Biosciences) and analyzed with the FlowJo software (Treestar).

2.5. Rt-pcr

Total RNA was isolated from 26 days-cultured cells using Trizol Reagent according to the manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA, USA). Following chloroform extraction, total RNA was precipitated in 2-propanol, washed with 70% ethanol, resuspended in nuclease-free water and used for cDNA synthesis. RNA concentrations and purity were measured by spectrophotometry (Nanodrop 1000, Peqlab). Total RNA isolated from mature Nile tilapia testes ($n = 2$) were used as reference for the gene expression pattern. cDNA was synthesized with oligo (dT) primer from total RNA by using M-MuLV Reverse Transcriptase (Thermo Scientific).

Polymerase chain reaction amplification was performed with the primers under the following conditions: 94 °C for 1 min, annealing temperature for 1 min, and 72 °C for 1 min completing a total of 30 cycles. The amount of cDNA per PCR reaction corresponded to 100 ng of total RNA. Constitutively expressed 18SrRNA was used as positive control. For negative control, reactions contained PCR components and specific primers but lacked the cDNA template. Oligonucleotide primers that have been used are listed in Table 1.

2.6. Spermatogonial transplantation

The cultured spermatogonia were maintained for different periods of time and after 30 days in culture were transplanted to the busulfan-treated Nile tilapia recipient testes ($n = 4$) (Lacerda et al., 2006, 2010). Before transplantation into the testes of sexually mature tilapia, spermatogonia cultivated for 30 days were isolated using StemPro Accutase and then incubated with the fluorescent membrane dye PKH26-GL (Sigma, St. Louis, MO), which served to identify transplanted cells in the recipient testes. Thus, three weeks after the first busulfan injection, recipient tilapias received the cultivated donor spermatogonia through the common spermatic duct according to techniques previously established (Lacerda et al., 2006, 2010). Following the germ cell transplantation, the recipient tilapia was maintained for 3–4 weeks, and their testes were then analyzed by confocal microscopy. Confocal images were obtained using a 20x objective and 488 nm Argon laser. The ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij/) software was used for the image analysis. Laser power and acquisition settings were adjusted to produce submaximal pixel values in the testis tissue. Background subtraction and contrast/brightness enhancement (up to 20% enhancement using the maximum slider in Image J) were identically performed for all the images in the same experiment.

3. Results

3.1. Expression of Gfra1 in the adult Nile tilapia testis

To study Gfra1 expression we used a commercial antibody developed against human GFRA1 peptide sequence that shows similarities with predicted Nile tilapia Gdnf receptor alpha 1 protein (GenBank: XP_003438304.1). The human and Nile tilapia Gfra1 amino acids sequence alignment is presented in Supplementary Fig. 1. In all fish investigated here, immunoreactivity of anti-GFRA1, evaluated in histological serial sections, was found exclusively in type A_{und} spermatogonia, which are large single cells showing a large nucleus and a prominent nucleolus (Fig. 1A–I). As it can be also evidenced in Fig. 1, early cysts of 2 to 8 spermatogonial cells, comprising type A differentiated spermatogonia (A_{diff}), did not show any evident labeling for Gfra1 (Fig. 1G–I'). More advanced germ cells such as type B spermatogonia, spermatocytes, spermatids and spermatozoa, as well as testicular somatic cells

Table 1
Sequences of primers pairs (5′-3′) used for RT-PCR analysis.

Gene	Database	Forward primer	Reverse primer	Amplicon
vasa	AB032467	CGATGAGATCTTGGTGGATG[a]	CATGAGATCCCTGCCAGCAGA[a]	175 pb
sox2	EF431920	TCCTACTCCCAGCAAACCAC[b]	GCACGGTGCTCTGGTAGTG[b]	247 pb
gfra1	XM_003438256	AGGTTCCTCCCAAGCACAGT[c]	CAAATTGCCTTGCCAGGAGAG[c]	800 pb
dmc1	AB182646	GAAGATGACTCTAGTTTCCAGGATGATG[a]	GCAGAGATGGCAAACGTAGCC[a]	974 pb
18SrRNA	AF497908	TGAAATTCTTGGACCGGCGC[a]	CCAGACAAATCGCTCCACCAAC[a]	407 pb

[a] Pfennig et al. (2012).
[b] Cnaani et al. (2007).
[c] Present work.

including Sertoli and Leydig cells likewise did not show immunoreactivity. Immunofluorescent analysis of dissociated testicular cells clearly demonstrated the expected membrane-associated labeling of Gfra1 in the Nile tilapia A_{und} spermatogonia (Fig. 1J–L), whereas other smaller testicular cells showed no staining (Fig. 1J‘ and K‘). The specificity of the GFRA1 antibody to recognize Nile tilapia Gfra1 protein was demonstrated by Western blot analysis using total lysates from adult testes. Therefore, the samples containing testicular protein showed a predominant band at the expected molecular weight (~50 kDa) of predicted tilapia Gfra1 protein (SignalP 4.1 Server and ExPASy/ProtParam) (Fig. 1M and Supplementary Fig. 2). Quantitative analysis using morphological criteria and Gfra1 immunolabeling showed that 96 ± 1.6 % ($p < 0.05$) of A_{und} spermatogonia in the adult Nile tilapia testis were Gfra1-positive (Fig. 1N).

Analysis of transverse sections from the Nile tilapia testis submitted to immunofluorescence and immunoperoxidase reactions allowed us to observe that a high density of Gfra1-positive spermatogonial cells were present at the distal regions of the seminiferous tubules, near to the tunica albuginea (Fig. 1O–P). Although at lower magnification these spermatogonial cells appeared as a group of cells, in fact they were single cells individually enveloped by Sertoli cells (Fig. 1O‘–P‘). Serial section images of distal regions of seminiferous tubules showing detailed single A_{und} spermatogonia enveloped by Sertoli cells are presented in Supplementary Fig. 3.

3.2. Expression of Nanos2 in the adult Nile tilapia testis

To evaluate the Nanos2 protein distribution in the adult tilapia testes we performed immunofluorescence and immunoperoxidase analysis using a commercial antibody developed against an immunogen sequence of human Nanos homolog 2 protein. Sequence alignment of human and predicted Nile tilapia Nanos2 protein (GenBank: XP_003439364.1) is shown in Supplementary Fig. 4. In all fish investigated, using histological serial sections, immunoreactivity for anti-Nanos2 was frequently observed in clusters of single type A_{und} spermatogonia located at the distal regions of the seminiferous tubules, near to the tunica albuginea (Fig. 2A–C). Nanos2 expression was also found in single A_{und} cells situated along the seminiferous tubules, set aside a certain distance from the tunica albuginea (Fig. 2D–E). Besides that, we could also observe Nanos2 positive spermatogonia along the tubules in cysts with germ cell clones of two (Fig. 2F), four (Fig. 2G) and eight spermatogonial cells (Fig. 2H), but not in larger cysts. As it can be also observed in Fig. 2 no evident labeling was found in more advanced germ cells such as type B spermatogonia, spermatocytes, spermatids and spermatozoa, or in somatic cells such as Sertoli and Leydig cells. Using the Nanos2 antibody, Western blot analysis of Nile tilapia testes lysates showed a predominant band of approximately 18 kDa, which corresponds to the expected size of this protein previously determined (SignalP 4.1,ProtParam) (Fig. 2J and Supplementary Fig. 2). Quantitative analysis using morphological criteria and Nanos2 immunolabeling indicated that 94.5 ± 1.5 % ($p < 0.05$) of single A_{und} spermatogonia in the adult tilapia testis were positive for Nanos2 (Fig. 2K).

3.3. Nile tilapia spermatogonia culture

Cells were harvested from adult tilapia testes and spermatogonia were enriched from dissociated testicular cells suspension using percoll gradient and differential plating (Lacerda et al., 2010). After 12 h in culture, the non-adherent cells, which usually form clumps (Fig. 3A), were seeded in gelatin-coated tissue culture plates in the specific culture medium. Until 2 days in culture, these cells were found to only barely attach to the gelatin-coated dish. However, increased cell attachment was observed at day 3 (Fig. 3B), and most of the attached cells displayed strong staining for DDX4/Vasa (Fig. 3C), a RNA helicase specifically expressed in the germ cells lineage of large number of animal taxa (Supplementary Fig. 7) (Gustafson and Wessel, 2010; Fujimura et al., 2011). DDX4/Vasa corresponding sequence alignment is presented in Supplementary Fig. 5. Besides that, these cultured cells also expressed the transcription factor Pou5f1/Oct4 (Fig. 3D), a specific pluripotency gene present in ES, ES-derived germ cells and primordial germ cells (PGC) of mammals and fish (Hong et al., 2004; Sánchez-Sánchez et al., 2010; Wang et al., 2011; Onichtchouk 2012; Takehashi et al., 2012, Froschauer et al., 2013) (Supplementary Figs. 6 and 7). The remaining negative cells were heterogeneous in morphology showing epithelioid and fibroblast-like appearance (Fig. 3C and D). Validation of DDX4/Vasa and Oct4 antibodies is shown in Supplementary Fig. 2. After 7 days (Fig. 3E), spermatogonial cells actively proliferate as demonstrated by BrdU incorporation (Fig. 3F). The nuclear BrdU labeling revealed the perinuclear chromatin distribution and a large nucleolus that is a typical characteristic of fish early spermatogonial cells (Nóbrega et al., 2010; Schulz et al., 2010). At ten days, tilapia spermatogonia continued to propagate *in vitro* (Fig. 3G) and the immunostaining showed that these cells retained the expression of the Gfra1 receptor (Fig. 3H), as well as Nanos2 protein (Fig. 3I), which are potential good markers of A_{und} spermatogonial cells.

