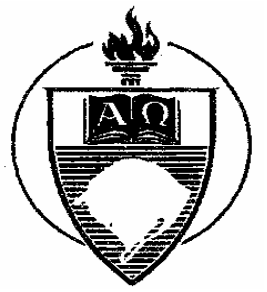

CRISTINA LUÍSA CONCEIÇÃO DE OLIVEIRA

ANÁLISE COMPARADA DA ULTRAESTRUTURA DOS ESPERMATOZÓIDES E MORFOLOGIA DA GLÂNDULA BRANQUIAL EM ESPÉCIES DE CHEIRODONTINAE (CHARACIFORMES: CHARACIDAE)

Tese apresentada ao Programa de Pós-Graduação em Biologia Animal, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do título de Doutora em Biologia Animal.

Área de Concentração: Biologia Comparada

Orientador: Prof. Dr. Luiz Roberto Malabarba

Co-orientador: Prof. Dr. John Robert Burns

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SUMÁRIO

RESUMO

A tese é divida em três capítulos, seguindo as regras da revista *Journal of Morphology*. O primeiro capítulo descreve a ultraestrutura dos espermatozóides de seis queirodontíneos inseminadores pertencentes à tribo Compsurini (*Kolpotocheirodon theloura, Compsura heterura, Acinocheirodon melanogramma, Saccoderma hastatus, "Odontostilbe" dialeptura* e *"Odontostilbe" mitoptera*), e de quatro espécies de fertilização externa, três destas pertencentes à tribo Cheirodontini (*Cheirodon interruptus*, *Serrapinnus calliurus,* e *Serrapinnus heterodon*) e uma espécie *incertae sedis* na subfamília (*Odontostilbe pequira*). Os espermatozóides de espécies de fertilização externa mostram estruturas conservativas e os espermatozóides de espécies inseminadoras apresentam mudanças estruturais, principalmente no núcleo espermático. As espécies da tribo Compsurini apresentam espermatozóides com peças intermediárias mais longas, que se estreitam progressivamente no sentido distal, e vesículas mais largas e em menor número. Em espécies de fertilização externa as peças intermediárias são mais curtas e terminam abruptamente, e as vesículas são menores e em maior número. Uma matriz foi construída baseada em dez caracteres da ultraestrutura dos espermatozóides. Obteve-se uma hipótese de relações parcialmente resolvida entre espécies inseminadoras. A análise somente de caracteres de ultraestrutura de espermatozóides, no entanto, não foi informativa para a hipótese de relações entre espécies de fertilização externa. As relações entre os membros de Cheirodontinae e a evolução da morfologia dos espermatozóides são discutidas. O segundo capítulo descreve a ultraestrutura dos espermatozóides da espécie inseminadora *Macropsobrycon uruguayanae.* Os espermatozóides apresentam núcleos moderadamente alongados e cromatina elétron-densa. Durante a espermiogênese a rotação nuclear acontece, deixando o flagelo posterior ao núcleo. Os centríolos são

paralelos, e o centríolo proximal é ligeiramente anterior ao distal. A ponta do centríolo proximal encontra-se dentro da rasa fossa nuclear. Estrias centriolares denominadas de rootlets partem de ambos centríolos. Nove microtúbulos acessórios circulam o axonema externamente. O flagelo tem axonema com a configuração típica (9+2). Além dos espermatozóides normais, são encontrados no lúmen testicular espermatozóides atípicos denominados paraespermatozóides. Estas células se assemelham ao espermatozóide em muitos aspectos, mas seu núcleo tem forma irregular e a cromatina é menos elétron-densa. Discutem-se as especializações vistas nos espermatozóides, as possíveis adaptações relacionadas ao hábito de inseminação e o fato de que a origem e função dos paraespermatozóides permanecem indeterminadas em *Macropsobrycon uruguayanae*. O terceiro capítulo descreve a glândula branquial de 17 espécies de Cheirodontinae. A glândula branquial está localizada na região anterior da cavidade branquial em ambos os lados do corpo. Esta estrutura foi encontrada em todos machos maduros. A glândula é pequena em espécies de fertilização externa ocupando no máximo 10 filamentos branquiais e em espécies inseminadoras, ela ocupa uma grande extensão ou o arco inteiro. Em algumas partes da glândula de *A Aphyocheirodon hemigrammus, C. heterura, K. theloura, M. uruguayanae* e *S. hastatus*, as lamelas não permanecem, ficando somente as células secretoras da glândula branquial. Um material não celular foi observado dentro das câmaras da glândula de *K. theloura.* Tanto espécies inseminadoras quanto de fertilização externa de Cheirodontinae apresentam glândula, não existindo relação entre a presença de glândula branquial e inseminação. A função da glândula não é conhecida, mas pela presença desta estrutura somente em machos maduros, esta pode ser usada para produção e liberação de secreção para a atração da fêmea durante o período reprodutivo ou na inibição de outros machos. As glândulas branquiais de queirodontíneos e de outros caracídeos são comparadas.

ABSTRACT

The thesis is divided in three chapters following the manuscript formatting rules of the *Journal of Morphology*. The first chapter describes the spermatozoa ultrastructure of six inseminating cheirodontines of the tribe Compsurini (*Kolpotocheirodon theloura, Compsura heterura, Acinocheirodon melanogramma, Saccoderma hastatus, "Odontostilbe" dialeptura and "Odontostilbe" mitoptera)*, and four externally fertilized species, three belonging to the tribe Cheirodontini (*Cheirodon interruptus, Serrapinnus calliurus*, and *Serrapinnus heterodon*) and one *incertae sedis* species (*Odontostilbe pequira*). Sperm ultrastructure of externally fertilized species has shown to be very conservative, while sperm of inseminating species have changes mostly related to sperm elongation. The species of Compsurini have spermatozoa with the midpiece longer progressively narrowing distally, and wider vesicles and in small number. In externally fertilized species the midpiece is shorter and it finishes abruptly, and the vesicles are smaller and in large number. A matrix was built based on ten characters of the spermatozoa ultrastructure. It was obtained a hypothesis of relationships partially solved among inseminating species, however the analysis of only characters of spermatozoa ultrastructure was uninformative to hypothesise relationship among externally fertilized species. The relationships among the species of the Cheirodontinae and the evolution of the morphology of the spermatozoa are discussed. Second chapter describes inseminated spermatozoa ultrastructure of *Macropsobrycon uruguayanae*. Spermatozoa have moderately elongate nuclei with electron dense chromatin. During spermiogenesis nuclear rotation takes place, leading the flagellum to be posterior to the nucleus. Centrioles are parallel one to another with the proximal centriole slightly anterior to the distal one. Proximal centriole anterior tip is into the shallow nuclear fossa. Striated centriolar rootlets radiate from both centrioles. Nine accessory microtubules surround the axoneme. The flagellum has a typical axoneme configuration (9+2). In

addition to regular sperm, atypical spermatozoa, named parasperm, are also found in the testicular lumen. These cells resemble spermatozoa in most aspects, except that their nuclei have irregular shape and the granular chromatin is less electron-dense than the one seen in spermatozoa. The specializations seen in the spermatozoa are discussed, as well as the possible adaptations related to insemination and the fact that the origin and function of paraspermatozoa remains undetermined. The third chapter describes the gill gland of 17 species of the Cheirodontinae. The gill gland is located in the anterior region of the gill cavity on either side of the midline of all analyzed mature males of the Cheirodontinae. The gill gland is small in externally fertilizing species of the Cheirodontinae, reaching up to 10 gill filaments. In the inseminating species of the tribe Compsurini, the gland occupies a large extension or almost the entire gill arch. In some portions of the gland of *Ap. hemigrammus, C. heterura, Kolpotocheirodon theloura, M. uruguayanae* and *S. hastatus*, the lamellae are not retained, remaining only secretor gill gland cells in these regions. *Kolpotocheirodon theloura* showed noncellular material within the gill gland chambers and they were turgid. There is no relation between the presence of a gill gland and insemination since both externally fertilizing and inseminating characids have or not gill glands. The function of the gill gland is not known yet, but presence of the gill gland in mature males and absence in females suggest that the secretion produced may be used to attract the females during the reproductive period or competition between males. The gill glands of cheirodontines and other characids are compared.

INTRODUÇÃO

Cheirodontinae

As espécies pertencentes à subfamília Cheirodontinae habitam ambientes lênticos e regiões de planície. Os Cheirodontinae são encontrados na maioria das bacias hidrográficas da América do Sul e Central, como bacia Amazônica, Orinoco, Paraná-Paraguai e São Francisco. Quatro espécies de *Cheirodon* são os únicos Characiformes encontrados no oeste dos Andes no Chile (Malabarba, 1998, 2003).

Quatro sinapomorfias são apresentadas por Malabarba (1998) para definir Cheirodontinae: a presença de dentes pedunculados e expandidos na região distal, a presença de uma única série de dentes na premaxila, a ausência de mancha umeral e a existência de uma falha da cobertura muscular na região umeral em ambos lados do corpo na região anterior da bexiga natatória, denominada pseudotímpano – supõe-se que esta ausência de musculatura na região umeral facilite a transmissão de ondas sonoras do ambiente para a bexiga natatória e desta para o ouvido interno através do aparelho de Weber (Malabarba 1998). Recentemente, a monofilia de Cheirodontinae apresentada por Malabarba (1998) foi confirmada por Bührnheim (2006, dados não publicados), baseando-se em uma nova analise filogenética com 53 táxons e 169 caracteres**.** A subfamília é redefinida por 15 sinapomorfias, duas correspondem as sinapomorfias encontradas por Malabarba (1998): a ausência da mancha umeral e a presença do pseudotímpano e cinco novas unicamente derivadas como o contorno do processo anteromedial do mesetmóide; o primeiro infraorbital subretangular com a porção anteroventral estendida; a porção posterior edêntula da maxila aproximadamente com tamanho equivalente ao da porção anterior com dentes da maxila; o perfil da nadadeira anal dos machos com o lobo anterior agudo e o perfil distal côncavo; e em geral a presença de dois ou três dentes na maxila (Bührnheim, 2006).

A subfamília Cheirodontinae compreende duas tribos, Cheirodontini e Compsurini, além de alguns gêneros considerados *incertae sedis*.

Tribo Cheirodontinae

A tribo Cheirodontini é reconhecida baseando-se principalmente em caracteres relacionados ao dimorfismo sexual secundário observado nos raios procorrentes ventrais da nadadeira caudal e nos raios da nadadeira anal dos machos. A tribo Cheirodontini é composta por 22 espécies (Malabarba, 2003) distribuídos nos seguintes seis gêneros: *Cheirodon* Girard, 1855 (distribuído no Sul do Brasil, Uruguai, Argentina e Chile); *Serrapinnus* Malabarba*,* 1998 (distribuído na bacia Amazônica, bacia do rio Uruguai, bacia do rio São Francisco, laguna dos Patos, bacia Paraná Paraguai); *Nanocheirodon* Malabarba*,* 1998 (distribuído na Colômbia no lago Maracaibo e bacia do rio Magdalena); *Heterocheirodon* Malabarba*,* 1999 (distribuído na bacia do rio Uruguai e laguna dos Patos); *Spintherobolus* Eigenmann, 1911 (distribuído no alto rio Tietê e nos rios costeiros do Sudeste do Brasil) e um novo gênero, ainda não descrito (denominado novo gênero e espécie C em Malabarba, 1998).

Tribo Compsurini

A tribo Compsurini inclui os queirodontíneos com inseminação, sendo caracterizada pela transferência de esperma dos testículos dos machos maduros para os ovários das fêmeas, como descrito em Burns et al. (1997). Os membros da tribo Compsurini apresentam também órgãos especializados na nadadeira caudal dos machos, que variam desde a presença de escamas modificadas até a presença de tecidos hipertrofiados aparentemente de função glandular (Malabarba & Weitzman, 1999, 2000). A tribo Compsurini é formada por nove espécies distribuídas nos seguintes cinco

gêneros: *Acinocheirodon* Malabarba & Weitzman, 1999 (distribuído nas bacias dos rios São Francisco e Jequitinhonha); *Compsura* Eigenmann*,* 1915 (gênero encontrado no Panamá, na bacia do rio São Francisco e rios costeiros do Nordeste); *Kolpotocheirodon* Malabarba & Weitzman, 2000 (cabeceiras do rio Paraná e São Francisco em Brasília e Bahia); *Macropsobrycon* Eigenmann*,* 1915 (laguna dos Patos, bacia do rio Uruguai, bacia do rio Tramandaí); e *Saccoderma* Schultz*,* 1944 (bacia do rio Maracaibo na Venezuela, bacia do rio Magdalena e rio Sinú na Colômbia)*.* "*Odontostilbe*" *dialeptura* (Fink & Weitzman, 1974) encontrado em rios da Costa Rica e Panamá, e "*O.*" *mitoptera* (Fink & Weitzman, 1974) distribuído no Panamá*,* são considerados pertencentes à tribo Compsurini e uma nova designação de gênero é necessária para estas espécies (Malabarba, 1998; Malabarba & Weitzman, 1999; Malabarba, 2003).