Because fish ES cells and spermatogonia are able to differentiate *in vitro* by enhanced cell–cell interactions (Hong et al., 1996, 2004), we kept the cells at high confluence, without subculture, in order to investigate the tilapia spermatogonia behavior in our cultivation system. Therefore, at 26 days of culture in high confluence, tilapia spermatogonia generated large colonies as shown in Fig. 4A and C. To further analyze the identity of the colony-forming cells we investigated the presence of the *vasa*, *sox2*, *gfra1* and *dmc1* gene transcripts in these groups of cells. Similar to *vasa*, the gene *sox2* is expressed in tilapia germ cells from spermatogonia to spermatocytes (Kobayashi et al., 2002; Pfennig et al., 2012), whereas *dmc1* is exclusively expressed in meiotic cells (Kajiura-Kobayashi et al., 2005). RT-PCR analysis confirmed that cultivated cells transcribed the germ line-specific genes *vasa, sox2* and the *gfra1* gene, but not the meiotic marker *dmc1* (Fig. 4B). Similar expression patterns of

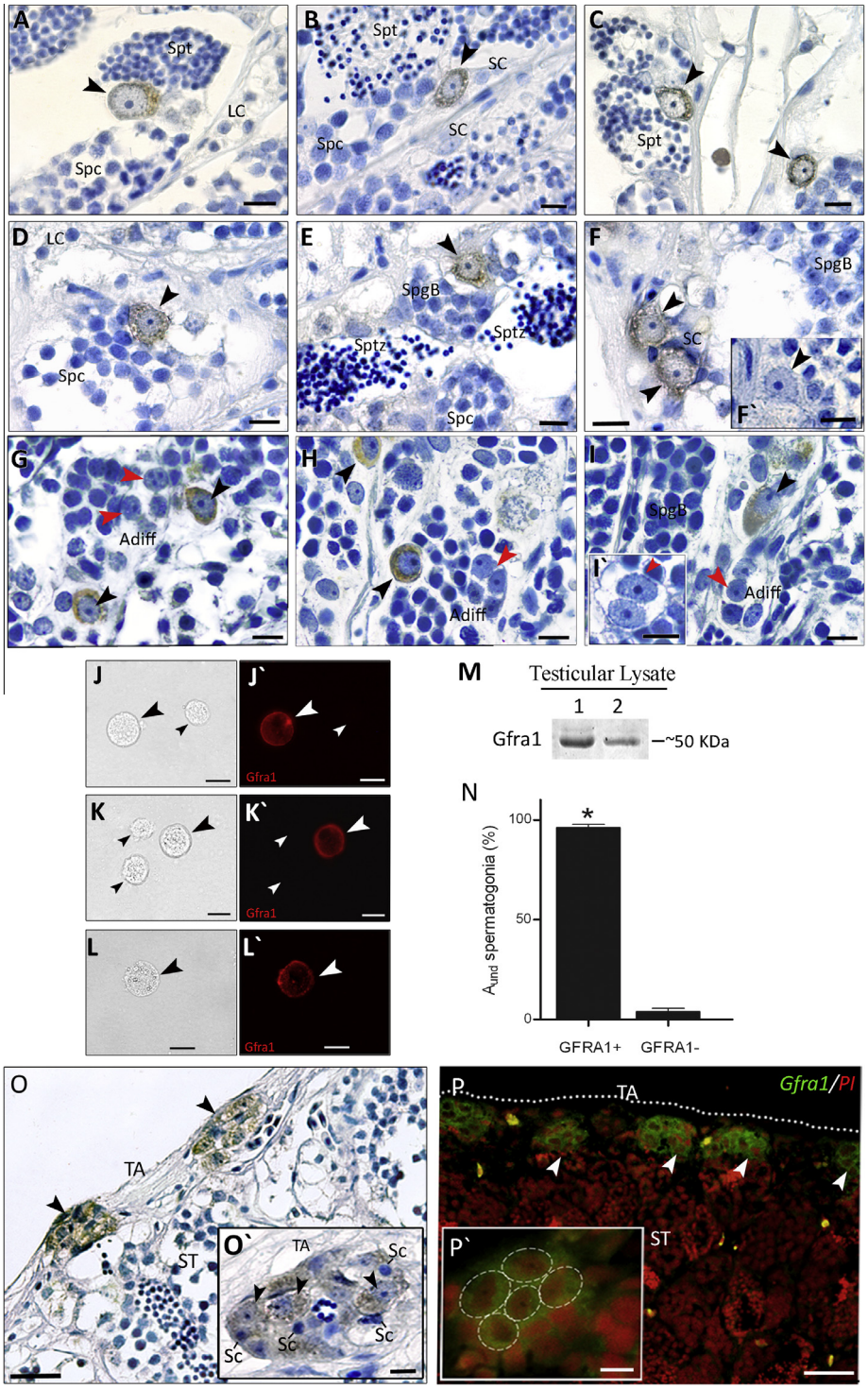


Fig. 1. Immunohistochemical localization of Gfra1 protein-positive spermatogonial cells in the adult Nile tilapia testis. (A–I) Gfra1 protein is detected in single type A_{und} spermatogonial cells that are morphologically characterized as larger cells showing a large nucleus and a prominent nucleolus (black arrowhead). In contrast, Gfra1 immunoreactivity was not detected in cysts of type A differentiated spermatogonia (A_{diff}; red arrowhead in G–I). Figure I′ shows at higher magnification a Gfra1-negative A_{diff} cyst containing 2-cells. Similar to A_{diff}, type B spermatogonia (SpgB), spermatocytes (Spc), spermatids (Spt) and spermatozoa (Sptz), as well as somatic cells such as Sertoli (Sc) and Leydig cells (Lc), did not show any evident labeling. Gfra1-negative A_{und} spermatogonia are rarely observed and a negative control is shown in F‘ (arrowhead). J–L and J‘–L‘ represent respectively bright field and fluorescent microscopy images. In these figures, immunofluorescence staining of isolated testicular cell evidencing the membrane-associated labeling of Gfra1 receptor in the Nile tilapia A_{und} spermatogonia is observed (large white arrowhead), whereas other smaller testicular cells show no staining (small arrowhead). (M) The suitability of GFRA1 antidody to recognize the Nile tilapia relative protein (molecular weight: ~50 kDa) in the adult testes evaluated was validated through immunoblotting analyses. (N) Quantitative analysis demonstrated that over 95% of A_{und} spermatogonia are Gfra1-positive cells in the Nile tilapia testis ($^*p < 0.05$). (O–P) Transversal (dorsoventral) sections of the Nile tilapia testis show that a high density of individual Gfra1-positive spermatogonial cells (arrowhead and white circle in P) is found at the distal region of the seminiferous tubules, near to the tunica albuginea (TA). The insert at higher magnification in O‘ shows that each individual Gfra1-positive cell (arrowhead) is enveloped by Sertoli cells (Sc). Green labeling indicates the Gfra1/Alexa Fluor488, whereas red denotes the propidium iodite. Bar:10 μm in A–L‘ and O‘–P‘; and 50 μm in O–P.

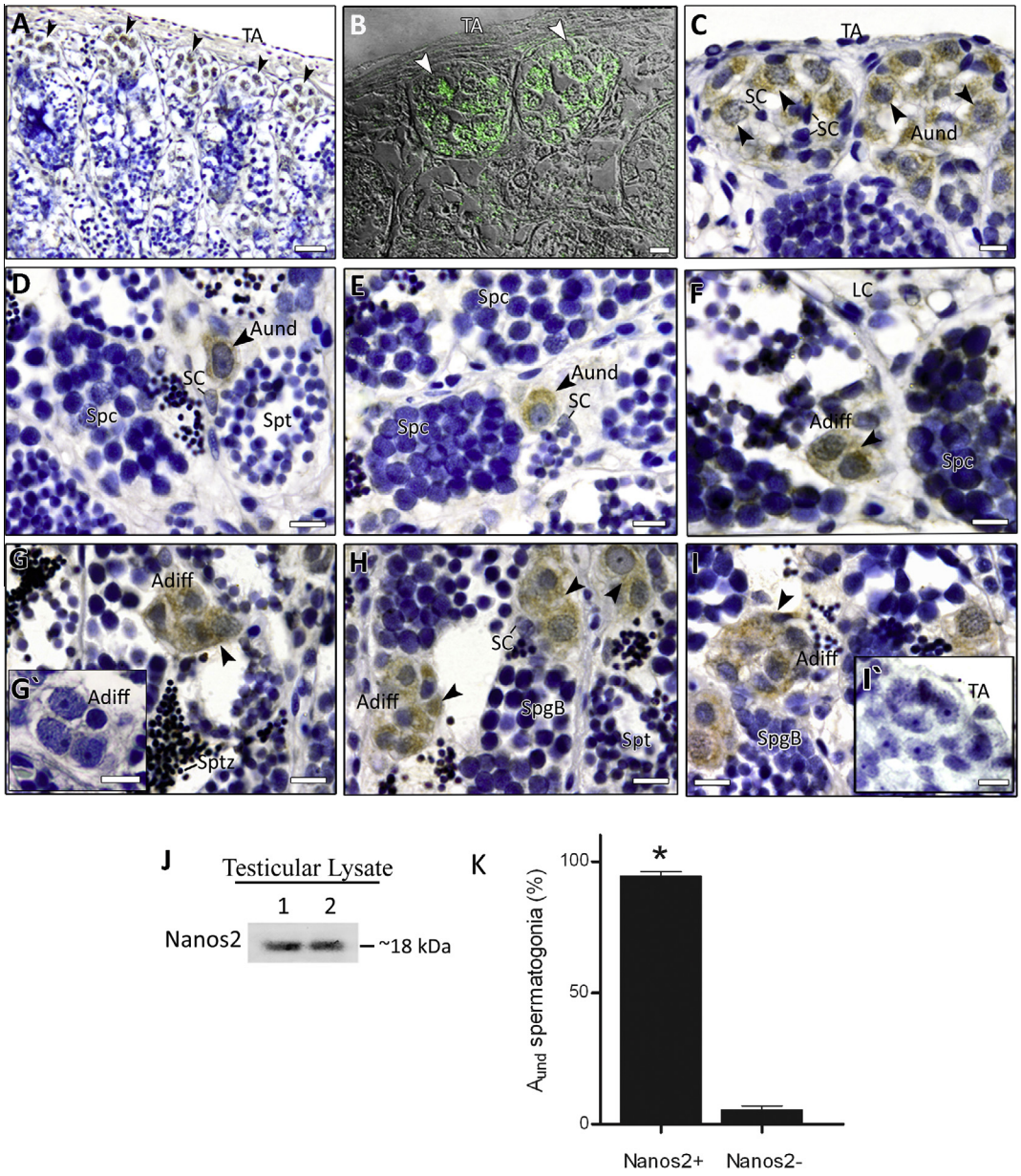


Fig. 2. Immunohistochemical localization of Nanos2 protein-positive spermatogonial cells in the adult Nile tilapia testis. (A and B) Immunoreactivity of anti-Nanos2 is frequently found in clusters of single type A_{und} spermatogonia (arrowheads) located near to the tunica albuginea (TA) either through immunoperoxidase (A) or immunofluorescence (B) reactions. Figure C is a representation at higher magnification of Figure A showing that each individual Nanos2-positive cell is enveloped by Sertoli cells (Sc). (D-I) Nanos2-positive spermatogonia (arrowheads) can also be observed along the seminiferous tubules as single cells (A_{und}; D and E), as well as in cysts of 2 (A_{diff}; F), 4 (A_{diff}; G) and 8-cells (A_{diff}; H-I). The insert in G′ illustrates at higher magnification a cyst of A_{diff} spermatogonia that did not express Nanos2 protein. All type B spermatogonia (SpgB; H and I), spermatocytes (Spc; D-F), spermatids (Spt; D and H), spermatozoa (Sptz; G), Sertoli cells (SC; D, E and H) and Leydig cells (LC; F) did not show any evident labeling. A negative control is shown in I′. (J) The specific expression of the Nanos2 protein (~18 kDa) in the testis of two adult Nile tilapia was confirmed by immunoblotting. (K) Quantitative analysis demonstrated that almost 95% of A_{und} spermatogonia in the Nile tilapia testis are Nanos2-positive cells ($^{*}p < 0.05$). Bar: 50 μm in A; and 10 μm in B-I‘.

mRNAs in adult testis strongly suggested that tilapia spermatogonial population was able to expand *in vitro* while conserving their original characteristics. Using immunofluorescence labeling we could observe *in situ* that at 30 days of culture the cells present in the colony also expressed Gfra1 protein (Fig. 4D). Thus, we could assume that the colony-forming cells contained tilapia A_{und} spermatogonial cells.