Gêneros *incertae sedis*

Os gêneros *Aphyocheirodon* Eigenmann*,* 1915 (distribuído no alto rio Paraná); *Cheirodontops* Schultz, 1944 (encontrado na bacia do rio Orinoco, Venezuela); *Odontostilbe* Cope, 1870 (distribuído na bacia Amazônica, bacia dos rios Mana, Maroni e Comté na Guiana Francesa, bacia do Panamá, bacia do rio Pilcomayo na Bolivia, bacia do rio Napo no Equador, bacia Paraná Paraguai e bacia do rio Uruguai); *Prodontocharax* Eigenmann & Pearson, 1953 (encontrado na bacia Amazônica, na Bolívia e no Peru); e *Pseudocheirodon* Meek & Hildebrand, 1916 (distribuído na costa rica e Panamá) não foram classificados em nenhuma das duas tribos, pois apresentavam caracteres de dimorfismo sexual pouco evidentes. Recentemente Bührnheim (2006) propôs a criação da tribo Odontostilbini. Esta tribo foi baseada em treze sinapomorfias encontradas nos canais sensoriais do parietal e do primeiro infraorbital, na forma do segundo e sexto infraorbitais, no bordo anterodorsal da maxila, no palatino, na protuberância lateral da maxila inferior, na parte lateral exposta do ramo

inferior do ângulo-articular, no branquiostegal mais posterior, no comprimento do raio não ramificado da nadadeira pélvica, no perfil da nadadeira anal e na extensão da linha lateral. Bührnheim propôs duas novas sinonímias, *Aphyocheirodon* e *Cheirodontops*, como sinônimos de *Holoshesthes*. Além disso, três gêneros são revalidados: *Holoshesthes* e *Lobodeuterodon* saem da sinonímia de *Odontostilbe*, e *Amblystilbe* da sinonímia de *Prodontocharax*. A tribo Odontostilbini é composta por 26 espécies distribuídas nos seguintes 6 gêneros: *Amblystilbe* Fowler, 1940; *Holoshesthes* Eigenmann 1903; *Lobodeuterodon* Fowler, 1945; *Odontostilbe* Cope 1870*, Prodontocharax* Pearson, 1924 *e Pseudocheirodon* Meek & Hildebrand, 1916.

Estudos realizados em Cheirodontinae

Entre os estudos recentes sobre sistemática, reprodução e caracteres sexuais primários e secundários em Cheirodontinae podemos citar: Burns et al. (1997) descrevem a morfologia dos espermatozóides; Malabarba (1998) apresenta definição e relações filogenéticas; Burns et al. (1998) descrevem a ultraestrutura de *M. uruguayanae* Eigenmann 1915; Gelain et al. (1999), estudam aspectos da reprodução de *Serrapinnus calliurus* (Boulenger, 1900)*;* Weitzman & Malabarba (1999) revisam *Spintherobolus*; Malabarba & Weitzman (1999) descrevem *Acinocheirodon*; Malabarba & Bertaco (1999) descrevem *Heterocheirodon;* Malabarba & Weitzman (2000) descrevem *Kolpotocheirodon;* Braun et al*.* (2000), descrevem a biologia reprodutiva de *Cheirodon ibicuhiensis* Eigenmann, 1915 da lagoa Fortaleza, RS; Oliveira et al*.* (2002), estimam o período reprodutivo, o tipo de desova e a fecundidade de *C. ibicuhiensis* do arroio Ribeiro, rio Grande do Sul; Malabarba (2003) caracteriza a subfamília e lista as espécies; Oliveira (2003) estima o período reprodutivo e fecundidade e também compara o desenvolvimento de caracteres sexuais secundários (glândula branquial e ganchos) com a maturação gonadal em duas espécies *Compsura heterura* Eigenmann, 1915 e *Odontostilbe* sp.; Silvano et al*.* (2003) estudam o período reprodutivo e fecundidade para *Serrapinnus piaba* (Lütken, 1874) do rio Ceará-Mirim, Rio Grande do

Norte; Gusmão-Pompiani (2003) descreve a ultraestrutura dos espermatozóides de *Serrapinnus notomelas* (Eigenmann 1915); Malabarba et al. (2004) descrevem *Kolpotocheirodon figueiredoi;* Azevedo (2004) estuda a biologia reprodutiva de *M. uruguayanae* do rio Ibicui Mirim-RS; Burns & Weitzman (2005) descrevem a ultraestrutura dos espermatozóides de *Serrapinnus kriegi* (Schindler, 1937); Bührnheim & Malabarba (2006) redescrevem a espécie tipo de *Odontostilbe* e descrevem três espécies novas *O. ecuadorensis, O. nareuda* e *O. pareci;* Bührnheim (2006) estuda a sistemática de *Odontostilbe* com a proposição de uma nova tribo Odontostilbini e redefinição dos gêneros *incertae sedis.*

Ultraestrutura dos espermatozóides

A análise da ultraestrutura de espermatozóides e da espermatogênese em teleósteos tem revelado importantes características morfológicas (Mattei, 1991; Spadella, 2004) e tem ajudado a esclarecer questões a respeito da filogenia das espécies de peixes (Jamieson, 1991; Mattei, 1991).

A forma, o comprimento e a largura do núcleo espermático apresentam variação entre as espécies e são freqüentemente associadas ao tipo de fecundação. A maioria das espécies de fecundação externa possui espermatozóides com núcleo arredondado, sendo denominado de aquasperma (Jamieson, 1991). O alongamento do núcleo é observado em espécies de fecundação interna ou inseminadoras (presença de espermatozóides nos ovários, sem conhecimento do exato momento da fecundação) (Burns et al., 1995, 1997; Burns & Weitzman, 2005). Algumas hipóteses são apresentadas para as possíveis vantagens seletivas do alongamento do núcleo. Núcleos espermáticos alongados são mais aerodinâmicos e podem facilitar a penetração na micrópila do ovócito (Fawcett, 1970; Jamieson, 1991) e podem passar pelo gonoporo da fêmea mais facilmente, aumentando o número de espermatozóides transferidos num determinado momento (Burns & Weitzman, 2005).

Outro aspecto relacionado ao núcleo é a posição deste em relação ao flagelo e isto

depende do tipo de espermiogênese, que pode ser do tipo 1 ou do tipo 2 segundo a classificação de Mattei (1970). Em ambos, o início do desenvolvimento do flagelo ocorre lateralmente ao núcleo. Na espermiogênese tipo I as mitocôndrias estão dispersas no citoplasma, o complexo centriolar está disposto lateralmente ao núcleo e preso à membrana plasmática e o centríolo distal forma o corpúsculo basal e desenvolve o flagelo. No processo tipo I os centríolos migram em direção ao núcleo e trazem junto à membrana plasmática e o flagelo inicial. Ocorre uma depressão no contorno nuclear sendo denominado de fossa nuclear. O núcleo faz uma rotação de 90° em relação ao eixo flagelar. No final do processo o flagelo fica perpendicular ao núcleo. A rotação nuclear tem sido observada em todas as espécies de Curimatidae e em quase todos os Characiformes (Quagio-Grassiotto et al*.,* 2003) com exceção de *Acestrorhynchus falcatus* (Bloch 1794) em Acestrorhynchidae (Matos et al., 2000), dos Glandulocaudinae (Burns et al., 1998; Pecio & Rafiński, 1999), Stevardiinae (Burns et al., 1998, Pecio et al., 2005), *Bryconamericus stramineus* Eigenmann 1908 em Characidae (Gusmão-Pompiani, 2003, Gusmão-Pompiani et al., submetido), em Cheirodontinae (Burns et al., 1997) e em três espécies consideradas *incertae sedis* em Characidae, *Bryconadenos tanaothoros* (Weitzman et al., 2005) e *Brittanichthys axelrodi* (Javonillo et al., 2007).

O processo de espermiogênese tipo II é semelhante ao tipo I, mas difere deste por não ocorrer rotação nuclear em relação ao flagelo, resultando num flagelo paralelo ao núcleo**.** Também ocorre a formação da fossa nuclear, mas os centríolos encontram-se fora da fossa nuclear. A espermiogênese do tipo III foi descrita recentemente para Siluriformes da família Pimelodidae (Quagio-Grassiotto & Carvalho, 2000). A espermátide jovem apresenta núcleo central e complexo centriolar medial ao núcleo e preso à membrana. Durante a espermiogênese a rotação nuclear não ocorre, os centríolos não migram permanecendo junto à membrana plasmática e o canal citoplasmático e a fossa nuclear não se formam. O espermatozóide apresenta flagelo

perpendicular ao núcleo. A espermiogênese do tipo III foi observada algumas espécies de Callichthyidae e Loricariidae (Spadella, 2004).

As espécies de fertilização externa geralmente apresentam uma peça intermediária pequena, enquanto que nas espécies inseminadoras esta estrutura é normalmente mais alongada (Jamieson, 1991; Mattei; 1991). Uma peça intermediária maior poderia abrigar um número maior de mitocôndrias, aumentando a capacidade de geração de energia da célula e viabilidade dos espermatozóides (Fawcett, 1970; Pecio & Rafiński, 1994).

A ultraestrutura de espermatozóides foi estuda em Glandulocaudinae [*Mimagoniates barberi* Regan 1907, por Pecio & Rafinski (1994); *Mimagoniates barberi* e *M. microlepis* (Steindachner 1877) por Burns et al. (1998)]; Stevardiinae [*Corynopoma riisei* Gill 1858*, Pseudocorynopoma doriae* Perugia 1891*, Diapoma speculiferum* Cope 1894, *Diapoma* sp. por Burns et al. (1998); *Tyttocharax cochui* (Ladiges 1950)*, T. tambopatensis* Weitzman & Ortega 1995 e *Scopaeocharax rhinodus* (Böhlke 1958) por Pecio et al. (2005)]; Aphyocharacinae [*Aphyocharax anisitsi* Eigenmann & Kennedy, 1903 por Burns et al. (1998) e por Gusmão-Pompiani (2003)]; Tetragonopterinae [*Tetragonopterus argenteus* Cuvier 1816 por Gusmão-Pompiani (2003)]; Stethaprioninae [*Poptella paraguayensis* (Eigenmann 1907) por Gusmão-Pompiani (2003)]; Characinae [*Roeboides bonariensis* (Reinhardt, 1851)*, Galeocharax humeralis* (Valenciennes, 1834) e *Galeocharax knerii* (Steindachner, 1879) por Gusmão-Pompiani (2003)]; espécies incertae sedis em Characidae [*Triportheus paranensis* (Kner, 1858)*, Bryconops affinis* (Günther, 1864)*, Hyphessobrycon eques* (Steindachner 1882)*, Moenkhausia sanctaefilomenae* (Steindachner, 1907)*, Bryconamericus stramineus* Eigenmann 1908*, Salminus maxillosus* (Cuvier, 1816), *Brycon microlepis* Perugia 1897 e *B. orbignyanus* (Valenciennes, 1850) por Gusmão-Pompiani (2003); *Hollandichthys* (Eigenmann, 1909) por Azevedo (2004); *S. maxillosus*, *B. microlepis* e *B. orbignyanus* por Veríssimo-Silveira et al. (2006); *Brittanichthys axelrodi* Géry 1965, por Javonillo et al. (2007). Em Cheirodontinae as informações são escassas, sendo disponíveis somente para três

espécies, *M. uruguayanae* (Burns & Weitzman, 1998, 2005)*, S. notomelas* (Gusmão-Pompiani, 2003) e *S. kriegi* (Burns & Weitzman, 2005).

Glândula branquial

Burns & Weitzman (1996) descreveram uma nova estrutura encontrada nas brânquias de *Corynopoma riisei*, um stevardiine. Esta estrutura foi denominada de glândula branquial e é formada pela união e modificação funcional dos filamentos mais ventrais da hemibranquia externa do primeiro arco branquial em ambos lado do corpo. Os filamentos da glândula branquial estão unidos externamente através de um tecido epitelial estratificado, mantendo a individualidade interiormente e formando câmaras, por onde a secreção é conduzida ao exterior (Burns & Weitzman, 1996; Bushmann et al., 2002). Entre as lamelas são encontradas células cilíndricas.

O desenvolvimento da glândula branquial parece iniciar com a multiplicação das células epiteliais que revestem os filamentos primários mais anteriores da porção mais ventral, estendendo-se para os filamentos seguintes. Esta expansão celular parece iniciar nas células que revestem a base dos filamentos, próximo ao arco, estendendo-se sobre os filamentos até quase a sua extremidade distal (Oliveira, 2003).

Os filamentos branquiais envolvidos na formação da glândula branquial provavelmente perdem a função respiratória. A função da glândula braquial ainda não está definida, mas por iniciar o seu desenvolvimento em machos em maturação gonadal, ser bem desenvolvida em machos maduros e estar ausente em fêmeas, esta pode estar relacionada à reprodução. A secreção produzida por esta glândula pode ser utilizada para atrair as fêmeas durante o período reprodutivo (Burns & Weitzman, 1996; Bushmann et al., 2002) ou pode estar associada à competição entre machos (Bushmann et al., 2002).

A glândula branquial foi encontrada em outras espécies de Stevardiinae (Bushmann et al., 2002) e em outras subfamílias de Characidae, como Cheirodontinae (Oliveira, 2000, 2003; Azevedo, 2004; Bührnheim, 2006); Aphyocharacinae (Gonçalves

et al., 2005), e algumas espécies consideradas incertae sedis em Characidae (Weitzman et al., 2005).

Esta tese é divida em três capítulos que tratam da descrição da ultraestrutura dos espermatozóides e descrição da glândula branquial de representantes de diferentes gêneros de Cheirodontinae. Os capítulos seguem as regras da revista *Journal of Morphology*.