In order to exclude eventual chromosomal changes, which are indicative of cell transformation, we further evaluated herein the DNA content of 30 days-cultivated cells, which also allow us to observe the germ cells progression through spermatogenesis. As a control, fresh dissociated adult testicular cells were used and the flow cytometry histogram displayed three peaks corresponding to haploid (c), diploid (2c) and tetraploid (4c) DNA content (Fig. 4E). In comparison to fresh dissociated cells, we found that the DNA content pattern of cultivated spermatogonia remained relatively unchanged once both histograms showed similar values of mean fluorescence intensity (*x*-axis; Fig. 4F). In addition, as expected for diploid cells in active proliferation, 30 days-cultivated cells exhibited prominent peaks of diploid (2c) and S-phase fraction (S), besides a modest tetraploid (4c) DNA content (Fig. 4F). Thus, we conclude that tilapia A_{und} cells remained euploid for at least 30 days under culture conditions and did not differentiate or formed haploid cells.

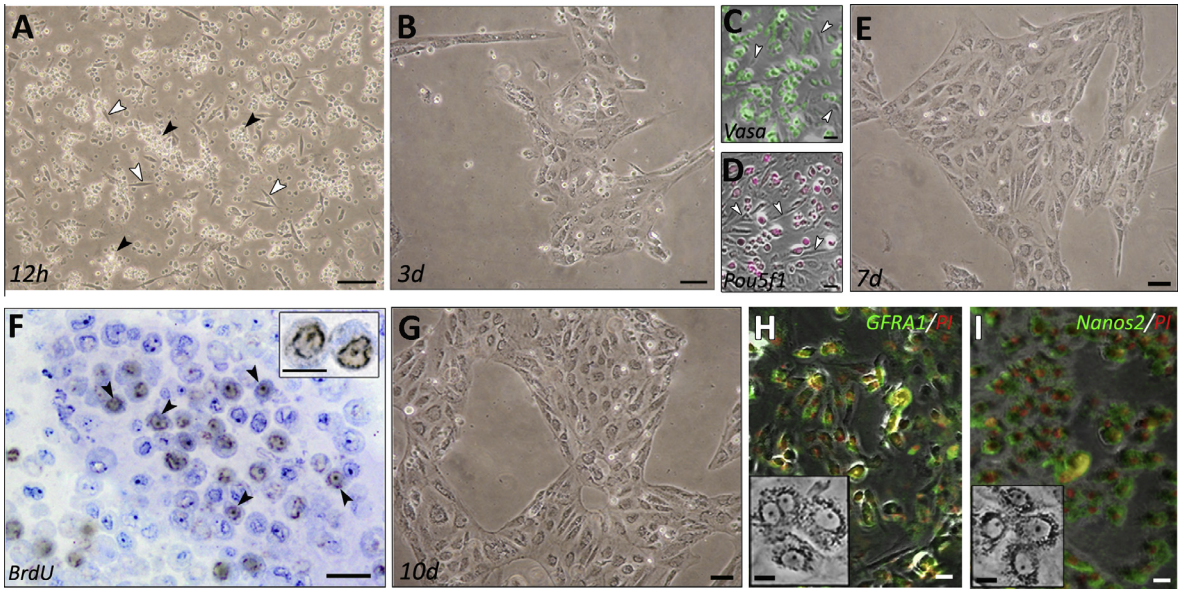


Fig. 3. Evaluation of cultured Nile tilapia spermatogonial cells. (A) After differential plating within 12 h in pre-culture to deplete testicular somatic cells, that firmly attach to the culture dishes (white arrowhead), germ cells remain in suspension and tend to form clumps (black arrowhead). (B) Germ cells attachment to the dish initiated within 3 days. Most of the attached cells are Vasa-positive (C; in green) and also express Pou5f1 (D; in pink). The remaining negative cells exhibit epithelioid and fibroblast-like appearance (C, D; white arrowhead). (E) At 7 days of culture, spermatogonial cells actively proliferate, as evidenced by cells confluence and BrdU incorporation (F; arrowhead). The insert in F shows the pattern of the nuclear BrdU-labeled chromatin distribution in two proliferating spermatogonia. (G) After 10 days of culture, attaching spermatogonia retain the expression of Gfra1 (H; in green) and Nanos2 (I; in green) proteins as demonstrated by fluorescent and brightfield DIC merged images (Figure H and I). The cell nuclei are stained with propidium iodite (PI; in red). Inserts in H and I show phase contrast images of 10 days-cultured cells evidencing the morphological details of Gfra1 and Nanos2-positive spermatogonia, which are not clearly observed in DIC images. Bar: 100 μm in A; and 20 μm in B–I.

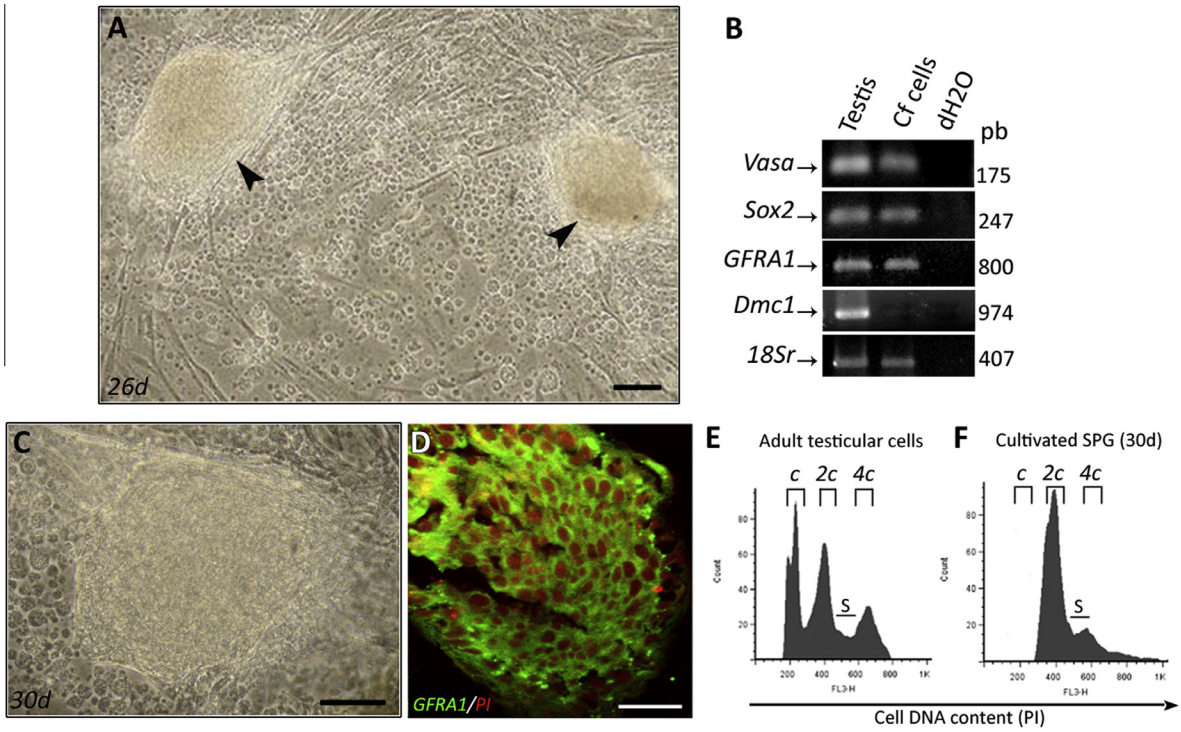


Fig. 4. Maintenance and propagation of the Nile tilapia spermatogonia *in vitro*. (A) At 26 days of culture, under high confluence, tilapia spermatogonia form large colonies (arrowhead). (B) Gene expression pattern evaluated by RT-PCR in the adult Nile tilapia testis and in colony-forming cells (Cf cells). As it can be observed, in comparison to the adult testis, cultivated cells expressed *vasa*, *sox2* and *gfra1* genes, but not the meiotic marker *dmc1*. *18Sr* mRNA is used as internal control for RT-PCR amplification, whereas the H_2O lane represents a negative control containing no cDNA template. (C and D) At 30 days of culture, immunofluorescence labeling shows that the cells present in the colony (C) expressed the A_{und} spermatogonia marker Gfra1 (D, in green) and that their nuclei are stained with propidium iodite (PI; in red). (E and F) Flow cytometry analysis of DNA content of the adult (E) and 30 days-cultured (F) testicular cells. In addition to diploid and tetraploid peaks (F; 2c and 4c), adult testicular cells histogram show a prominent haploid peak (E; c). After 30 days in culture, tilapia spermatogonial cells apparently maintain their identity (ploidy) as well as their proliferation pattern, showing diploid (F; 2c) and tetraploid peaks (F; 4c). The S-phase of the cell cycle (S) is observed in both histograms. Bar: 60 μm.

3.4. Transplantation of cultivated spermatogonia

Fish SSCs are only retrospectively identified by their activity to colonize recipient seminiferous tubules after germ cell transplantation. We previously established in our laboratory all the necessary techniques for spermatogonial transplantation in the adult Nile tilapia and demonstrated that transplanted SSCs were able to colonize, proliferate and produce functional sperm in the recipient tilapia testes (Lacerda et al., 2010). Taking advantage of this system, we examined whether Gfra1-positive cultured spermatogonia maintained their ability to colonize the recipient tilapia gonads. Thus, these cells were maintained under optimized conditions for at least 1 month, labeled with the PKH26 fluorescent dye (Fig. 5A–C) in order to trace them in the recipients and subsequently transplanted into de testis of adult busulfan-treated recipient fish (Fig. 5D). Three (Fig. 5E and G) to four weeks (Fig. 5I and K) after transplantation, evaluation of the testes *in toto* revealed that PKH26-positive cultured SSCs were incorporated into the recipients gonads. Therefore, many clusters/colonies of fluorescent-positive cells were observed in the seminiferous tubules of all transplanted tilapias (Fig. 5E, G, I, K), but not in the gonads of non-transplanted ones (Fig. 5F, H, J, L). We also observed that the quantity of donor cells present in the fluorescent clusters/colonies increased from three to four weeks post-transplantation, indicating therefore the functional viability of the transplanted SSCs to survive, colonize and proliferate in the recipient testes.