OBJETIVOS

- Descrever a ultraestrutura dos espermatozóides em espécies de Cheirodontinae utilizando o método de microscopia eletrônica de transmissão (MET);

- Comparar a morfologia dos espermatozóides ao tipo de estratégia reprodutiva apresentada pelas espécies (inseminação ou fecundação externa);

- Comparar e analisar possíveis homologias na ultraestrutura dos espermatozóides entre as espécies de queirodontíneos e outros caracídeos, formulando hipóteses acerca da evolução destes caracteres e da filogenia dos táxons envolvidos;

- Investigar a presença e comparar a morfologia da glândula branquial em espécies de Cheirodontinae;

- Comparar e analisar possíveis homologias entre a glândula branquial nos queirodontíneos e outros caracídeos, formulando hipóteses acerca da evolução destes caracteres em Characidae e mais especificamente em Cheirodontinae.

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Capítulo 1:

Sperm ultrastructure of the species of Cheirodontinae (Teleostei: Characidae): description and implications on the phylogeny of the inseminating compsurins

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Keywords: sperm ultrastructure, insemination, Compsurini, Cheirodontinae

ABSTRACT

The spermatozoa ultrastructure is described in six inseminating cheirodontines of the tribe Compsurini (*Kolpotocheirodon theloura, Compsura heterura, Acinocheirodon melanogramma, Saccoderma hastatus, "Odontostilbe" dialeptura* and *"Odontostilbe" mitoptera*), and four externally fertilized species, three belonging to the tribe Cheirodontini (*Cheirodon interruptus, Serrapinnus calliurus*, and *Serrapinnus heterodon*) and one *incertae sedis* species (*Odontostilbe pequira*). Sperm ultrastructure of externally fertilized species has shown to be very conservative, while sperm of inseminating species presented several changes mostly related to sperm elongation. The aquasperm of the inseminating species of *Kolpotocheirodon* is also differentiated regarding the aquasperm of the externally fertilized ones. A phylogenetic analysis is performed using ten characters derived from sperm ultrastructural analysis, resulting in a partially solved hypothesis of relationships among inseminating species, but being uninformative to hypothesize relationships among externally fertilized species. Hypotheses of character evolution in the sperm morphology are discussed based on the resulting phylogeny.

INTRODUCTION

Cheirodontines are small characid fishes largely distributed in freshwater drainages from Central and South America, constituting the only characiform group found in the western slope of the Andes, in Chile (Malabarba, 1998; 2003). This subfamily is characterized by the presence of pedunculate jaw teeth largely compressed and expanded distally, absence of a humeral spot and presence of a gap on the muscles covering the anterior portion of the swim bladder, referred to as pseudotympanum (Malabarba, 1998).

Cheirodontinae contains two tribes, the Cheirodontini and Compsurini, plus some genera considered *incertae sedis* in the subfamily by Malabarba (1998). These *Incertae Sedis* genera, however, were hypothesized to belong to a single tribe, the Odontostilbini, in the unpublished work of Bührnheim (2006). The species of Cheirodontini are characterized by the presence of secondary sexually dimorphic characters associated with the anal-fin rays and ventral procurrent caudal-fin rays of males. The species of Compsurini are characterized by the presence of modified anal-fin hooks, modified caudal-fin rays and/or scales in mature males, and transference of sperm from the testis to ovaries, that has been denominated insemination (Burns et al., 1997, 1998). The tribe Odontostilbini as proposed by Bührnheim (2006) is characterized by thirteen synapomorphies, related to osteological modifications of the cranial bones, the elongation of the unbranched pelvic-fin ray, the absence of sexual dimorphism in the anal-fin profile; and the completeness of the lateral line.

Previous studies on the sperm ultrastructure of cheirodontines are limited to two externally fertilized species of the tribe Cheirodontini, *Serrapinnus notomelas* (from Gusmão-Pompiani, 2003) and *S. kriegi* (from Burns and Weitzman, 2005), and one inseminating species of the tribe Compsurini, *Macropsobrycon uruguayanae* (Burns et

al., 1998; Burns and Weitzman, 2005; Oliveira et al., this volume).

Characters of spermatozoa ultrastructure have been demonstrated useful in phylogenetic studies (Jamieson, 1991; Mattei, 1991). The purpose of this study is to provide a comparative analysis of sperm ultrastructure among the species of all cheirodontine genera and discuss their putative relationships.

MATERIAL AND METHODS

Examined specimens belong to ANSP - Academy of Natural Sciences, Philadelphia, USA; MCP - Museu de Ciências e Tecnologia, Porto Alegre, Brazil; MNRJ - Museu Nacional, Rio de Janeiro, Brazil; UFRGS - Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; and USNM - National Museum of Natural History, Washington D.C., USA. Testis of mature males of *Acinocheirodon melanogramma* Malabarba and Weitzman, 1999 (MCP 19142, 34.97mm SL); *Cheirodon interruptus* (Jenyns, 1842) (UFRGS 8977, 33. 50 mm SL; USNM 176077, 31.2 mm SL); *Compsura heterura* Eigenmann, 1915 (UFRGS 8979, 27.20 mm SL); *Kolpotocheirodon theloura* Malabarba and Weitzman, 2000 (MNRJ 18081, 26.11 mm SL); *Macropsobrycon uruguayanae* Eigenmann, 1915 (MCP 18588, 39.0 mm SL; UFRGS 8792, SL 29 mm UFRGS 8791, 31 mm); *"Odontostilbe" dialeptura* (Fink and Weitzman, 1974) (USNM 348763, 35.5 mm SL; USNM 209500, 27.27 mm SL); "*O*"*. mitoptera* (Fink and Weitzman, 1974) (USNM 208541, 34.33 mm SL; USNM 348762, 35.5 mm SL); *Odontostilbe pequira* (Steindachner, 1882) (UFRGS 8990, 36.9 mm SL); *Saccoderma hastatus* (Eigenmann, 1913) (ANSP 139487, 30.0 mm SL); *Serrapinnus calliurus* (Boulenger, 1900) (MCP 18500, 28.5 mm SL) and *Serrapinnus heterodon* (Eigenmann, 1915) (UFRGS 8793, 29.7 mm SL) were removed for transmission electron microscopy (TEM) and scanning electron microscopy (SEM) analysis. The specimens of *K. theloura, S. hastatus* and *A. melanogramma* were obtained from museum collections; therefore the whole fishes had been fixed in formalin. Testis of *C. heterura, C. interruptus, M.*

uruguayanae, "O". dialeptura, "*O*"*. mitoptera*, *C. interruptus, O. pequira*, *S. calliurus, S. heterodon* and were removed from fresh collected specimens and fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The testis of *Macropsobrycon uruguayanae* is described and discussed in a separate article (Oliveira et al., this volume).

For SEM, testes were dehydrated in ethanol series, critical-point dried, crushed with needle and spread on stubs with carbon double-stick tape and sputter-coated with carbon and gold. Samples obtained were viewed in a Philips XL 30 scanning electron microscopy from Pontificia Universidade Católica do Rio Grande do Sul, Brazil; in a Jeol 6060 scanning electron microscopy from Universidade Federal do Rio Grande do Sul, Brazil or in LEO 1430VP scanning electron microscopy from George Washington University, USA.

For TEM, testes were cut in small pieces (1 mm^3), rinsed in phosphate buffer and post-fixed in 1% osmium tetroxide in phosphate buffer. Afterwards, these samples were dehydrated in acetone series, infiltrated and embedded in Araldite-Epon (Embed-812) or in Araldite 502. Ultrathin sections were stained with a saturated of uranyl acetate in 50% alcohol and lead citrate. The resulting sections were viewed in a JEOL 1200 from Universidade Federal do Rio Grande do Sul, Brazil, in a Philips CM100 transmission electron microscope from Universidade Estadual Paulista, Botucatu, Brazil and in a JEOL JEM 1200 from George Washington University, Washington DC, USA.

The phylogenetic analysis was performed with Hennig86, and the data entered with Tree Gardener. A matrix was constructed with a total of 10 characters based on spermatozoa ultrastructure. The ingroup included 7 species and all genera of the tribe Compsurini (*A. melanogramma, C. heterura, K. theloura, M. uruguayanae, "O". dialeptura, "O". mitoptera* and *S. hastatus*), 1 species of Odontostilbini (*O. pequira*) and 3 species of Cheirodontini (*C. interruptus, S. calliurus* and *S. heterodon*). Character polarity was determined through outgroup comparison. One hypothetical outgroup was established based on the analysis of the sperm ultrastructure of the following taxa:

Aphyocharax anisitsi Eigenmann and Kennedy 1903 (MCP 18583, 33.0 mm SL)*; Charax stenopterus* (Cope, 1894) (MCP 18474, 77.0 mm SL); *Cyanocharax itaimbe* Malabarba and Weitzman, 2003 (MCP 18496, 36.5 mm SL); *Bryconamericus stramineus* (Eigenmann, 1908) (data from Gusmão-Pompiani, 2003) and *Brycon microlepis* (Perugia, 1897) (data from Veríssimo-Silveira et al., 2006). *Aphyocharax anisitsi, C. stenopterus* and *C. itaimbe* were fixed with the same fixative. Character states found in both the ingroup and outgroup were considered primitive and assigned state 0 in the hypothetical outgroup. Character states found in the ingroup and absent in the outgroup were considered derived and assigned state 1 (or 1+n). In the case of multiple character states present in both ingroup and outgroup, it was considered uninformed in the hypothetical outgroup, except when noted. All characters have the same weight and were considered unordered, except when noted.

Measurements of the nucleus were taken from TEM micrographs, being considered the longest longitudinal axis and the longest transversal length.

RESULTS

Sperm morphology of inseminating species

Kolpotocheirodon theloura

Spermatozoa are typical aquasperm with a spherical nucleus (Figs. 1A, 2). There is no acrosomal vesicle. Nuclei measure approximately 1.6 μm in diameter. The chromatin is homogeneous and electron-dense. A narrow cytoplasmic strip surrounds the nucleus and organelles are absent in this thin area. The cytoplasmic canal length measures approximately 1.5 μm (Figs. 2B, D, E). There is a shallow nuclear fossa (Figs. 2A, B) and both centrioles are located outside it. Distal and proximal centrioles are parallel (Figs. 2A, C) and the proximal centriole is slightly anterior to the distal one. The midpiece is elongated and narrow. There are at least five mitochondria. They are

spherical, small and are located posterior to the nucleus, in the anterior and medial portion of the midpiece (Fig. 2). It is possible to identify vesicles in the posterior end of the midpiece (Figs. 2B, E). There are few and large vesicles. A single flagellum is present posterior to the nucleus, lacking fins or membranous compartment. It was not possible to see more details of sperm ultrastructure due to fixation with formalin.

Compsura heterura

Compsura heterura has spermatozoa with a slightly elongate nucleus (Figs. 1B, 3). Acrosomal vesicle is absent. Nucleus reaches approximately 2.7 μm in length and 1.4 μm in width. Chromatin is homogeneous and highly electron dense. A narrow cytoplasmic strip surrounds the nucleus and no organelles are viewed in this area. The cytoplasmic canal length measures approximately 1.6 μm in length. The anterior portion of the midpiece contains six large globular mitochondria, which surround the proximal portion of the flagellum (Figs. 3A, C, E). Large vesicles are found in the posterior region of the midpiece in small number (Figs. 3A, C, F). Centrioles are parallel one to another and the anterior tip of distal centriole is inside a shallow nuclear fossa. The flagellum is posterior to the nucleus and it does not have fins or membranous compartment. Axoneme has the usual 9+2 arrangement.

Acinocheirodon melanogramma and *Saccoderma hastatus*

Acinocheirodon melanogramma and *Saccoderma hastatus* have similar spermatozoa. Both have elongate nuclei and no acrosomal vesicle. In *A. melanogramma* reaches approximately 3.1 μm in length and 1.3 μm in width (Figs. 1C, 4A) and in *S. hastatus,* the nucleus reaches approximately 3.5 μm in length and 0.9 μm in width (Figs. 1D, 5A). The nucleus is lateral and has condensed chromatin. A long lateral cytoplasmic canal remains attached along of the cell in both species and reach approximately 2.9 μm in *A. melanogramma* and 4.5 μm in *S. hastatus* (Figs. 4A ,B, 5A-

C). The distal centriole is outside of the nuclear fossa and the anterior tip of the proximal centriole is located inside. This centriole is anterior and oblique to the distal one (Figs. 4A, 5B). Large globular mitochondria are located in the end of the nucleus (Figs. 4A, C, 5A). *Acinocheirodon melanogramma* has three mitochondria (Fig. 4C). It was not possible to count the mitochondria in *S. hastatus.* Details of mitochondria, axoneme and vesicles were not viewed because of formalin fixation.