4. Discussion

The lack of SSC molecular markers and the small number of SSCs in the testis have been a great hurdle to identify or purify SSCs in fish. Therefore, to date the SSCs biology in this group of vertebrate is poorly known and the promising applications of SSCs in several valuable biotechnologies involving fish production are

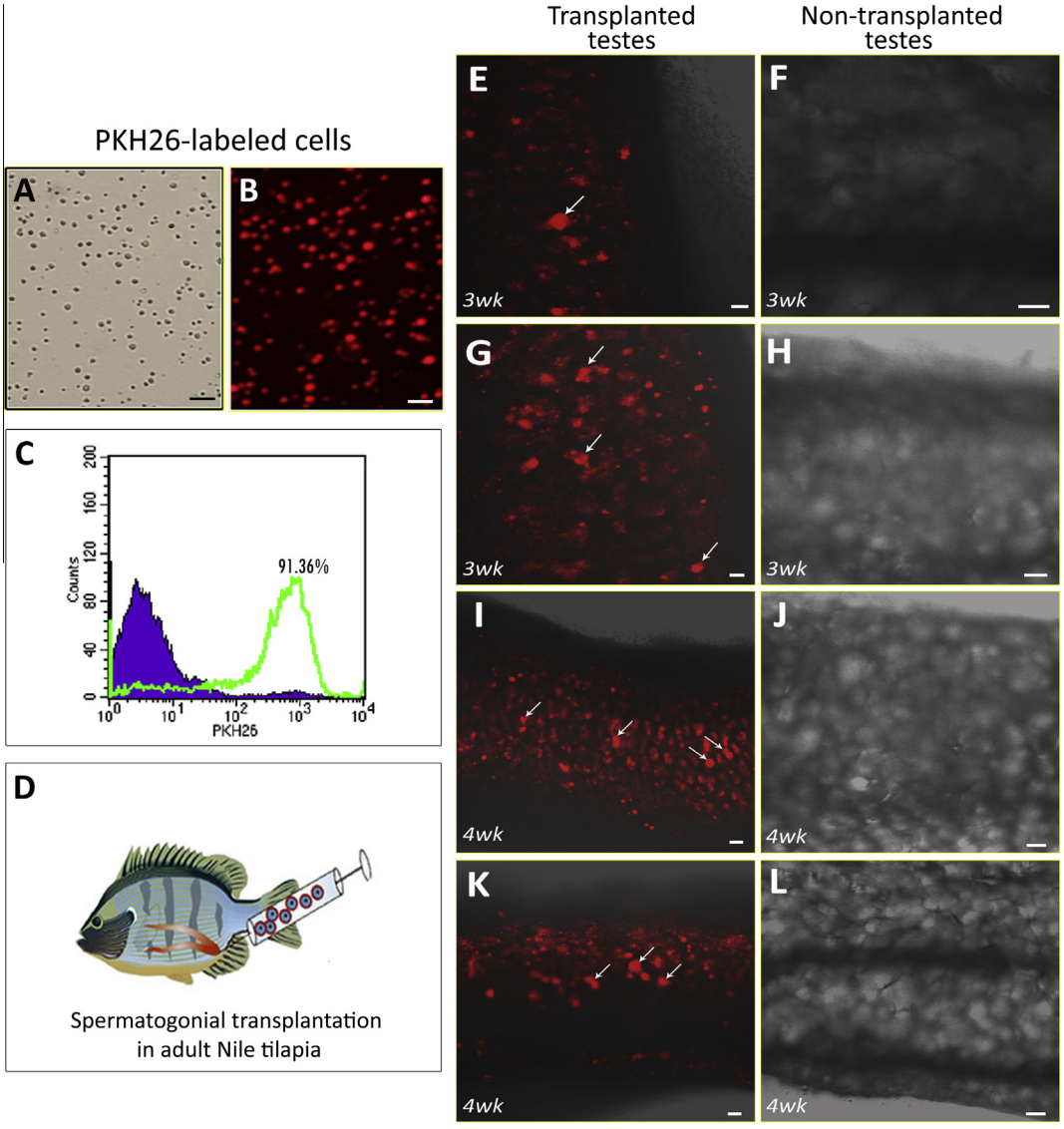


Fig. 5. Isolation and transplantation of 30 days-cultured A_{und} spermatogonia in the adult Nile tilapia recipient testis. The isolated spermatogonial cells (A) plasma membrane was labeled with PKH26 fluorescent dye (B; in red) that, as shown in the histogram (C), represented almost 92% of the cells (shown in green line) in comparison to the peak in blue depicting the negative control (non-labeled cells). (D) Schematic drawing illustrating the transplantation procedure by which cultured A_{und} spermatogonia were injected into the testis of busulfan-treated adult Nile tilapia. (E, G, I, K) Merged brightfield and fluorescent images of a recipient testis 3 weeks (E–G) and 4 weeks (I–K) post-transplantation, where A_{und} cultured spermatogonia were able to colonize the recipient gonad, as demonstrated by the presence of several PKH26-positive clusters/colonies of cells (arrow) in the recipient seminiferous tubules of transplanted tilapia. (F, H, J, L) Merged brightfield and fluorescent images of non-transplanted Nile tilapia testis. No fluorescent cells were present in the seminiferous tubules of non-transplanted testes. Bar: 60 μm in A and B; and 100 μm in E–L.

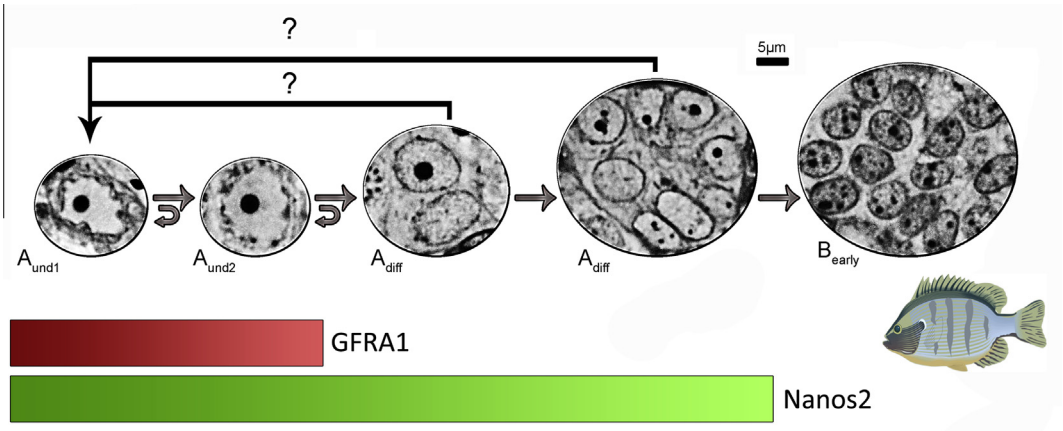


Fig. 6. Illustration of Gfra1 and Nanos2 protein expression in the different types of spermatogonial cells in the Nile tilapia testis. As it can be noticed, the expression of Gfra1 (in red) is restricted to single A_{und} spermatogonia (A_{und1} and A_{und2}) that contain the spermatogonial stem cell population, which supposedly present the capacity of self-renewing (curved arrow) or differentiation (straight arrow). Although Nanos2 (in green) is observed in both single A_{und} spermatogonia, its expression is also found in type A differentiated spermatogonia (A_{diff}) that form clones/cysts of 2–8 germ cells. Nanos2 expression is no longer observed after the type A_{diff} divide to give rise to type B spermatogonia (B_{early}). However, it is not yet known whether the Nile tilapia A_{diff} spermatogonia (2 to 8-cell clones) possess the ability to dedifferentiate (?; long arrows) and become single cells with stemness capacity.

not yet well explored. In the present study we report the establishment of reliable markers for single type A undifferentiated spermatogonia in the Nile tilapia, the Gfra1 receptor, as well as another useful marker for early spermatogonia, the protein Nanos2. Our data also demonstrate the development of effective culture conditions for the propagation of the Nile tilapia A_{und} spermatogonia for at least 1 month. The spermatogonial identity in culture was characterized by several evidences that included the A_{und} spermatogonial phenotype, expression of germ cell markers and, more convincingly, the ability of these cells to colonize the recipient testis when transplanted into the adult Nile tilapia.

The GFRA1 membrane receptor is considered one of the most useful markers of A_{und} cells in mammals (Hofmann et al., 2005) and its expression on these germ cells was already described in mice (Baloh et al., 2000; Dettin et al., 2003; Hofmann et al., 2005; Jing et al., 1996; Von Schönfeldt et al., 2004), rats (Gassei et al., 2009), hamster (Sato et al., 2011), pigs (Kuijk et al., 2009), domestic cats (Powell et al., 2012; Silva et al., 2012), peccaries (Campos-Junior et al., 2012), equids (Costa et al., 2012), rhesus monkeys (Gassei et al., 2010; Hermann et al., 2010) and humans (Gassei et al., 2010, He et al., 2012). Different from fish, in which A_{und} spermatogonia are characterized as single cells surrounded by Sertoli cells (Leal et al., 2009; Nóbrega et al., 2010; Schulz et al., 2010), in most investigated mammalian species A_{und} spermatogonia correspond to a group of cells comprising A single (A_s), A paired (A_{pr}) and A aligned (A_{al}) spermatogonia, the latter usually being clones of 4, 8, or 16 cells (De Rooij and Russell, 2000). Although in mammals GFRA1 expression is observed in all A_{und} spermatogonial types, its expression gradually decreases in mice as spermatogonial clones become larger (Nakagawa et al., 2010; Suzuki et al., 2009). In the present investigation, even using histological serial sections, we could not observe cysts of 2 or more spermatogonial cells immunolabeled for Gfra1, but rather single cells (A_{und}) were found to express Gfra1 exclusively in the testis of adult Nile tilapia, contrasting therefore with the expression pattern observed in mammals. Also, single A_{und} spermatogonia do not uniformly express Gfra1 in the Nile tilapia testis. We still do not know the functional significance of this finding. However, Gfra1-negative A_{und} spermatogonia are less abundant, accounting for only about 4% of the total number of A_{und} spermatogonia. In rodents, morphometric analysis demonstrated that about 10% of A_s spermatogonia did not express GFRA1 but were able to form colonies after germ cell transplantation assays (Grisanti et al., 2009). So, it remains controversial as to whether the heterogeneous expression of markers in spermatogonia in fact reflects different temporal stage (e.g. phases of cell cycle) within a functionally homogenous cellular population and not functionally distinct populations (Izadyar et al., 2002; Lok et al., 1982; Schulz et al., 2010).

It is currently widely accepted that A_s spermatogonia represents the actual SSC population in mammalian testes (Yoshida et al., 2007; Oatley et al., 2011). Considering this aspect, we could speculate that Gfra1-positive spermatogonia, or at least a large fraction of this population, represent the SSCs in the adult Nile tilapia testis. While the expression of other candidate molecules has been reported in fish, including Ly75 (Nagasawa et al., 2010, 2012), Notch1 (Yano et al., 2009), Plzf (Ozaki et al., 2011), Pou5f1 (Sanchez-Sanchez et al., 2010) and SGSA-1 (Kobayashi et al., 1998), to our knowledge Gfra1 represents the first described molecular marker to distinguish A_{und} cell from other progenitor spermatogonia in the teleost testis. Therefore, since Gfra1 receptor is a selective surface marker it might be feasible to employ it as molecular target in immunomagnetic or immunofluorescent based cell sorting in order to isolate or obtain enriched A_{und} spermatogonia population in Nile tilapia or other fish species.