"Odontostilbe" dialeptura and *"O". mitoptera*

"Odontostilbe" dialeptura and *"O". mitoptera* exhibit very similar spermatozoa. Both species have elongate nucleus reaching approximately 2.6 μm in length and 1.2 μm in width (Figs. 1E,F, 6, 7). Acrosome is absent. Chromatin of the nucleus is condensed. The nucleus is lateral and parallel to the flagellum (Figs. 6A, 7A). The cytoplasmic canal is very long, measuring approximately 3.3 μm in length in *"O". dialeptura* and 3.2 μm in *"O". mitoptera*. Centriolar complex is located in the anterior tip of the nucleus. The proximal centriole is anterior and oblique to the distal one and both are located outside the nuclear fossa (Figs. 6B, 7B). The anterior border of the nucleus is clearly oblique to the longest axis of the nucleus. Mitochondria are located on the anterior tip of the nucleus. *"Odontostilbe" dialeptura* has at least two big globular mitochondria (Fig. 6A) and *"O". mitoptera* has three (Fig. 7C). The mitochondria show electron dense crystals. Vesicles are found at one side of the nucleus, attached to the flagellum and along all nucleus extension, but they are more concentrated at the anterior tip of the cell, after mitochondria region. Flagellar axoneme has typical 9 + 2 microtubules doublets. There is no fins or membranous compartment.

Sperm morphology of externally fertilization species

Spermatozoa of *C. interruptus, S. heterodon, S. calliurus* and *O. pequira* are very

similar (Fig. 8). They have head without acrosomal vesicle, midpiece and flagellum. The nucleus is spherical, typical aquasperm. The nucleus measures approximately 2.2 μm in diameter in *C. interruptus* (Fig. 8A, 9A), 1.9 μm in *O. pequira* (Fig. 8B, 9C), 2.1 μm in *S. calliurus* (Fig. 8C, 10A) and 2.1 μm in *S. heterodon* (Fig. 8D, 10C). The chromatin is electron-dense and homogeneous in all species. A narrow cytoplasmic strip surrounds the nucleus and organelles are absent in this thin area. In *C. interruptus* the cytoplasmic strip that surrounds the nucleus has irregular width and it is a little thick in some regions. Midpiece and cytoplasmic canal are short. The nuclear fossa is shallow, has one arch and is ramified. The centrioles are parallel and located outside of the shallow nuclear fossa, except in *O. pequira* that has both centrioles partially inside the nuclear fossa (Fig. 9C). There are at least six mitochondria. They are spherical, small and are located posterior to the nucleus, in the anterior portion of the midpiece. In the end of midpiece there is a large number of small and globular vesicles. They are well developed and can be connected or unconnected (Fig. 9B, 9D, 10B, 10D). A single flagellum is present, posterior to the nucleus, and there is not fins or membranous compartment. The flagellar axoneme is formed by nine peripheral microtubules.

DISCUSSION

Monophyly of the Cheirodontinae and included tribes (Cheirodontini, Compsurini and Odontostilbini) have been well corroborated based on gross morphology. Relationships and diagnosis of included genera have been also well studied and supported for Cheirodontini (Malabarba, 1998) and Odontostilbini (Bührnheim, 2006), but relationships and diagnosis of inseminating compsurin genera (except for *Acinocheirodon* Malabarba and Weitzman, 1999 and *Kolpotocheirodon* Malabarba and Weitzman, 2000) remain to be thoughtfully investigated.

Phylogenetic analyses based on ten ultrastructural sperm morphology characters (Appendix I) further contributes to the recognition of the Compsurini as monophyletic and allows hypothesize the relationships among included taxa. Sperm morphology of the externally fertilized species of Cheirodontini and Odontostilbini, however, seems to be very conservative (based on the analysis of the specimens available) and has shown uninformative to hypothesize relationships among included species (Fig. 11).

Although conservative, the aquasperm of the species of Cheirodontini and Odontostilbini is clearly distinct of those of other externally fertilized characiforms. The vesicles found in the midpiece present a large variability in shape and size among the Characiformes, or even are lacking in some of it representatives. Among externally fertilized cheirodontines, there is a pattern found in the externally examined species that comprises a large number of small globular vesicles fulfilling most of the midpiece (Character 10, state 1). Such a pattern has not been found in other Characiformes, except for a similar pattern described for *Citharinus* sp. (Mattei et al., 1995), but with clearly smaller vesicles. The sperm cells of the inseminating species of the Compsurini (including the aquasperm of *Kolpotocheirodon*) have a small number of larger and somewhat elongated vesicles, but there is no clear recognized pattern different of those of other characiforms (e.g. *Aphyocharax anisitsi* – Burns et al., 1998:237, fig. 1, and our observation).

The positioning of *Kolpotocheirodon* species within the Compsurini has been supported previously only by the presence of insemination, lacking any morphological evidence of their affinity to the remaining species of the tribe (Malabarba, 1998). Regardless the fact *Kolpotocheirodon* species have an aquasperm with a spherical nucleus, the longer cytoplasmic canal and elongated midpiece (Character 4, state 1) support a close relationship with the inseminating compsurins. Aquasperm of externally fertilized cheirodontines have a short midpiece, truncated posteriorly. Aquasperm of *Kolpotocheirodon* species and introsperm of other compsurins have the midpiece

elongated with longer cytoplasmic canal, progressively narrow posteriorly (not truncate posteriorly). Thus, although classified as an aquasperm due to the spherical nucleus, the spermatozoon of *Kolpotocheirodon* is derived and different of those externally fertilized species.

Elongate midpiece is a characteristic associated with insemination (Jamieson, 1991; Mattei, 1991). The resulting enlargement of midpiece is believed to make possible an increase in the sperm capacity of generate energy, especially because of the greater amount of mitochondria (Koya et al., 2002). This could provide energy for sperm dispersal throughout the ovary and could also prolong viable sperm storage within the ovary (Pecio and Rafinski, 1994; Yao et al, 1995). Most of the externally fertilizing teleosts have a short midpiece (Burns and Weitzman, 2005). Although *Brycon* and *Salminus* considered two primitive genera in Characidae have long midpiece and have externally fertilizing (Veríssimo-Silveira et al., 2006).

Two of the three putative synapomorphies that supports a monophyletic clade containing *C. heterura*, *M. uruguayanae*, *A. melanogramma*, *S. hastatus*, "*Odontostilbe*" *mitoptera* and "*O*." *dialeptura* are related to nuclear elongation (Characters 1 and 2). *Compsura heterura* and *M. uruguayanae* exhibit very similar bullet shaped spermatozoa (Character 1, state 1), while *S. hastatus, A. melanogramma, "O". mitoptera* and *"O". dialeptura* have a clearly elongated nucleus (Character 1, state 2). Regardless the fact this character was considered unordered in the analysis, the resulting hypothesis supports the bullet shaped spermatozoa as a synapomorphy to the clade containing *C. heterura*, *M. uruguayanae*, *A. melanogramma*, *S. hastatus*, "*Odontostilbe*" *mitoptera* and "*O*." *dialeptura* and an intermediary stage in the evolution of the elongate spermatozoa of the clade containing *A. melanogramma*, *S. hastatus*, "*Odontostilbe*" *mitoptera* and "*O*." *dialeptura*.

Nucleus size and shape, related to the relation between its length and width

(Character 2) also allowed the recognition of three different stages in spermatozoa elongation. The primitive condition in Cheirodontinae seems to be that found in all examined externally fertilized species and in *Kolpotocheirodon theloura*, that presents an spherical nucleus, whose width mean ranges from 1.67 to 2.17 μm and nucleus length mean ranges from 1.59 to 2.18μm (Character 2, state 0). Two discrete derived states related to nucleus elongation were found, corresponding to nucleus width mean ranging from 1.03 to 1.40 μm and nucleus length mean ranging from 2.40 to 2.96μm (Character 2, state 1) found in *C. heterura*, *M. uruguayanae*, *A. melanogramma*, "*Odontostilbe*" *mitoptera* and "*O*." *dialeptura*, and nucleus width mean 0.7 μm and nucleus length mean 3.17μm (Character 2, state 2) observed in *S. hastatus*. Again, even considered unordered in the analysis, the elongation of the sperm cells (Character 2, state 1) consists in a synapomorphy of the Clade containing *C. heterura*, *M. uruguayanae*, *A. melanogramma*, *S. hastatus*, "*Odontostilbe*" *mitoptera* and "*O*." *dialeptura* and an intermediary stage in the evolution of the extremely elongate and narrow spermatozoa of *S. hastatus* (Character 2, state 2).

Nuclear elongation is the spermatozoon morphological specialization most associated with insemination (Jamieson, 1991; Burns et al., 1995, 1997; Burns and Weitzman, 2005). There are many possibilities why insemination and nuclear elongation could be an adaptive advantage (Ginzburg, 1968; Gardiner, 1978; Jamieson, 1991; Burns and Weitzman, 2005) all emphasizing the less energy spend in fertilization. According to Burns and Weitzman (2005), the elongate nuclei allows sperm cells to be thinner, streamlined and to have lesser head area, being able to pass through female gonopore in more number at the same time, moving easily through female reproductive tract and suffering less resistance to cross fluids, tending to move at forward direction (Gardiner, 1978). In addition, it enables side-to-side sperm alignment and clumping, facilitating spermatozoa to flow together (Burns and Weitzman, 2005), possibly facilitating flow and reducing losses of cells to the environment (Ginzburg, 1968).

The third character supporting the monophyly of the clade *C. heterura*, *M. uruguayanae*, *A. melanogramma*, *S. hastatus*, "*Odontostilbe*" *mitoptera* and "*O*." *dialeptura* is related to the position of the centriolar complex in relation to the nuclear fossa. In externally fertilized species and *Kolpotocheirodon theloura* both centrioles are located outside the nuclear fossa (Character 5, state 0), while *C. heterura*, *M. uruguayanae*, *A. melanogramma* and *S. hastatus* possess the anterior tip of the proximal centriole located inside the nuclear fossa and the distal centriole outside (Character 5, state 1). "*Odontostilbe*" *mitoptera* and "*O*." *dialeptura* have both centrioles located outside the nuclear fossa, but this is considered under parsimony a secondary reversal and a synapomorphy of these two species.

Acinocheirodon melanogramma, "O". dialeptura, "O". mitoptera and *S. hastatus* constitute a monophyletic subclade of the Compsurini, supported by four characters shared by all species: the shape of the nucleus (Character 1, state 2, discussed above), the absence of nuclear rotation (Character 3), the relative position of the centrioles (Character 6), and the position of the mitochondria (Character 7).

Mattei (1970) has classified two types of spermiogenesis, according to the presence or absence of nuclear rotation. In both types, the flagellum develops laterally to the nucleus. In the type I, nuclear rotation takes place and the flagellum is located perpendicular to the nucleus (Character 3, state 0). In the type II, nucleus does not rotate and flagellum remains lateral to the nucleus (Character 3, state 1). The nuclear rotation occurs at most of Characiformes. Spermiogenesis type I was observed in the externally fertilized cheirodontins and odontostilbins and some compsurins species as *C. heterura, M. uruguayanae* and *K. theloura.* Spermiogenesis type II is found in *S. hastatus, A. melanogramma, "O". mitoptera* and *"O". dialeptura* and constitutes a synapomorphy of those species. Glandulocaudinae (Burns et al., 1998; Pecio and Rafinski, 1999), Stevardiinae (Burns et al., 1998; Pecio et al., 2005), one Acestrorhynchidae *Acestrorhyncus falcatus* (Matos et al., 2000), one Tetragonopterinae,

Bryconamericus stramineus (Gusmão-Pompiani et al., in press) and three species *incertae sedis* in Characidae, *"Cheirodon" stenodon* (Gusmão-Pompiani et al., in press) *Bryconadenos tanaothoros* (Weitzman et al., 2005) and *Brittanichthys axelrodi* (Javonillo et al., 2007) also have type II spermiogenesis, but independently acquired from those of some inseminating compsurins.

The perpendicular position of the centrioles (Character 6, state 1) and the mitochondria lodged in the posterior tip of the nucleus aside a small portion near the midlength and distant from the origin of the flagellum (Character 7, state 1) are also synapomorphic for the clade containing *A. melanogramma, "O". dialeptura, "O". mitoptera* and *S. hastatus*. The mitochondria position in these species is not a consequence and redundant with the absence of nuclear rotation. In glandulocaudines and stevardiines without nuclear rotation, the mitochondria are usually located in a certain extension along the flagellum, aside and behind the nucleus.

The last clade supported by sperm ultrastructural characters is formed by "*O*"*. dialeptura* and "*O*"*. mitoptera*. Both species are provisionally allocated in *Odontostilbe*, awaiting for a new generic allocation in the Compsurini (Malabarba, 1998). The results obtained herein supports their recognition in a new and monophyletic genus, but information on the ultrastructure of the spermatozoa are still lacking in four species of the compsurins. Monophyly of this clade is supported by the position of both centrioles outside the nuclear fossa (character 5, state 0 - see discussion above), by the shape of the anterior border of the nucleus, asymmetric, clearly oblique to the longest axis of the nucleus (Character 8, state 1), and in the position of the vesicles, located after the mitochondria, in the basal region of the midpiece and along the cytoplasmic collar in one side of the nucleus (Character 10, state 1). The anterior border of the nucleus is semicircular or emarginated in other Cheirodontinae, inseminating or not. The oblique anterior anterior border of the nucleus observed in *"O". dialeptura* and *"O". mitoptera* is unique among described spermatozoa of characiforms. Likewise, the presence of

vesicles along the nucleus inside the cytoplasmic collar was observed only in *"O". dialeptura* and *"O". mitoptera*, and has not been found in other characiforms.

The analysis presented herein supported the monophyly of the Compsurini and allowed hypothesize relationships among representatives of all genera, as well as with "*O*"*. dialeptura* and "*O*"*. mitoptera*. These results conflict the preliminary hypothesis of relationships among compsurins presented in Malabarba (1998), suggesting further investigation is needed in gross morphology of these taxa.

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Appendix A

List of Characters

Two autapomorphies of *M. uruguayanae*, presence of accessory microtubules and striated rootlet (see Oliveira et al., this volume) were not used in analyses, because autapomorphies are not informative to hypothesize relationships.

Character 1 (CI = 1.00) - Nucleus shape (unordered) [modified from character 70 in Malabarba, 1998]:

0= spherical

1= bullet shaped

2= elongated

The spherical nucleus that constitutes the typical aquasperm (State 0) was assigned to the hypothetical outgroup since a spherical nucleus represent the primitive condition among externally fertilized characiforms. The elongate nucleus found in some other representatives of the Characidae have been considered as non-homologous to the condition found in Compsurini (Malabarba, 1998; Weitzman and Menezes, 1998; Weitzman et al., 2005). This character was considered unordered because we consider the bullet shaped cannot be assumed as an intermediary step in the evolution of the elongate nucleus.

Character 2 (CI = 1.00) - Relation between length and width of nucleus (ordered).

0= nucleus width mean ranging from 1.67 to 2.17 μm; and nucleus length mean ranging from 1.59 to 2.18 μm.

1= nucleus width mean ranging from 1.03 to 1.40 μm; and nucleus length mean ranging from 2.40 to 2.96 μm.

2= nucleus width mean 0.7 μm; and nucleus length mean 3.17 μm.

The analysis of the means of the length and width of the nucleus per species allowed the recognition of three distinct classes of spermatozoa shape, based on both three distinct and discontinuous ranges of nucleus length and three distinct and discontinuous ranges of nucleus width (Fig. 12). The first class includes the typical aquasperm with similar measurements of length and width, shared by all externally fertilized species and the inseminating species of *Kolpotocheirodon.* The second class includes elongated sperm shared by *Compsura heterura, Acinocheirodon melanogramma, M. uruguayanae uruguayanae, "Odontostilbe" dialeptura* and *"O". mitoptera.* The third class includes only *Saccoderma* that possess an even more elongate and narrow sperm nucleus.

The character was considered ordered in the analysis and state 0 corresponding to aquasperm assigned to the hypothetical outgroup.

Character 3 (CI = 1.00) - Nuclear rotation (ordered):

0= present with flagellum posterior to the nucleus

1= absent with flagellum attached laterally to the nucleus

The character was considered ordered in the analysis and state 0 corresponding to aquasperm assigned to the hypothetical outgroup.

Characters 4 (CI = 1.00) - Shape of midpiece and length of citoplasmatic canal (ordered):

0 = midpiece roundish nearly truncated posteriorly and citoplasmatic canal length approximately half nucleus length

1 = midpiece elongated not truncated posteriorly; citoplasmatic canal length is longer than half nucleus length

The character was considered ordered in the analysis, and state 0, corresponding to aquasperm, was assigned to the hypothetical outgroup.

Character 5 (CI = 1.00) - Position of centriolar complex in relation to the nuclear fossa (unordered):

0= both centrioles are outside the nuclear fossa.

1= anterior tip of the proximal centriole is located inside the nuclear fossa and the distal centriole is outside.

2= both centrioles are partially inside the nuclear fossa.

The position of the centriolar complex was found in two states in the ingroup, but it is also variable in the outgroup. In the outgroup, the state 0 was observed in *Knodus*, and state 1 in *Charax. Brycon microlepis* (=*Brycon hilarii*) has both centrioles inside the nuclear fossa and *Bryconamericus stramineus* has proximal centriole is inside the nuclear fossa and distal centriole is outside. It is not possible to order this character and it was considered uninformed in the hypothetical outgroup.

Character 6 (CI = 0.50) - Centrioles (unordered):

0= parallel

1= perpendicular

This Character is variable in the outgroup and was considered uninformed in the hypothetical outgroup. It is not possible to order this character.

Character 7 (CI = 1.00) - Mitochondria position (ordered):

0= in the midpiece, posterior to the nucleus and around the origin of the flagellum

1= near the tip of nucleus (without nuclear rotation) and distant from the origin of the flagellum

The mitochondria location in *A. melanogramma, "O". dialeptura, O mitoptera* and *Saccoderma* (state 1) is clearly derived and not similar to the mitochondria position in other inseminating characid species that do not present nuclear rotation. In these species mitochondria is lodged in the posterior tip of the nucleus aside a small portion near the midlength of flagellum. In glandulocaudines and stevardiines without nuclear

rotation, the mitochondria are usually located in a certain extension along the flagellum, aside and behind the nucleus.

Character 8 (CI = 1.00) - Anterior border of the nucleus (ordered):

0= semicircular or emarginated

1= asymmetric, clearly oblique to the longest axis of the nucleus

The anterior border of the nucleus in *"O". dialeptura* and *"O". mitoptera* is oblique, with the lateral of the nucleus opposite to the flagellum clearly longer than the lateral of the nucleus where the flagellum is attached. In comparative taxa it is observed only the state 0.

Character 9 (CI = 1.00) - Vesicles (ordered):

0= elongated vesicles

1= large number of small and globular vesicles

The vesicles found in the midpiece present a large variability in shape and size among the Characiformes, or even are lacking in some of it representatives. Among cheirodontines, there is a pattern found in the externally examined species that comprises a large number of small and globular vesicles fulfilling most of the midpiece. Such a pattern has not been found in other Characiformes, except for a similar pattern described for *Citharinus* sp. (Mattei et al., 1995), but with clearly smaller vesicles.

The inseminating species of the Cheirodontinae have a small number of larger and somewhat elongated vesicles. There is no clear recognized pattern common to all inseminating species of the Cheirodontinae and different of those of all outgroup characiforms (e.g. *Aphyocharax anisitsi* - Burns et al., 1998:237, fig. 1) being assigned State 0 for these taxa.

Character 10 (CI = 1.00) - Vesicles position (ordered):

0 = located only after the mitochondria, in the basal region of the midpiece.

1 = located after the mitochondria, in the basal region of the midpiece and along the cytoplasmic collar in one side of the nucleus.

The presence of vesicles along the nucleus inside the cytoplasmic collar was observed only in *“O”. dialeptura* and *“O”. mitoptera*, and has not been found in other characiforms.

Appendix B. Characters matrix of 10 characters for the 11 taxa of Cheirodontinae.

	1	2	3	4	5	6	7	8	9	10
outgroup	0	0	0	0	0	0	0	0	0	0
Kolpotocheirodon theloura	0	0	0	1	0	0	0	0	0	0
Compsura heterura	1	1	0	1	1	0	0	0	0	0
Macropsobrycon uruguayanae	1	1	0	1	1	0	0	0	0	0
Acinocheirodon melanogramma	2	1	1	1	1	1	1	0	0	0
Saccoderma hastatus	2	2	1	1	1	1	1	0	0	0
“Odontostilbe” dialeptura	2	1	1	1	0	1	1	1	0	1
“Odontostilbe” mitoptera	2	1	1	1	0	1	1	1	0	1
Cheirodon interruptus	0	0	0	0	0	0	0	0	1	0
Odontostilbe pequira	0	0	0	0	2	0	0	0	1	0
Serrapinnus calliurus	0	0	0	0	0	0	0	0	1	0
Serrapinnus heterodon	0	0	0	0	0	0	0	0	1	0

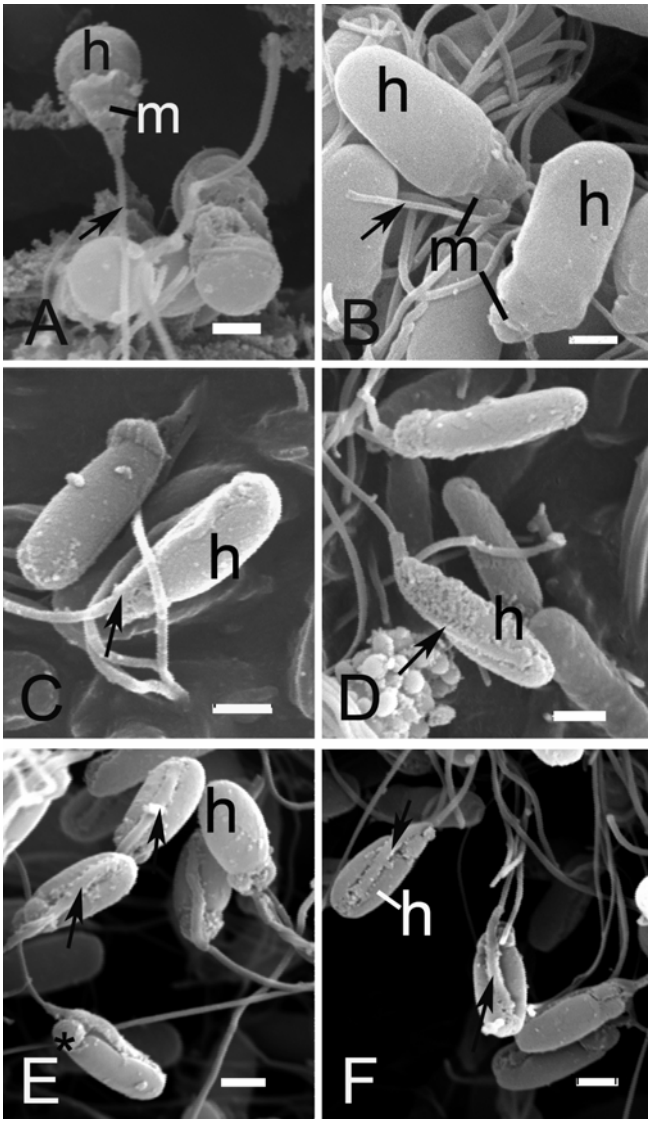

Fig. 1. SEM of mature spermatozoa of *Kolpotocheirodon theloura* (a)*, Compsura heterura* (b)*, Acinocheirodon melanogramma* (c)*, Saccoderma hastatus* (d)*, "Odontostilbe" dialeptura* (e) and "*O." mitoptera* (f). arrow, flagellum; asterisk, mitochondria; h, sperm head; m, midpiece;. Scale bars: 1 µm.

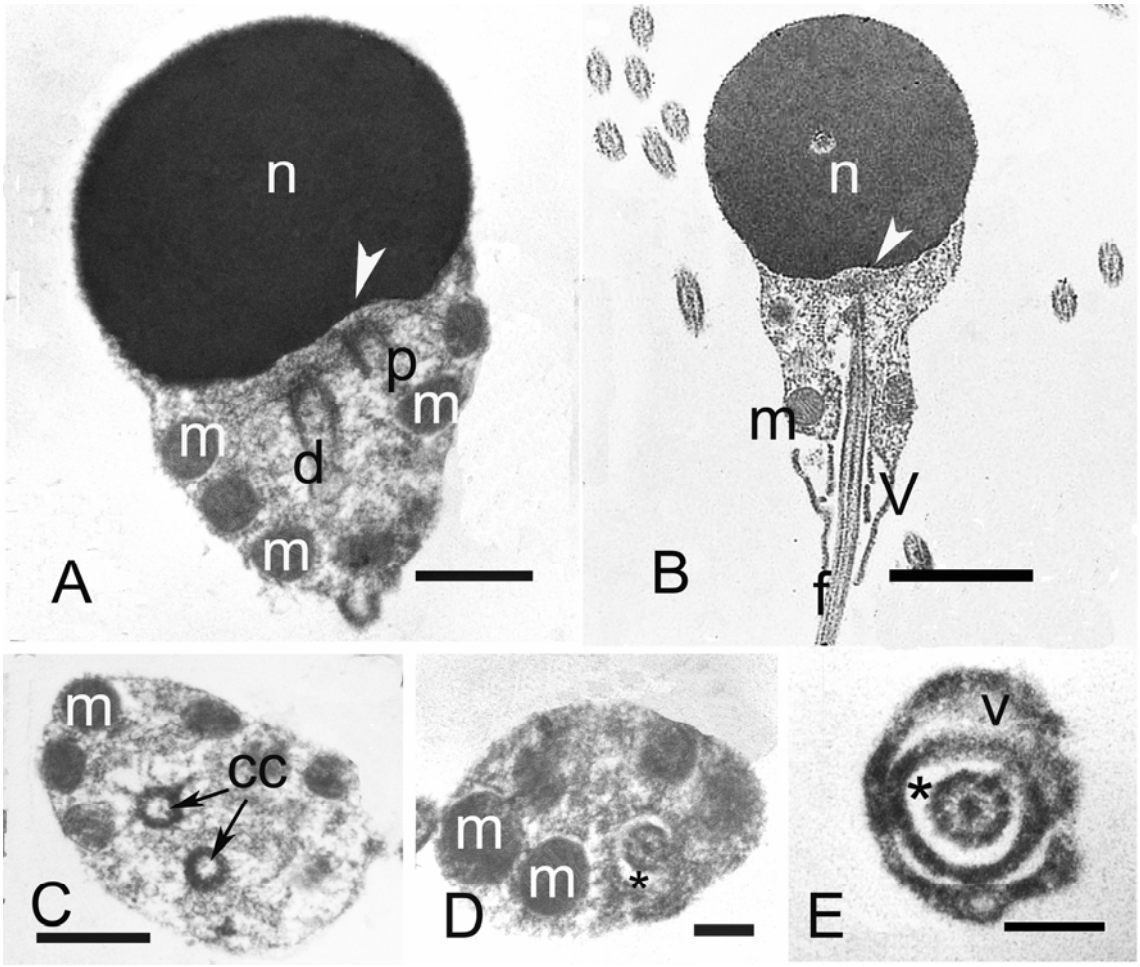

Fig. 2. TEMs of sections through mature spermatozoa of *Kolpotocheirodon theloura*. A: longitudinal sections of spermatozoa showing spherical nuclei with condensed chromatin, a long midpiece with parallel centrioles and spherical mitochondria. B: longitudinal sections of spermatozoa showing an elongate cytoplasmic canal and vesicles in the end of midpiece. C, D, E: successively posterior transverse sections through spermatozoa. C: section through proximal portion of midpiece showing small spherical mitochondria and centriolar arrangement. D: section through more distal portion of midpiece showing mitochondria and axoneme. E: section through tubular-vesicular compartment showing axoneme in the center. arrowhead, nuclear fossa; asterisk, cytoplasmic canal; cc, centriolar complex; d, distal centriole; f, flagellum; m, mitochondrion; n, nucleus; p, proximal centriole; v, tubular-vesicular compartment. Scale bars: 0.5 µm (A, C; B); 1 µm (D); 0.2 µm (E).