Nanos2 is a key stem cell regulator that is expressed in self-renewing SSCs and maintains the stem cell state during murine spermatogenesis (Sada et al., 2009; Suzuki et al., 2009). The GDNF/GFRA1 signaling pathway is one of the candidates to induce or maintain Nanos2 expression in the mouse testis (Sada et al., 2012). In fish, Nanos2 protein has been previously demonstrated in zebrafish PGCs at early stages of sex differentiation (Beer and Draper, 2012) and in medaka (*Oryzias latipes*) spermatogonia and oogonia (Aoki et al., 2009). Similar to the pattern observed in rodents in which A_s to A_{al} undifferentiated spermatogonia are Nanos2-positive (Sada et al., 2009, 2012; Barrios et al., 2010; Suzuki et al., 2009), in the testis of the adult Nile tilapia Nanos2 was found to be expressed in both type A_{und} and A_{diff} spermatogonia up to the 8-cell clone. Using double immunostaining, a previous study showed that in mouse about two-thirds of Nanos2-positive A_s to A_{al} cells co-expressed GFRA1. However, all of the GFRA1-positive cells were also Nanos2-positive (Suzuki et al., 2009). Although we did not perform this kind of analysis in the present investigation, based on the high percentage of Gfra1-positive (~96%) and Nanos2-positive (~94%) single A_{und} observed here, we could

expect that, similar to mice, in the Nile tilapia testis most if not all of the Gfra1-positive spermatogonia were also Nanos2-positive. Interestingly, since we detected Gfra1 expression only in single A_{und} cells and Nanos2 expression in A_{und} and A_{diff} cells, the percentage of spermatogonia co-expressing these markers in the Nile tilapia testis was probably lower than that observed in mice. However, as shown in the *Gfra1*-knockout mice testis (Sada et al., 2012), we could speculate that in the Nile tilapia Nanos2 could also repress differentiation of both Gfra1-positive cells and cells that have lost Gfra1 expression (A_{diff} 2 to 8-cell clones) during initial spermatogonial divisions (see below in Fig. 6).

Recently, it was demonstrated that undifferentiated murine spermatogonia, from A_s to A_{al8} (8-cell clones), maintain their stemness status, being able to differentiate into subsequent spermatogonial cells (A_1) or becoming a new A_s by fragmentation of the spermatogonial clone (Nakagawa et al., 2010). Considering that in the murine spermatogenic stem cell compartment A_{pr} to A_{al8} show reversibility and potential stemness capacity, it would be of great value to investigate if in Nile tilapia A_{diff} spermatogonia (2 to 8-cell clones), Nanos2-positive cells show such a reversibility as well, or if they are definitely committed to differentiation. Based on the results found here, Fig. 6 summarizes the expression pattern of the two proteins evaluated in the Nile tilapia spermatogonial cells.

In the present study we also developed functional *in vitro* culture conditions for supporting the survival, mitotic activity and self-renew of tilapia A_{und} spermatogonia. So far, the basic techniques for the long-term *in vitro* culture of spermatogonia have been established only for zebrafish (Kawasaki et al., 2012), rainbow trout (Shikina et al., 2008; Shikina and Yoshizaki, 2010) and medaka (Hong et al., 2004). Indicating the maintenance of the undifferentiated status of the spermatogonial cells, we observed that after 26 days of culture and under high confluence the Nile tilapia spermatogonia formed large colonies of Gfra1-positive cells. In contrast, at full confluence without subculture, medaka spermatogonial cells underwent meiosis and spermiogenesis, generating motile sperm *in vitro* (Hong et al., 2004). Similar to zebrafish and mammalian spermatogonial culture systems (Aponte et al., 2008; Kala et al., 2012; Kawasaki et al., 2012; Mirzapour et al., 2012; van der Wee et al., 2001), some testicular somatic cells derived from dissociated testicular cells could be observed in our culture system, acting as a feeder layer for tilapia A_{und} spermatogonia. Although we have observed Gfra1 receptor expression in cultured spermatogonia in different time periods, the recombinant mammalian growth factor GDNF was not used as a supplement in our culture medium. Shikina and Yoshizaki (2010) have previously reported no effect of rat GDNF or rat GFRA1-Fc fusion protein on the proliferation of rainbow trout type A spermatogonia, whereas recombinant human GDNF was shown to enhance zebrafish spermatogonia proliferation *in vitro*. However, in comparison to mouse SSC cultures, a 5- to 10-fold higher concentration of this growth factor was used for zebrafish cells (Kawasaki et al., 2012). Surely, this important aspect deserves a better investigation in our culture system in future studies.

It is widely accepted that only SSCs are able to colonize the available niches and re-establish spermatogenesis in the recipient testis. Therefore, germ cell transplantation provides a functional approach to study the stem cell niche in the testis and to unequivocally detect SSCs (Nagano et al., 2013; Tang et al., 2012). Using germ cell transplantation assay, we demonstrated that the Nile tilapia A_{und} spermatogonia, maintained for at least 1 month in culture, were able to colonize busulfan-depleted seminiferous tubules of recipient fish. While further studies are still necessary to determine whether normal sperm and offspring can be obtained from cultured cells, the wide spread of PHK26-positive colonies/clusters observed 3–4 weeks after transplantation functionally demonstrate the regenerative capacity of cultured A_{und} spermatogonia, which also suggest their stemness potencial.

Based on our findings, it is reasonable to assume that important aspects related to SSCs physiology are phylogenetically conserved among vertebrates, or at least in teleosts and mammals. However, further investigations are still required to determine if any differences from patterns already known in mammals do exist, which would allow discovering potentially novel (fish specific?) signaling pathways. Despite that, besides providing a better knowledge of fish SSCs biology, the findings reported here represent crucial steps for the progress toward the development of new biotechnologies in fish production. Therefore, we expect that these results will be valuable tools furthering the development of better methods for *in vitro* SSCs expansion, which represent an important prerequisite for SSC-based genetic manipulations in fish.

Acknowledgments

Technical help from Mara Lívia Santos and financial support from FAPEMIG and CNPq are fully appreciated.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2013.06.013.

References

Aoki, Y., Nakamura, S., Ishikawa, Y., Tanaka, M., 2009. Expression and syntenic analyses of four nanos genes in medaka. Zool. Sci. 26, 112–118.
Aponte, P.M., Soda, T., Teerds, K.J., Mizrak, S.C., van de Kant, H.J., de Rooij, D.G., 2008. Propagation of bovine spermatogonial stem cells in vitro. Reproduction 136, 543–557.
Baloh, R.H., Enomoto, H., Johnson Jr., E.M., Milbrandt, J., 2000. The GDNF family ligands and receptors – implications for neural development. Curr. Opin. Neurobiol. 10, 103–110.
Barrios, F., Filipponi, D., Pellegrini, M., Paronetto, M.P., Di Siena, S., Geremia, R., Rossi, P., De Felici, M., Jannini, E.A., Dolci, S., 2010. Opposing effects of retinoic acid and FGF9 on Nanos2 expression and meiotic entry of mouse germ cells. J. Cell Sci. 123, 871–880.
Beer, R.L., Draper, B.W., 2012. Nanos3 maintains germline stem cells and expression of the conserved germline stem cell gene nanos2 in the zebrafish ovary. Dev. Biol. 374, 308–318.
Buageaw, A., Sukhwani, M., Ben-Yehudah, A., Ehmcke, J., Rawe, V.Y., Pholpramool, C., Orwig, K.E., Schlatt, S., 2005. GDNF family receptor alpha1 phenotype of spermatogonial stem cells in immature mouse testes. Biol. Reprod. 73, 1011–1016.
Campos-Junior, P.H., Costa, G.M., Lacerda, S.M., Rezende-Neto, J.V., de Paula, A.M., Hofmann, M.C., de França, L.R., 2012. The spermatogonial stem cell niche in the collared peccary (*Tayassu tajacu*). Biol. Reprod. 86, 1–10.
Cnaani, A., Lee, B.Y., Ozouf-Costaz, C., Bonillo, C., Baroiller, J.F., D'Cotta, H., Kocher, T., 2007. Mapping of sox2 and sox14 in tilapia (Oreochromis spp.). Sex Dev. 1, 207–210.
Conrad, S., Renninger, M., Hennenlotter, J., Wiesner, T., Just, L., Bonin, M., Aicher, W., Bühring, H.J., Mattheus, U., Mack, A., Wagner, H.J., Minger, S., Matzkies, M., Reppel, M., Hescheler, J., Sievert, K.D., Stenzl, A., Skutella, T., 2008. Generation of pluripotent stem cells from adult human testis. Nature 456, 344–349.
Costa, G.M., Avelar, G.F., Rezende-Neto, J.V., Campos-Junior, P.H., Lacerda, S.M., Andrade, B.S., Thomé, R.G., Hofmann, M.C., Franca, L.R., 2012. Spermatogonial stem cell markers and niche in equids. PLoS One 7, e44091.
de Rooij, D.G., Russell, L.D., 2000. All you wanted to know about spermatogonia but were afraid to ask. J. Androl. 21, 776–798.
Dettin, L., Ravindranath, N., Hofmann, M.C., Dym, M., 2003. Morphological characterization of the spermatogonial subtypes in the neonatal mouse testis. Biol. Reprod. 69, 1565–1571.
Draper, B.W., McCallum, C.M., Moens, C.B., 2007. Nanos1 is required to maintain oocyte production in adult zebrafish. Dev. Biol. 305, 589–598.
Froschauer, A., Khatun, M.M., Sprott, D., Franz, A., Rieger, C., Pfennig, F., Gutzeit, H.O., 2013. Oct4-EGFP reporter gene expression marks the stem cells in embryonic development and in adult gonads of transgenic medaka. Mol. Reprod. Dev. 80, 48–58.
Fujimura, K., Conte, M.A., Kocher, T.D., 2011. Circular DNA intermediate in the duplication of Nile tilapia vasa genes. PLoS One 6, e29477.
Gassei, K., Ehmcke, J., Schlatt, S., 2009. Efficient enrichment of undifferentiated GFRalpha1+ spermatogonia from immature rat testis by magnetic activated cell sorting. Cell Tissue Res. 337, 177–183.