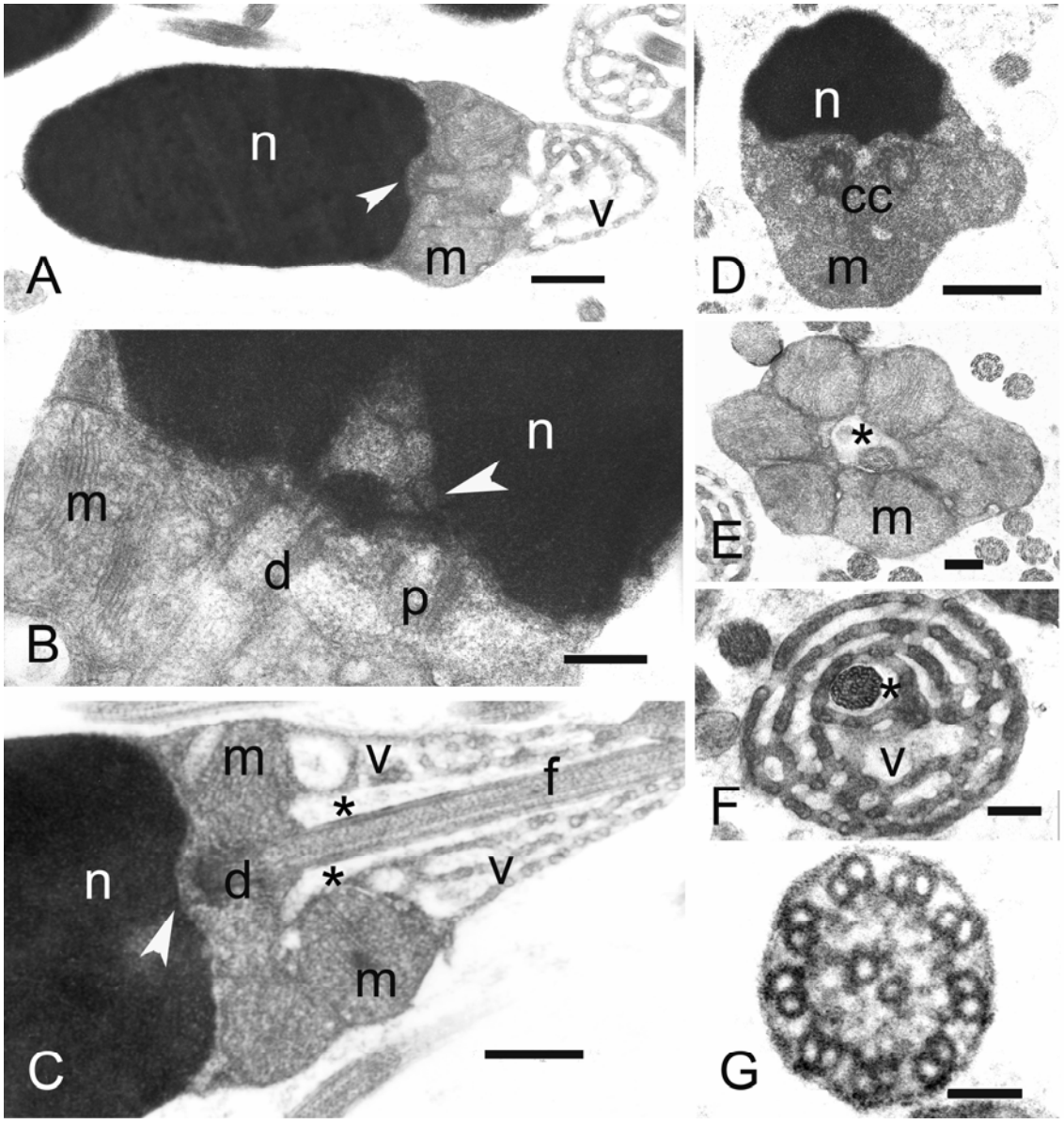

Fig.3. TEMs of sections through mature spermatozoa of *Compsura heterura*. A-C: longitudinal sections of spermatozoa. D-G: transversal sections of spermatozoa. A: spermatozoon with slightly elongate nuclei and highly condensed chromatin. B: parallel centrioles. C: elongate cytoplasmic canal and vesicles in the end of midpiece. D: oblique section through proximal portion of midpiece showing the centriolar arrangement. E: section through more distal portion of midpiece showing large mitochondria and

axoneme. F: section through tubular-vesicular compartment showing axoneme in the center. G: section through typical axoneme. arrowhead, nuclear fossa; asterisk, cytoplasmic canal; cc, centriolar complex; d, distal centriole; f, flagellum; m, mitochondrion; n, nucleus; p, proximal centriole; v, tubular-vesicular compartment. Scale bars: 0.5 µm (A, C, D); 0.2 µm (B, E, F); 50 nm (G).

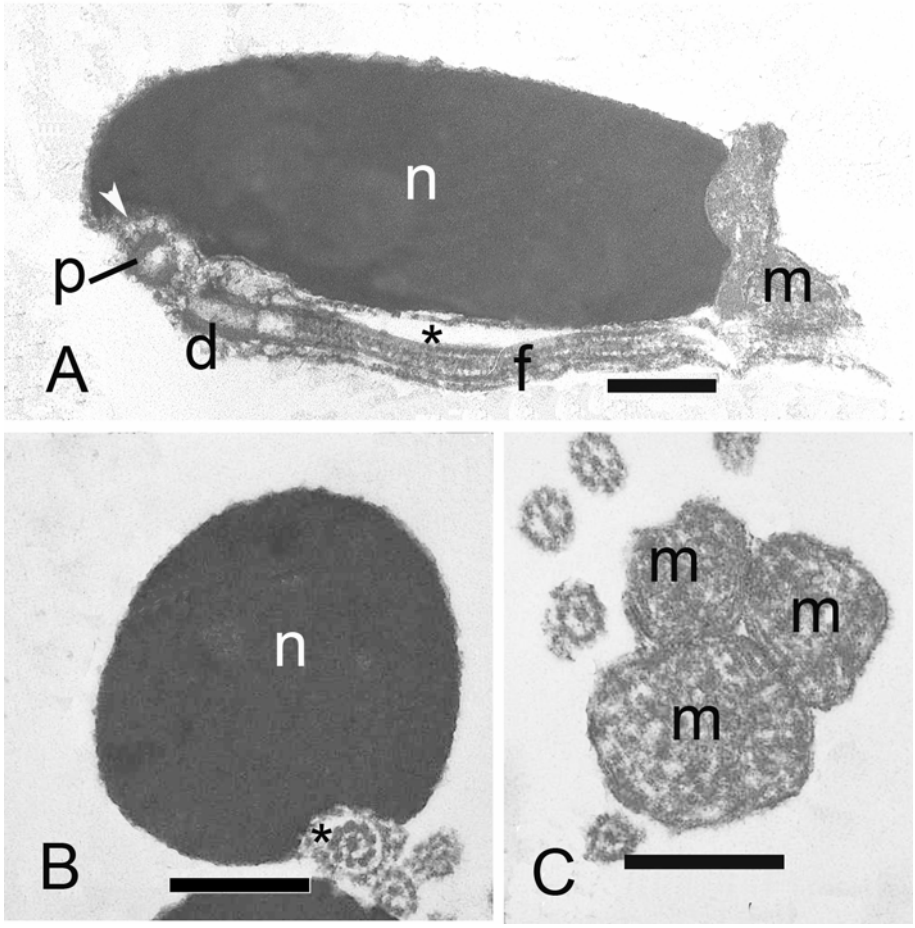

Fig. 4. TEMs of sections through mature spermatozoa of *Acinocheirodon melanogramma*. A: longitudinal sections of spermatozoa showing elongate nuclei with highly condensed chromatin. B: section through medial portion of nucleus showing the axoneme and the cytoplasmic canal surrounding flagellum. C: section through region posterior of spermatozoon showing the three mitochondrial. arrowhead, nuclear fossa; asterisk, cytoplasmic canal; d, distal centriole; f, flagellum; m, mitochondrion; n, nucleus; p, proximal centriole. Scale bars: 0.5 µm

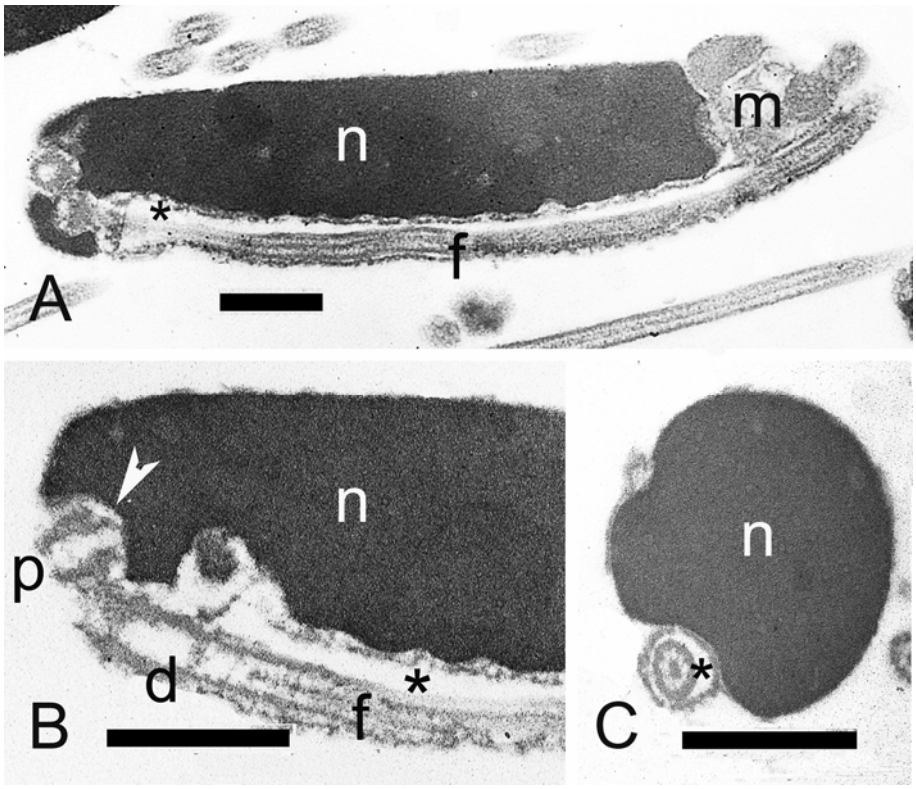

Fig. 5. TEMs of sections through mature spermatozoa of *Saccoderma hastatus*. A, B: longitudinal sections of spermatozoa showing elongate nuclei with highly condensed chromatin. B: perpendicular centrioles. C: section through medial portion of nucleus showing the axoneme and the cytoplasmic canal surround flagellum. arrowhead, nuclear nucleus; asterisk, cytoplasmic canal; d, distal centriole; f, flagellum; m, mitochondrion; n, nucleus; p, proximal centriole. Scale bars: 0.5 µm

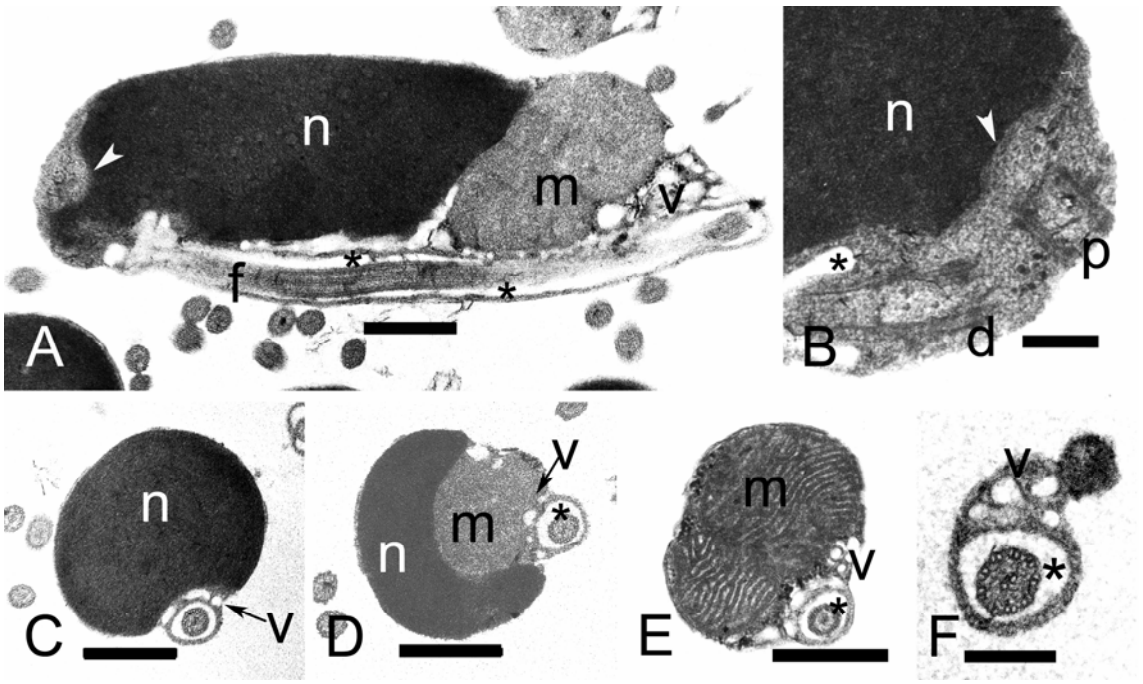

Fig. 6. TEMs of sections through mature spermatozoa of *"Odontostilbe" dialeptura.* A, B: longitudinal sections of spermatozoa. C-F: transverse sections of spermatozoa. A: spermatozoon with elongate nuclei, highly condensed chromatin and lateral flagellum. B: proximal centriole is anterior and oblique to the distal one and both are located outside the nuclear fossa. C - F: successively more posterior transverse sections of spermatozoon. C, D: section through medial portion of nucleus showing the axoneme and the cytoplasmic canal surround flagellum. E: section through of mitochondria and axoneme. F: section through tubular-vesicular compartment showing axoneme in the center. arrowhead, nuclear fossa; asterisk, cytoplasmic canal; d, distal centriole; f, flagellum; m, mitochondrion; n, nucleus; p, proximal centriole; v, tubular-vesicular compartment. Scale bars: 0.5 µm (A, C, D, E); 0.2 µm (B, F).

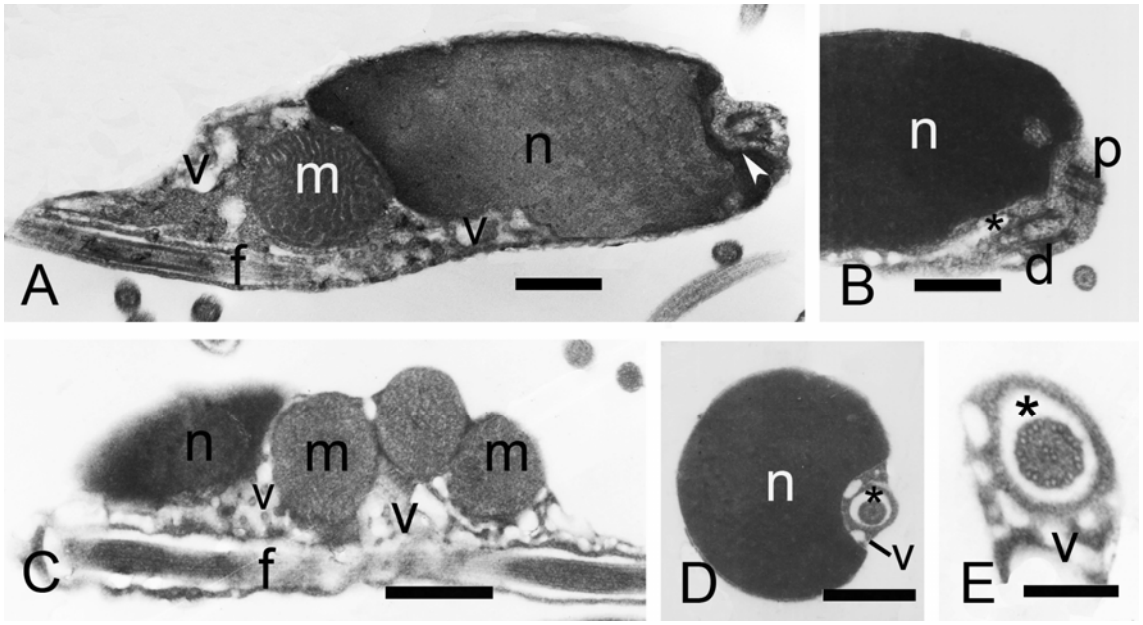

Fig. 7. TEMs of sections through mature spermatozoa of *"Odontostilbe" mitoptera.* A, B, C: longitudinal sections of spermatozoa. D, E: transverse sections of spermatozoa. A: elongate lateral nucleus with highly condensed chromatin, a big mitochondrion and vesicles in one side of the nucleus and surround the mitochondrion. B: proximal centriole is anterior and oblique to the distal one and both are located outside the nuclear fossa. C: three mitochondria located after the tip of nucleus, lateral flagellum and vesicles. D: section through medial portion of nucleus showing the axoneme and the cytoplasmic canal surround flagellum. E: section through tubular-vesicular compartment showing axoneme in the center. arrowhead, nuclear fossa; asterisk, cytoplasmic canal; d, distal centriole; f, flagellum; m, mitochondrion; n, nucleus; p, proximal centriole; v, tubular-vesicular compartment. Scale bars: 0.5 µm (A, B, C, D); 0.2 µm (E).

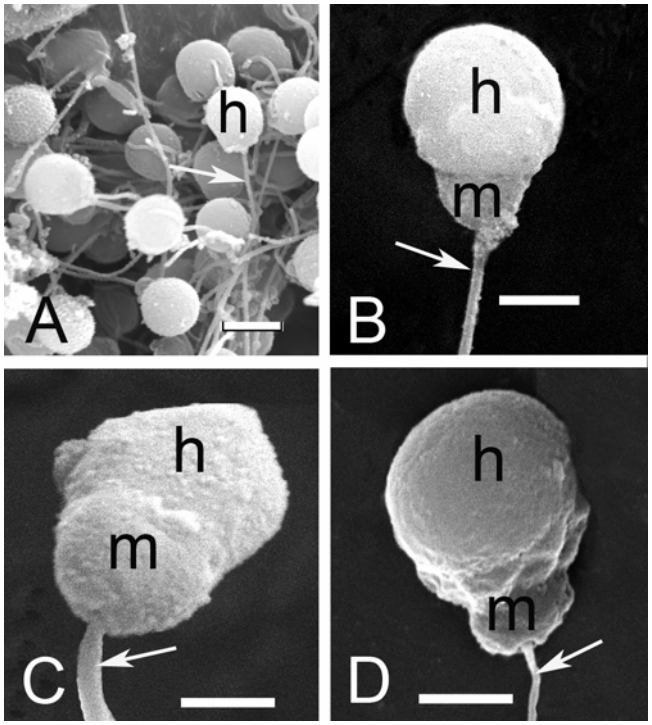

Fig. 8. SEM of mature spermatozoa of *Cheirodon interruptus* (A)*, Odontostilbe pequira* (B)*, Serrapinnus calliurus* (C)*, Serrapinnus heterodon* (D)*.* arrow, flagellum; h, sperm head; m, midpiece. Scale bars: 2 µm (A); 1 µm (B-D).

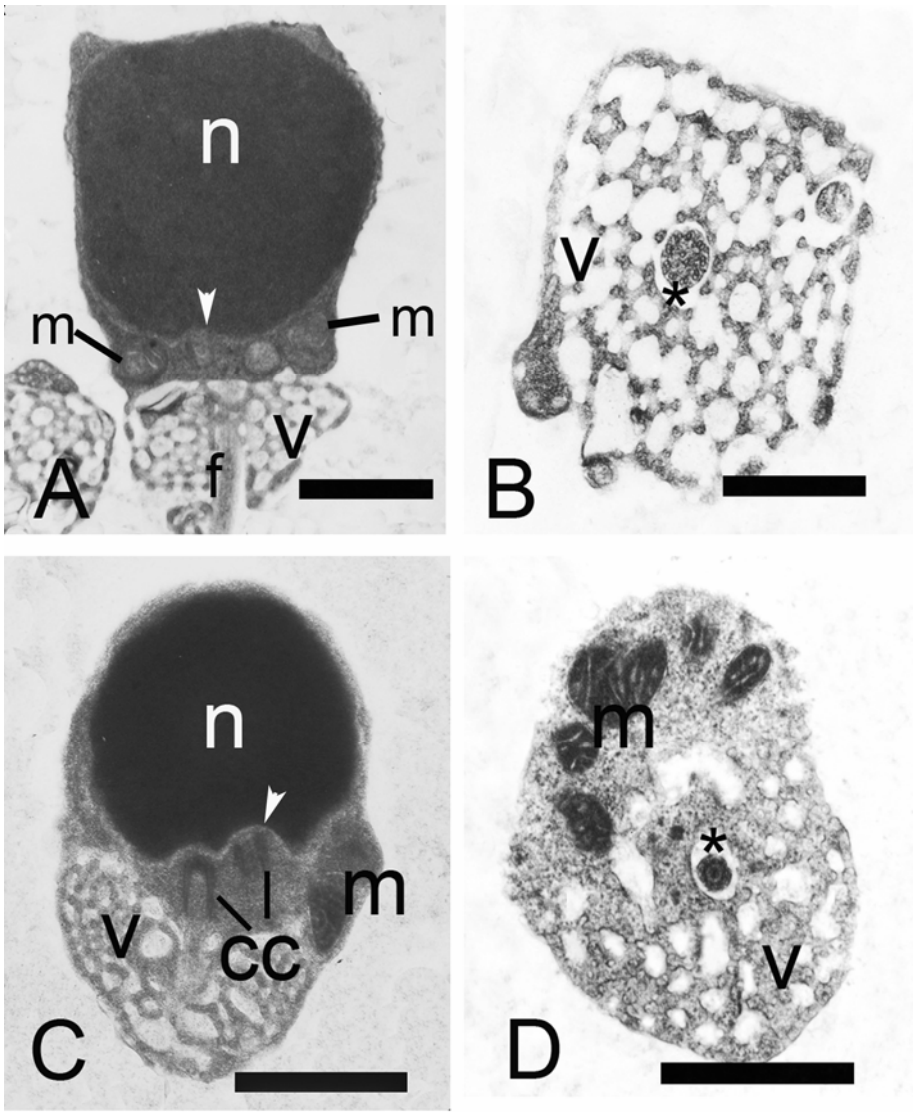

Fig. 9. TEMs of sections through mature spermatozoa of *Cheirodon interruptus* (A, B) and *Odontostilbe pequira* (C, D). A, C: longitudinal sections of spermatozoa showing spherical nuclei with condensed chromatin, short midpiece, spherical mitochondria and vesicles in the end of midpiece. C: both centrioles are partially inside nuclear fossa. B, D: transverse sections of spermatozoa showing the tubular-vesicular compartment and axoneme in the center. arrowhead, nuclear fossa; asterisk, cytoplasmic canal; cc, centriolar complex; f, flagellum; m, mitochondrion; n, nucleus; v, tubular-vesicular compartment. Scale bars: 1 µm (A, C, D), 0.5 µm (B).

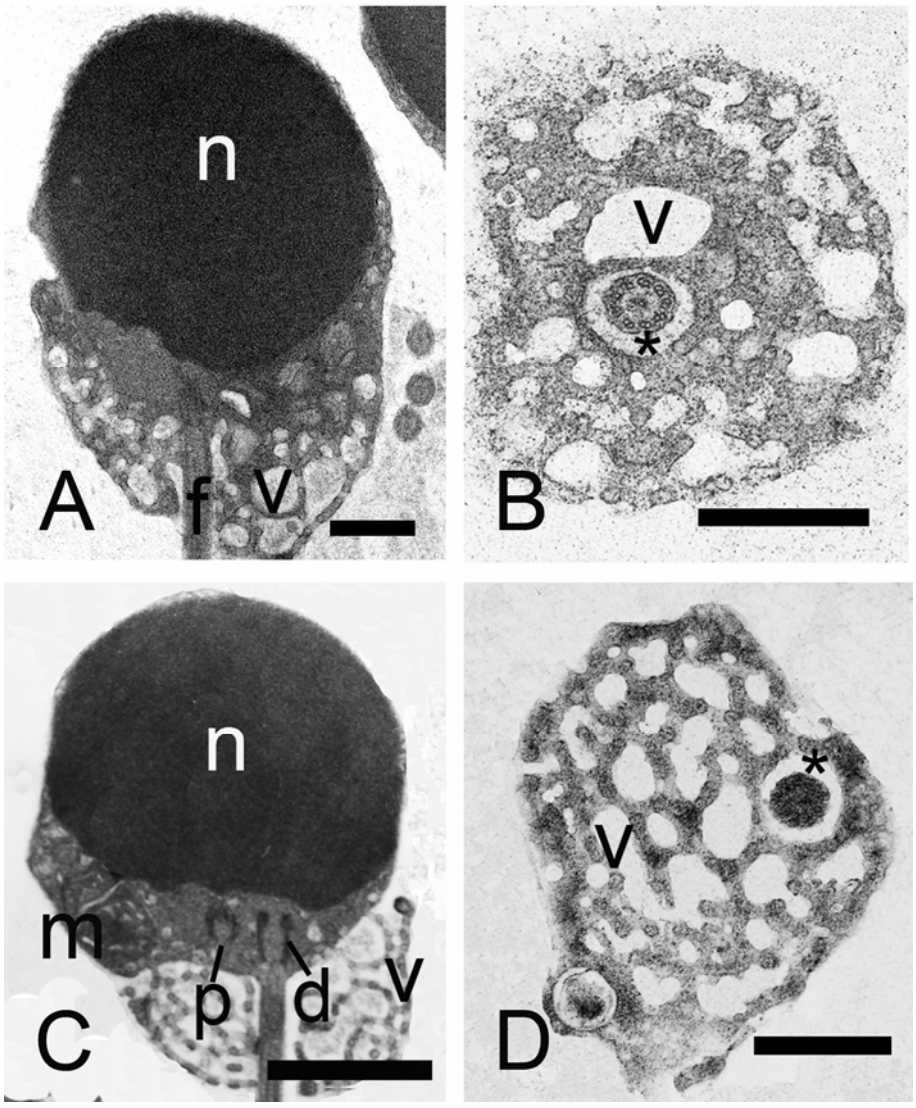

Fig. 10. TEMs of sections through mature spermatozoa of *Serrapinnus calliurus* (A, B), and *Serrapinnus heterodon* (C, D). A, C: longitudinal sections of spermatozoa showing spherical nuclei with condensed chromatin, short midpiece and vesicles in the end of midpiece. B, D: transverse sections of spermatozoa showing the tubular-vesicular compartment and axoneme in the center. asterisk, cytoplasmic canal; c, centriolar complex; d, distal centriole; f, flagellum; m, mitochondrion; n, nucleus; p, proximal centriole; v, tubular-vesicular compartment. Scale bars: 0.5 µm (A, B, D), 1 µm (C).