Gassei, K., Ehmcke, J., Dhir, R., Schlatt, S., 2010. Magnetic activated cell sorting allows isolation of spermatogonia from adult primate testes and reveals distinct GFRa1-positive subpopulations in men. J. Med. Primatol. 39, 83–91.
Grisanti, L., Falciatori, I., Grasso, M., Dovere, L., Fera, S., Muciaccia, B., Fuso, A., Berno, V., Boitani, C., Stefanini, M., Vicini, E., 2009. Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. Stem Cells 27, 3043–3052.
Gustafson, E.A., Wessel, G.M., 2010. Vasa genes: emerging roles in the germ line and in multipotent cells. Bioessays 32, 626–637.
Hayashi, M., Sato, M., Iwasaki, Y., Terasawa, M., Tashiro, M., Yokoyama, S., Katayama, N., Sadaie, S., Miwa, M., Yoshizaki, G., 2012. Combining next-generation sequencing with microarray for transcriptome analysis in rainbow trout gonads. Mol. Reprod. Dev. 79, 870–878.
He, Z., Kokkinaki, M., Jiang, J., Dobrinski, I., Dym, M., 2010. Isolation, characterization, and culture of human spermatogonia. Biol. Reprod. 82, 363–372.
He, Z., Kokkinaki, M., Jiang, J., Zeng, W., Dobrinski, I., Dym, M., 2012. Isolation of human male germ-line stem cells using enzymatic digestion and magnetic-activated cell sorting. Methods Mol. Biol. 825, 45–57.
Hermann, B.P., Sukhwani, M., Hansel, M.C., Orwig, K.E., 2010. Spermatogonial stem cells in higher primates: are there differences from those in rodents? Reproduction 139, 479–493.
Hofmann, M.C., 2008. Gdnf signaling pathways within the mammalian spermatogonial stem cell niche. Mol. Cell Endocrinol. 288, 95–103.
Hofmann, M.C., Braydich-Stolle, L., Dym, M., 2005. Isolation of male germ-line stem cells; influence of GDNF. Dev. Biol. 279, 114–124.
Honaramooz, A., Yang, Y., 2010. Recent advances in application of male germ cell transplantation in farm animals. Vet. Med. Int. Pii 657860.
Hong, Y., Liu, T., Zhao, H., Xu, H., Wang, W., Liu, R., Chen, T., Deng, J., Gui, J., 2004. Establishment of a normal medaka fish spermatogonial cell line capable of sperm production in vitro. Proc. Natl. Acad. Sci. USA 101, 8011–8016.
Hong, Y., Winkler, C., Schartl, M., 1996. Pluripotency and differentiation of embryonic stem cell lines from the medaka fish (*Oryzias latipes*). Mech. Dev. 60, 33–44.
Hong, Y., Schartl, M., 1996. Isolation and differentiation of medaka embryonic stem cells. In: Turksen, K. (Ed.), Embryonic Stem Cell Protocols. Methods in Molecular Biology. Humana Press Inc, New Jersey, pp. 5–16.
Izadyar, F., Spierenberg, G.T., Creemers, L.B., den Ouden, K., de Rooij, D.G., 2002. Isolation and purification of type A spermatogonia from the bovine testis. Reproduction 124, 85–94.
Jaruzelska, J., Kotecki, M., Kusz, K., Spik, A., Firpo, M., Reijo Pera, R.A., 2003. Conservation of a Pumilio-Nanos complex from Drosophila germ plasm to human germ cells. Dev. Genes Evol. 213, 120–126.
Jijiwa, M., Kawai, K., Fukihara, J., Nakamura, A., Hasegawa, M., Suzuki, C., Sato, T., Enomoto, A., Asai, N., Murakumo, Y., Takahashi, M., 2008. GDNF-mediated signaling via RET tyrosine 1062 is essential for maintenance of spermatogonial stem cells. Genes Cells 13, 365–374.
Jing, S., Wen, D., Yu, Y., Holst, P.L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R., Louis, J.C., Hu, S., Altrock, B.W., Fox, G.M., 1996. GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. Cell 85, 1113–1124.
Kajiura-Kobayashi, H., Kobayashi, T., Nagahama, Y., 2005. Cloning of cDNAs and the differential expression of A-type cyclins and Dmc1 during spermatogenesis in the Japanese eel, a teleost fish. Dev. Dyn. 232, 1115–1123.
Kala, S., Kaushik, R., Singh, K.P., Kadam, P.H., Singh, M.K., Manik, R.S., Singla, S.K., Palta, P., Chauhan, M.S., 2012. In vitro culture and morphological characterization of prepubertal buffalo (*Bubalus bubalis*) putative spermatogonial stem cell. J. Assist. Reprod. Genet. 29, 1335–1342.
Kawasaki, T., Saito, K., Sakai, C., Shinya, M., Sakai, N., 2012. Production of zebrafish offspring from cultured spermatogonial stem cells. Genes Cells 17, 316–325.
Kobayashi, T., Kajiura-Kobayashi, H., Nagahama, Y., 1998. A novel stage-specific antigen is expressed only in early stages of spermatogonia in Japanese eel, *Anguilla japonica* testis. Mol. Reprod. Dev. 51, 355–361.
Kobayashi, T., Kajiura-Kobayashi, H., Nagahama, Y., 2002. Two isoforms of vasa homologs in a teleost fish: their differential expression during germ cell differentiation. Mech. Dev. 111, 167–171.
Kolasa, A., Misiakiewicz, K., Marchlewicz, M., Wiszniewska, B., 2012. The generation of spermatogonial stem cells and spermatogonia in mammals. Reprod. Biol. 12, 5–23.
Kuijk, E.W., Colenbrander, B., Roelen, B.A., 2009. The effects of growth factors on in vitro-cultured porcine testicular cells. Reproduction 138, 721–731.
Lacerda, S.M., Aponte, P.M., Costa, G.M., Campos-Junior, P.H., Segatelli, T.M., Silva, M.A., França, L.R., 2012. An overview of spermatogonial stem cell physiology, niche and transplantation in fish. Anim. Reprod. 9, 798–808.
Lacerda, S.M., Batlouni, S.R., Costa, G.M., Segatelli, T.M., Quirino, B.R., Queiroz, B.M., Kalapothakis, E., França, L.R., 2010. A new and fast technique to generate offspring after germ cells transplantation in adult fish: the Nile tilapia (*Oreochromis niloticus*) model. PLoS One 5, e10740.
Lacerda, S.M., Batlouni, S.R., Silva, S.B., Homem, C.S., França, L.R., 2006. Germ cells transplantation in fish: the Nile-tilapia model. Anim. Reprod. 3, 146–159.
Lacerda, S.M., Costa, G.M., Campos-Junior, P.H., Segatelli, T.M., Yazawa, R., Takeuchi, Y., Morita, T., Yoshizaki, G., França, L.R., 2013. Germ cell transplantation as a potential biotechnological approach to fish reproduction. Fish Physiol. Biochem. 39, 3–11.
Leal, M.C., Cardoso, E.R., Nóbrega, R.H., Batlouni, S.R., Bogerd, J., França, L.R., Schulz, R.W., 2009. Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. Biol. Reprod. 81, 177–187.
Lok, D., Weenk, D., De Rooij, D.G., 1982. Morphology, proliferation, and differentiation of undifferentiated spermatogonia in the Chinese hamster and the ram. Anat. Rec. 203, 83–99.
Mirzapour, T., Movahedin, M., Tengku Ibrahim, T.A., Koruji, M., Haron, A.W., Nowroozi, M.R., Rafieian, S.H., 2012. Effects of basic fibroblast growth factor and leukaemia inhibitory factor on proliferation and short-term culture of human spermatogonial stem cells. Andrologia 44, 41–55.
Mochizuki, K., Sano, H., Kobayashi, S., Nishimiya-Fujisawa, C., Fujisawa, T., 2000. Expression and evolutionary conservation of nanos-related genes in Hydra. Dev. Genes Evol. 210, 591–602.
Nagano, M.C., Yeh, J.R., 2013. The identity and fate decision control of spermatogonial stem cells: where is the point of no return? Curr. Top. Dev. Biol. 102, 61–95.
Nagasawa, K., Miwa, M., Yazawa, R., Morita, T., Takeuchi, Y., Yoshizaki, G., 2012. Characterization of lymphocyte antigen 75 (Ly75/CD205) as a potential cell-surface marker on spermatogonia in Pacific bluefin tuna *Thunnus orientalis*. Fish Sci. 78, 791–800.
Nagasawa, K., Shikina, S., Takeuchi, Y., Yoshizaki, G., 2010. Lymphocyte antigen 75 (Ly75/CD205) is a surface marker on mitotic germ cells in rainbow trout. Biol. Reprod. 83, 597–606.
Nakagawa, T., Sharma, M., Nabeshima, Y., Braun, R.E., Yoshida, S., 2010. Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science 328, 62–67.
Naughton, C.K., Jain, S., Strickland, A.M., Gupta, A., Milbrandt, J., 2006. Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. Biol. Reprod. 74, 314–321.
Nóbrega, R.H., Greebe, C.D., van de Kant, H., Bogerd, J., de França, L.R., Schulz, R.W., 2010. Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. PLoS One 5, e12808.
Oatley, J.M., Brinster, R.L., 2012. The germline stem cell niche unit in mammalian testes. Physiol. Rev. 92, 577–595.
Oatley, M.J., Kaucher, A.V., Racicot, K.E., Oatley, J.M., 2011. Inhibitor of DNA binding 4 is expressed selectively by single spermatogonia in the male germline and regulates the self-renewal of spermatogonial stem cells in mice. Biol. Reprod. 85, 347–356.
Okutsu, T., Suzuki, K., Takeuchi, Y., Takeuchi, T., Yoshizaki, G., 2006. Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. Proc. Natl. Acad. Sci. USA 103, 2725–2729.
Onichtchouk, D., 2012. Pou5f1/oct4 in pluripotency control: insights from zebrafish. Genesis 50, 75–85.
Ozaki, Y., Saito, K., Shinya, M., Kawasaki, T., Sakai, N., 2011. Evaluation of Sycp3, Plzf and Cyclin B3 expression and suitability as spermatogonia and spermatocyte markers in zebrafish. Gene Expr. Patterns 11, 309–315.
Pfennig, F., Kurth, T., Meissner, S., Standke, A., Hoppe, M., Zieschang, F., Reitmayer, C., Göbel, A., Kretzschmar, G., Gutzeit, H.O., 2012. The social status of the male Nile tilapia (*Oreochromis niloticus*) influences testis structure and gene expression. Reproduction 143, 71–84.
Phillips, B.T., Gassei, K., Orwig, K.E., 2010. Spermatogonial stem cell regulation and spermatogenesis. Philos. Trans. R. Soc. Lond. B Biol. Sci. 365, 1663–1678.
Powell, R.H., Biancardi, M.N., Galiguis, J., Qin, Q., Pope, C.E., Leibo, S.P., Wang, G., Gómez, M.C., 2012. 286 expression of pluripotent stem cell markers in domestic cat spermatogonial cells. Reprod. Fertil. Dev. 25, 290–291.
Sada, A., Hasegawa, K., Pin, P.H., Saga, Y., 2012. NANOS2 acts downstream of glial cell line-derived neurotrophic factor signaling to suppress differentiation of spermatogonial stem cells. Stem Cells 30, 280–291.
Sada, A., Suzuki, A., Suzuki, H., Saga, Y., 2009. The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. Science 325, 1394–1398.
Sánchez-Sánchez, A.V., Camp, E., García-España, A., Leal-Tassias, A., Mullor, J.L., 2010. Medaka Oct4 is expressed during early embryo development, and in primordial germ cells and adult gonads. Dev. Dyn. 239, 672–679.
Sato, T., Aiyama, Y., Ishii-Inagaki, M., Hara, K., Tsunekawa, N., Harikae, K., Uemura-Kamata, M., Shinomura, M., Zhu, X.B., Maeda, S., Kuwahara-Otani, S., Kudo, A., Kawakami, H., Kanai-Azuma, M., Fujiwara, M., Miyamae, Y., Yoshida, S., Seki, M., Kurohmaru, M., Kanai, Y., 2011. Cyclical and patch-like GDNF distribution along the basal surface of Sertoli cells in mouse and hamster testes. PLoS One 6, e28367.
Schulz, R.W., de França, L.R., Lareyre, J.J., Le Gac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411.
Shikina, S., Yoshizaki, G., 2010. Improved in vitro culture conditions to enhance the survival, mitotic activity, and transplantability of rainbow trout type a spermatogonia. Biol. Reprod. 83, 268–276.
Shikina, S., Ihara, S., Yoshizaki, G., 2008. Culture conditions for maintaining the survival and mitotic activity of rainbow trout transplantable type A spermatogonia. Mol. Reprod. Dev. 75, 529–537.
Silva, R.C., Costa, G.M., Lacerda, S.M., Batlouni, S.R., Soares, J.M., Avelar, G.F., Böttger, K.B., Silva Jr., S.F., Nogueira, M.S., Andrade, L.M., França, L.R., 2012. Germ cell transplantation in felids: a potential approach to preserving endangered species. J. Androl. 33, 264–276.
Suzuki, H., Sada, A., Yoshida, S., Saga, Y., 2009. The heterogeneity of spermatogonia is revealed by their topology and expression of marker proteins including the germ cell-specific proteins Nanos2 and Nanos3. Dev. Biol. 336, 222–231.
Takehashi, M., Tada, M., Kanatsu-Shinohara, M., Morimoto, H., Kazuki, Y., Oshimura, M., Tada, T., Shinohara, T., 2012. Hybridization of testis-derived stem cells with

somatic cells and embryonic stem cells in mice. Biol. Reprod. 86, 178, 10.1095/biolreprod.112.098988.
Takeuchi, Y., Yoshizaki, G., Takeuchi, T., 2004. Biotechnology: surrogate broodstock produces salmonids. Nature 430, 629–630.
Tang, L., Rodriguez-Sosa, J.R., Dobrinski, I., 2012. Germ cell transplantation and testis tissue xenografting in mice. J. Vis. Exp. 6 (60), pii: 3545. 10.3791/3545.
Thoma, E.C., Wagner, T.U., Weber, I.P., Herpin, A., Fischer, A., Schartl, M., 2011. Ectopic expression of single transcription factors directs differentiation of a medaka spermatogonial cell line. Stem Cells Dev. 20, 1425–1438.
Tokuda, M., Kadokawa, Y., Kurahashi, H., Marunouchi, T., 2007. CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. Biol. Reprod. 76, 130–141.
van der Wee, K.S., Johnson, E.W., Dirami, G., Dym, T.M., Hofmann, M.C., 2001. Immunomagnetic isolation and long-term culture of mouse type A spermatogonia. J. Androl. 22, 696–704.
von Schonfeldt, V., Wistuba, J., Schlatt, S., 2004. Notch-1, c-kit and GFRalpha-1 are developmentally regulated markers for premeiotic germ cells. Cytogenet. Genome Res. 105, 235–239.
Wang, D., Manali, D., Wang, T., Bhat, N., Hong, N., Li, Z., Wang, L., Yan, Y., Liu, R., Hong, Y., 2011. Identification of pluripotency genes in the fish medaka. Int. J. Biol. Sci. 7, 440–451.
Wang, Z., Lin, H., 2004. Nanos maintains germline stem cell self-renewal by preventing differentiation. Science 303, 2016–2019.
Westerfield, M., 1995. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), third ed. Eugene, OR.
Yano, A., von Schalburg, K., Cooper, G., Koop, B.F., Yoshizaki, G., 2009. Identification of a molecular marker for type A spermatogonia by microarray analysis using gonadal cells from pvasa-GFP transgenic rainbow trout (*Oncorhynchus mykiss*). Mol. Reprod. Dev. 76, 246–254.
Yoshida, S., Sukeno, M., Nabeshima, Y., 2007. A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 317, 1722–1726.
Yoshizaki, G., Okutsu, T., Morita, T., Terasawa, M., Yazawa, R., Takeuchi, Y., 2012. Biological characteristics of fish germ cells and their application to developmental biotechnology. Reprod. Domest. Anim. 47, 187–192.

9. ANEXO II

RSC Advances

COMMUNICATION

View Article Online
View Journal | View Issue

CrossMark click for updates

Cite this: *RSC Adv.*, 2014, **4**, 37985

Received 11th June 2014
Accepted 4th August 2014

DOI: 10.1039/c4ra05621c

www.rsc.org/advances

Gene delivery to Nile tilapia spermatogonial stem cells using carboxi-functionalized multiwall carbon nanotubes†

F. M. P. Tonelli,[a] S. M. S. N. Lacerda,[b] M. A. Silva,[b] E. S. Ávila,[c] L. O. Ladeira,[c] L. R. França[b] and R. R. Resende*[a]

Carboxi-functionalized multiwall carbon nanotubes (fMWCNTs), when complexed with DNA, can promote gene delivery to Nile tilapia spermatogonial stem cells with higher transfection efficiency than cationic lipids or electroporation, causing also less cell death.

Studies on gene transfer to fish cells have been made since the 80s (ref. 1) using different techniques such as electroporation,[2] proteins conjugated to polyamine,[3] cationic lipids,[4] microinjection of newly fertilized eggs,[5] among others. However, despite significant and recent progress, frequently, these methods have shown limited success due to technical difficulties, resulting in a low rate of transfection and consequently high cost. With the aim of finding different gene delivery systems to deliver genes or plasmids to cells, alternative gene carriers have been developed.[6–8] Among them, are the functionalized carbon nanotubes whose potential as gene transfer vectors has been explored and generated promising results.[9–12] However, this important ability of carbon nanotubes has not yet been evaluated in fish cells.

When it comes to fish cells, spermatogonial stem cells (SSCs) are noteworthy. Because of its unique ability to transmit genetic information to offspring through the male gamete, the genetic modification of SSC represents an excellent alternative strategy to produce transgenic animals, particularly if we consider that usually a single spermatogonial stem cell gives rise to several hundred sperm.[13] Fish production through transgenic techniques offers many potential economic advantages for commercial aquaculture production, including introduction of new or novel traits and increased response to selection for faster growth.

Here, we report for the first time the use of carboxi-functionalized multiwall carbon nanotubes (fMWCNTs) to transfect Nile tilapia (*Oreochromis niloticus*) SSCs, achieving higher transcription level of transgene and less cell death when compared to cationic lipids (Lipofectamine 2000® – Life Technologies) and electroporation, conventional methods to perform the gene delivery.

MWCNTs (Fig. 1a and 2a) were produced by the chemical vapor deposition method (CVD) using cobalt and iron as

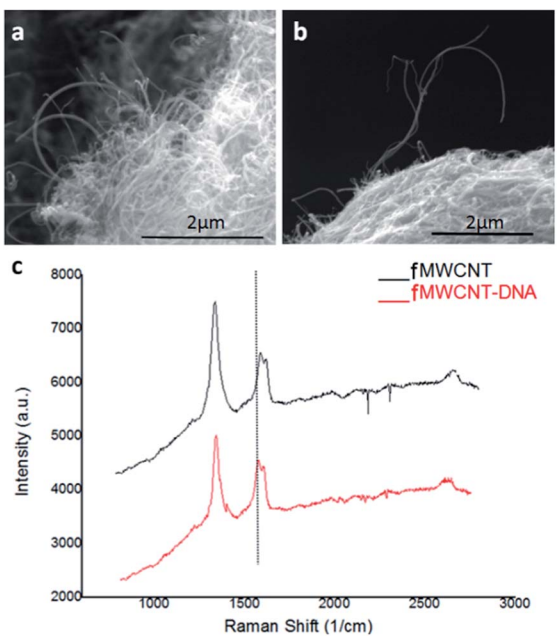


Fig. 1 (a) Field Emission Gun Scanning Electron Microscopy (FEG-SEM) image of MWCNTs. (b) FEG-SEM image of fMWCNTs. (c) Raman spectra obtained for nanotubes (fMWCNTs), and the complex (fMWCNT-DNA). The G band downshift induced by the complex formation is highlighted.

[a]*Cell Signaling and Nanobiotechnology Laboratory, Department of Biochemistry and Immunology, Federal University of Minas Gerais, Bloco N4 112, Av Antônio Carlos, 6627, 31270-901, Belo Horizonte, Brazil. E-mail: rrresende@hotmail.com*

[b]*Cell Biology Laboratory, Department of Morphology, Federal University of Minas Gerais, Belo Horizonte, Brazil*

[c]*Nanomaterials Laboratory, Department of Physics and Center of Microscopy, Federal University of Minas Gerais, Belo Horizonte, Brazil*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c4ra05621c

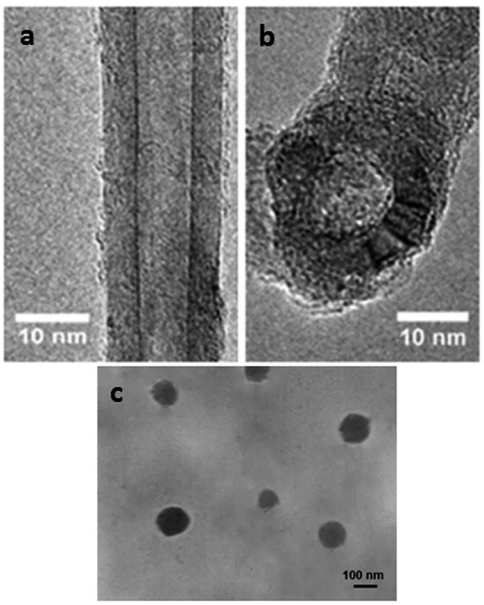


Fig. 2 (a) Transmission Electron Microscopy (TEM) image of MWCNT. (b) fMWCNT. (c) Lipofectamine 2000®.

catalysts, and ethylene as the carbon source. Subsequently, carbon nanotubes underwent acid treatment to remove impurities, catalyst and amorphous materials. They were functionalized through oxidation in nitric/sulfuric acid[14,15] for 15 minutes, using microwaves. The fMWCNTs were then washed in neutral pH, and dried. Carboxyl group functionalization of MWCNTs was confirmed by FT-NIR (Fourier Transform Near-Infrared spectroscopy). The CNTs had an average length of 200 nm, and an average outer diameter of 25 nm; through thermal analysis of fMWCNTs it was determined the weight loss related to carboxylic function: 6.36%.

The plasmid DNA (6121 pb – 20 nM) containing the endogenous *O. niloticus* β-actin promoter, and the gene of AmCyan1 fluorescent protein was conjugated to the fMWCNT (Fig. 1b and 2b) (0.25 mg mL^{-1}) through ultrasonic bath (25 kHz, 100 W for 30 minutes). The attachment was confirmed through resonant Raman spectroscopy assisted by optical microscopy (Fig. 1c). The complex fMWCNT-DNA formation induced a downshift (at ~8 cm^{-1}) of the tangential G band (localized at ~1580 cm^{-1}). This behavior is essentially attributed to the charge transfer between the oxygen atoms on the surface of the fMWCNTs and the electron donating groups present in the DNA, such as amine groups.[16]

The complex fMWCNT-DNA was added to primary culture of Nile tilapia SSCs (10^6 cell per dish) obtained by density gradient centrifugation and differential plating.[17] The gene delivery was also performed by Lipofectamine 2000® (Fig. 2c) and electroporation (225 V and 50 μF). Cells were then incubated for 24 hours at 28 °C and 5% CO_2. After this period, internalization of plasmid DNA into cells and induction of transgene expression were accessed by fluorescence microscopy (Fig. 3), and cells viability through flow cytometry (Fig. 4) using the Annexin-V-FLUOS staining kit (Roche).

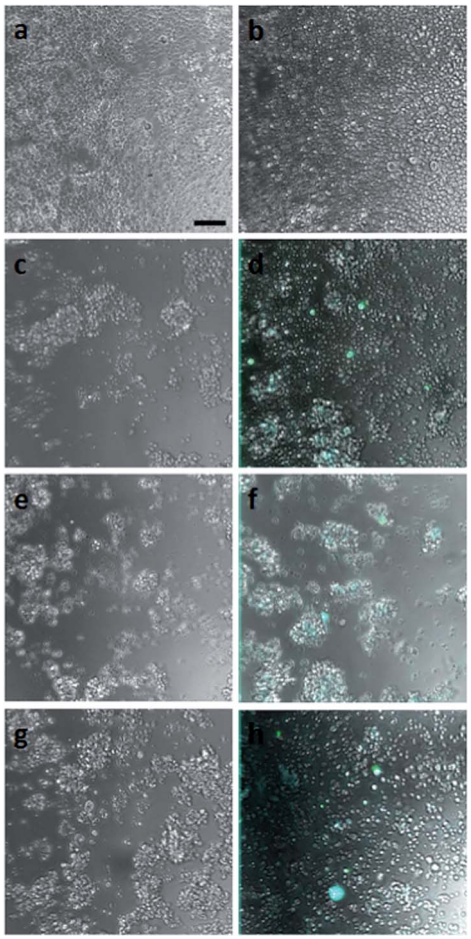


Fig. 3 Fluorescence microscopy images of SSCs culture; (a) control; (b) DNA 20 nM and no vehicle; (c) fMWCNTs; (d) fMWCNTs + DNA 20 nM; (e) electroporation; (f) electroporation + DNA 20 nM; (g) Lipofectamine 2000®; (h) Lipofectamine 2000® + DNA 20 nM. Bar = 50 μm.

The cyan fluorescence in SSCs was observed for all techniques/vehicles of gene delivery investigated (Fig. 3d, f and h), and in the absence of plasmid DNA no fluorescence was

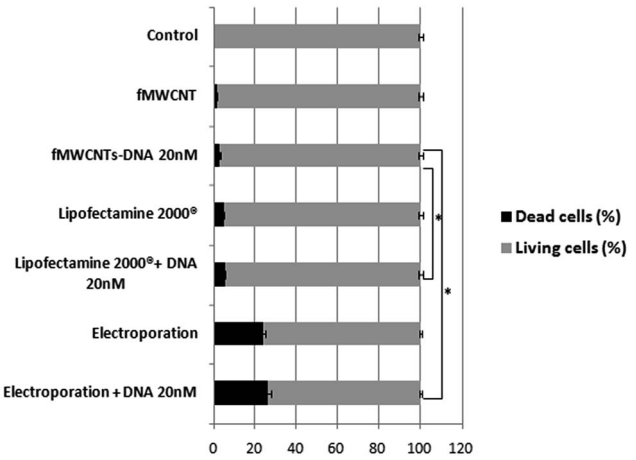


Fig. 4 Dead and living cells percentage obtained through flow cytometry for the different methods of gene delivery tested (asterisk, $p < 0.05$, t test).

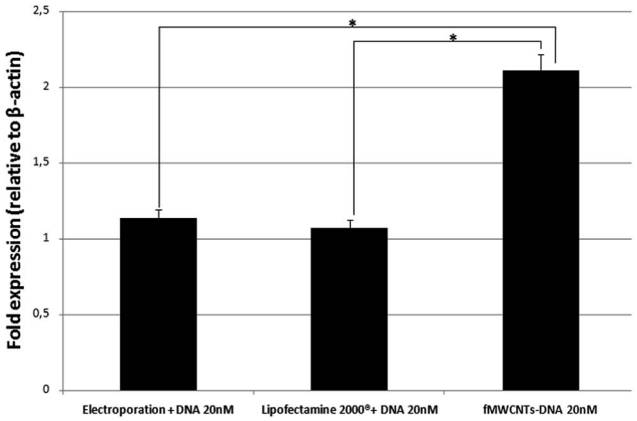


Fig. 5 Dosage of transgene's expression by qPCR. The values correspond to expression of AmCyan1 mRNA in relation to β-actin's in Nile SSCs using different methods of transfection (asterisk, $p < 0.05$, t test).

detected for these techniques (Fig. 3c, e and g). As expected, when only the DNA (20 nM), without any delivery vehicle, was added to SSCs culture (Fig. 3b) it did not induced AmCyan1 expression, showing therefore no passive incorporation of plasmids by the fish cells.

Cell death rate observed in transfection using fMWCNTs was significantly lower compared to those in which Lipofectamine or electroporation were applied to gene delivery. These results indicate that fMWCNTs have low cytotoxic effect on Nile tilapia SSCs at the concentration used. Similarly to results obtained for zebrafish neurons,[18] higher cytotoxicity was induced by electroporation.

SSCs RNA were extracted with TRIzol® reagent (Life) and underwent RT-PCR using oligodT primer. To access transcription level of AmCyan1 fluorescent protein, q-PCR (Fig. 5) was performed using the cDNA generated, and primers: for the housekeeping gene β-actin (FWD-CGGTATGGAGTCTTGTGGTA TC; REV-AGCACAGTGTTGGCGTATAA) and for the AmCyan1 gene (FWD-TTCGAGAAGATGACCGTGTG; REV-AGGTGTG GAAC TGGCATCTGTA).

Our experimental data revealed that the transcription level of AmCyan1 gene is higher when the gene delivery is performed using the fMWCNT. This expression was 2.1 (±0.1) fold higher than the β-actin and also significantly superior to the expression achieved by electroporation or Lipofectamine. In zebrafish fibroblasts, low efficiency of transgene expression was previously observed using Lipofectamine. This limitation could be due to intracellular barriers as, for example, poor endocytosis.[19]

Conclusions

In conclusion, we reported for the first time success in the use of carbon nanotubes to perform the gene delivery to a fish cell. The fMWCNTs were able to transfect the Nile tilapia SSCs inducing low cytotoxicity. Cell death percentage obtained was lower than those observed for electroporation and cationic lipids, which are commonly used for gene transfection. Moreover, fMWCNTs were able to deliver plasmid DNA more efficiently than the other methods tested. The expression level of cyan fluorescent protein gene related to β-actin, was significantly higher when exposing SSCs to the complex fMWCNT-DNA, instead of using Lipofectamine 2000® or electroporation.

Notes and references

1 G. Brem, B. Brenig, G. Hörstgen-Schwark and E. Winnacker, *Aquaculture*, 1998, **68**, 209–219.
2 K. Schuster and A. Ghysen, *Cold Spring Harbour Protocols*, 2013, **12**, 1169–1171.
3 Y. Yan, N. Hong, T. Chen, M. Li, T. Wang, G. Guan, Y. Qiao, S. Chen, M. Schartl, C. M. Li and Y. Hong, *PLoS One*, 2013, **8**, e59400.
4 A. M. Sandbichler, T. Aschberger and B. Pelster, *BioRes. Open Access*, 2013, **2**, 20–27.
5 L. Zhang, C. Sun, X. Ye, S. Zou, M. Lu, Z. Liu and Y. Tian, *Fish Physiol. Biochem.*, 2014, **40**, 221–233.
6 K. C. F. Leung, C. P. Chak, S. F. Lee, J. M. Y. Lai, X. M. Zhu, Y. X. J. Wang, K. W. Y. Sham and C. H. K. Cheng, *Chem. Commun.*, 2013, **49**, 549–551.
7 Y. Tao, E. Ju, J. Ren and X. Qu, *Chem. Commun.*, 2013, **49**, 9791–9793.
8 Q. Peng, F. Chen, Z. Zhong and R. Zhuo, *Chem. Commun.*, 2010, **46**, 5888–5890.
9 D. Pantarotto, R. Singh, D. McCarthy, M. Erhardt, J. P. Briand, M. Prato, K. Kostarelos and A. Bianco, *Angew. Chem.*, 2004, **116**, 5354–5358.
10 A. Bianco, K. Kostarelos, C. D. Partidos and M. Prato, *Chem. Commun.*, 2005, **5**, 571–577.
11 V. Ramos-Perez, A. Cifuentes, N. Coronas, A. de Pablo and S. Borrós, *Nanomaterial Interfaces in Biology*, 2013, vol. 1025.
12 M. S. Ladeira, V. A. Andrade, E. R. Gomes, C. J. Aguiar, E. R. Moraes, J. S. Soares, E. E. Silva, R. G. Lacerda, L. O. Ladeira, A. Jorio, P. Lima, M. F. Leite, R. R. Resende and S. Guatimosim, Highly efficient siRNA delivery system into human and murine cells using single-wall carbon nanotubes, *Nanotechnology*, 2010, **21**(38), 385101, DOI: 10.1088/0957-4484/21/38/385101.
13 B. T. Phillips, K. Gassei and K. E. Orwig, *Philos. Trans. R. Soc., B*, 2010, **365**, 1663–1678.
14 N. G. Sahoo, H. Bao, Y. Pan, M. Pal, M. Kakran, H. K. F. Cheng, L. Li and L. P. Tan, *Chem. Commun.*, 2011, **47**, 5235–5237.
15 E. Lorençon, A. S. Ferlauto, S. de Oliveira, D. R. Miquita, R. R. Resende, R. G. Lacerda and L. O. Ladeira, Direct production of carbon nanotubes/metal nanoparticles hybrids from a redox reaction between metal ions and reduced carbon nanotubes, *ACS Appl. Mater. Interfaces*, 2009, **1**(10), 2104–2106, DOI: 10.1021/am900424m.
16 Z. Li, Z. Wu and K. Li, *Anal. Biochem.*, 2009, **387**, 267–270.
17 S. M. S. N. Lacerda, S. R. Batlouni, S. B. G. Silva, C. S. P. Homem and L. R. França, *Anim. Reprod.*, 2006, **3**, 146–159.
18 M. Hendricks and S. Jesuthasan, *Neural Dev.*, 2007, **2**, 634–642.
19 R. Badakov and A. Jaźwińska, *Cytotechnology*, 2006, **51**, 105–110.