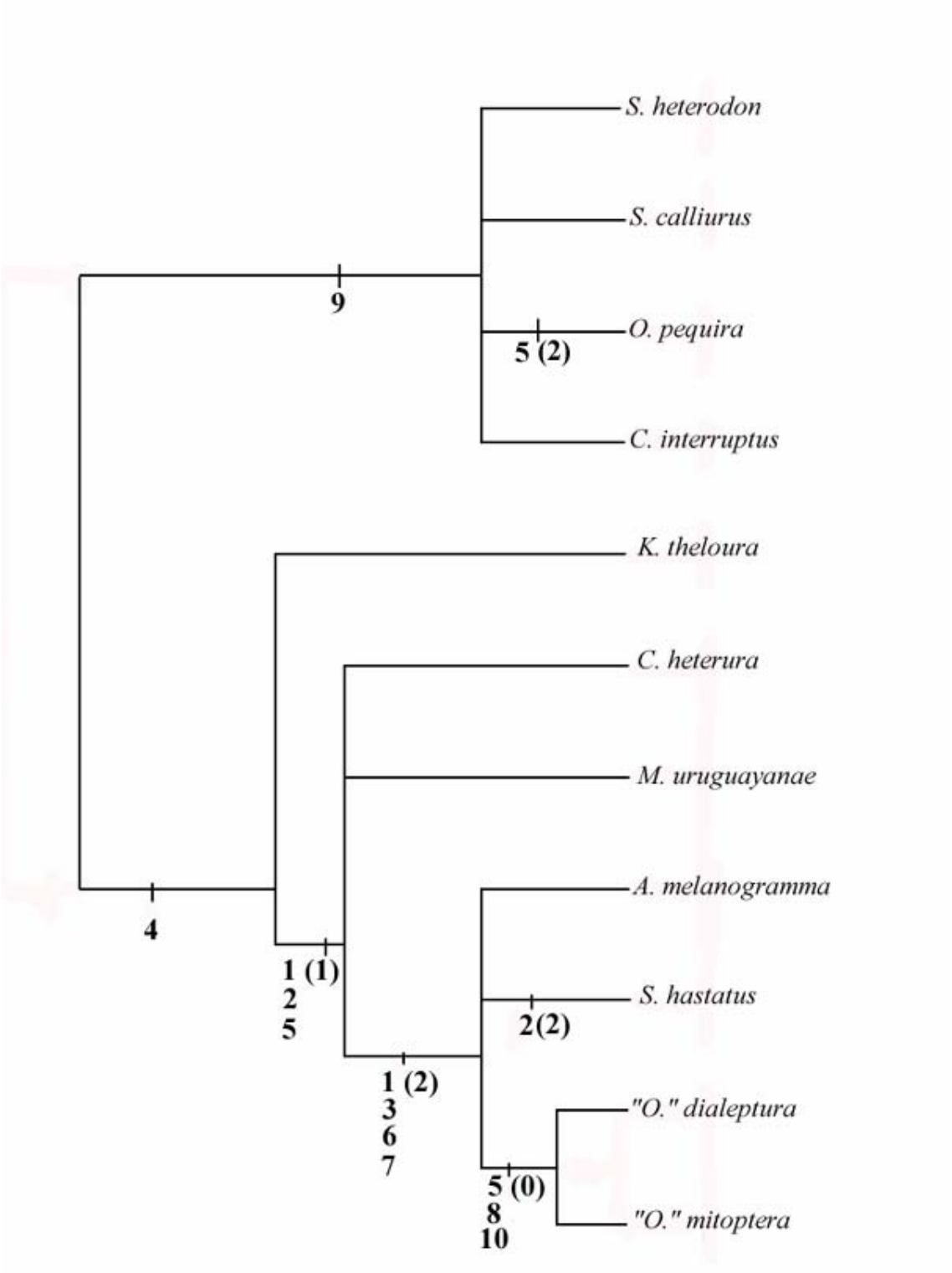

Fig. 11. Consensus cladogram with a hypothesis of relationships among the species of Cheirodontinae based on the analysis of 10 characters of the sperm ultrastructure. Consensus of 3 equally parsimonious trees, length 14, Consistence Index (CI) 0.92, and Retention Index (RI) 0.96.

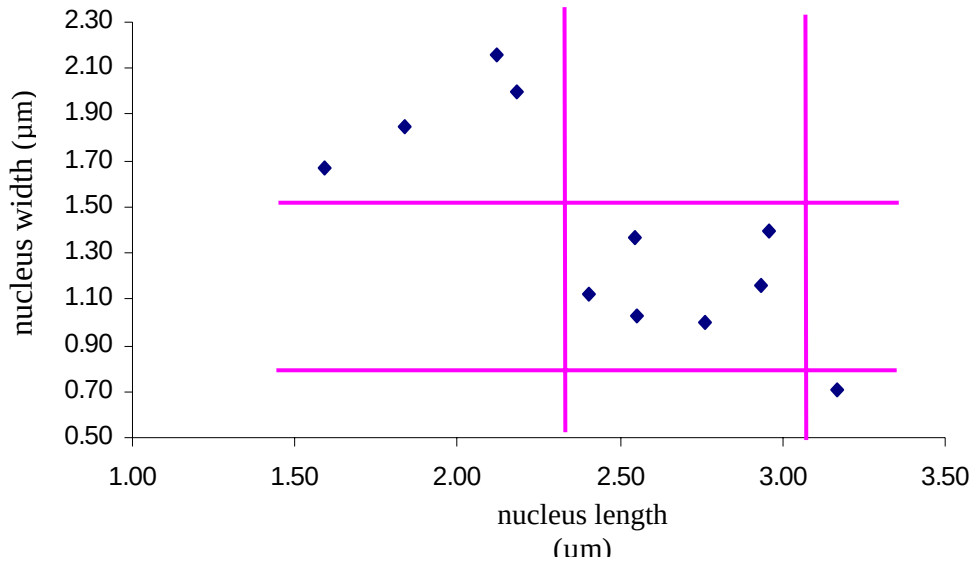

Fig 12. Relation between length and width of nucleus of species of Cheirodontinae. The horizontals bars represents the width of nucleus intervals in micrometers and the verticals bars represents the length of nucleus intervals.

Capítulo 2:

Sperm ultrastructure in the inseminating *Macropsobrycon uruguayanae* (Teleostei: Characidae: Cheirodontinae)

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Keywords: insemination, sperm ultrastructure, parasperm, accessory microtubules, striated rootlet.

ABSTRACT

Macropsobrycon uruguayanae is a small inseminating characid (tetra) of the tribe Compsurini. Although spermatozoa can be found within the ovarian cavity close to oocytes, the exact moment of fertilization has not yet been determined. Mature spermatozoa have moderately elongate nuclei with electron dense chromatin. During spermiogenesis nuclear rotation takes place. Elongate mitochondria with lamellar cristae are found posterior to the nucleus. Centrioles are parallel to one another with the proximal centriole slightly anterior to the longer distal one. The anterior tip of the proximal centriole is located within a shallow nuclear fossa. Electron-dense spurs are associated within the anterior and posterior ends of the distal centriole. Striated centriolar rootlets radiate both anteriorly and posteriorly from the distal centriole. Nine longitudinal accessory microtubules surround the axoneme in the proximal flagellum. The flagellum has a typical 9+2 axoneme with no intratubular differentiation. Atypical spermatozoa are also found in the testicular lumen. These cells resemble spermatozoa in most aspects, except that their nuclei are variable in shape, with the granular chromatin less electron-dense than that seen in spermatozoa. The origin and function of these cells could not be determined. The specializations seen in the spermatozoa are discussed as possible adaptations related to the habit of insemination.

INTRODUCTION

Species of the subfamily Cheirodontinae, family Characidae, are characterized by the presence of pedunculated jaw teeth, absence of a humeral spot and a gap between the muscles covering the anterior portion of the swim bladder, referred to as the pseudotympanum. This subfamily comprises two tribes, the Cheirodontini and the Compsurini, plus several *incertae sedis* genera. *Macropsobrycon uruguayanae* belongs to the Compsurini whose species are characterized by the presence of modified anal-fin hooks, and modified caudal-fin rays and/or scales in mature males, and the ability of males to transfer sperm to the ovary of the female, i.e. insemination (Burns et al., 1997; Malabarba, 1998, 2003). Insemination has often been associated with modifications of the sperm cells that appear to relate to this unique mode of reproduction (Jamieson, 1991; Burns and Weitzman, 2005). Additionally, since insemination is a necessary first step toward viviparity, examination of the morphological specializations of the spermatozoa of inseminating species may be useful in understanding the initial steps toward live-bearing.

Characters obtained from sperm ultrastructure have been useful in hypothesizing phylogenetic relationships among fishes (Jamieson, 1991; Mattei, 1991). Prior to Jamieson (1991), no information on sperm ultrastructure was available for any species of the family Characidae, which is estimated to contain over 962 species of freshwater fishes (Nelson, 2006). Since that time at least some information on sperm ultrastructure for over 35 characid species has become available and the data indicate marked morphological variation, particularly among those species that are inseminating (Burns and Weitzman, 2005). For cheirodontine species, data are available for two externally fertilizing species, *Serrapinnis notomelas* (from Gusmão-Pompiani, 2003) and *S. kriegi* (from Burns and Weitzman, 2005), and very limited data for one inseminating species, *Macropsobrycon uruguayanae* (Burns et al., 1998; Burns and Weitzman, 2005). The main purpose of the present study is to provide a detailed analysis of sperm

ultrastructure in *Macropsobrycon uruguayanae*. The morphological specializations found are discussed as possible adaptations related to the reproductive habit of insemination.

MATERIALS AND METHODS

The three mature males analyzed in this study are catalogued in the fish collections of the Museu de Ciências e Tecnologia (MCP) and Departamento de Zoologia, Universidade Federal do Rio Grande do Sul (UFRGS), both from Porto Alegre, Brazil. The specimens are representative of two populations from Rio Grande do Sul State, Brazil. One specimen (MCP 18588, SL 39 mm) was collected in a pond near the margin of the Rio Jacuí, Cachoeira do Sul, laguna dos Patos drainage, 30°03'S, 52 °54'W. The other two specimens were collected in two different localities from the Rio Uruguay drainage. One specimen (UFRGS 8792, SL 29 mm) was from the Rio Ibicuí da Faxina between Santana do Livramento and Rosário do Sul, 30°47'22"S, 55°12'41"W, and the other (UFRGS 8791, 31 mm) from the Sanchuri dam, Uruguaiana, 29°32'41"S, 56 °49'06"W.

The fishes were sacrificed by immersion in a lethal dose of tricaine methanesulfonate (MS222) and/or a cut through the spinal cord. Immediately afterwards, whole testes or smaller pieces were placed in modified Karnovsky's fixative (Ito and Karnosvsky, 1968) and kept under refrigeration until the start of sample processing.

For scanning electron microscopy (SEM), whole testes were dehydrated in an ethanol series and critical-point dried. The dried tissue was then attached to stubs with carbon double-stick tape and teased apart with needles. The sample was sputter-coated with carbon and gold and viewed in a Philips XL 30 scanning electron microscope.

For transmission electron microscopy (TEM), testes were cut into smaller pieces (1 mm^3), rinsed in phosphate buffer, and postfixed in 1% osmium tetroxide in phosphate buffer. These were then rinsed in phosphate buffer, dehydrated in an ethanol series,

infiltrated, and embedded in Araldite 502. Ultrathin sections were stained with aqueous uranyl acetate and lead citrate and examined with a JEOL JEM 1200 transmission electron microscope.

RESULTS

In early spermatids (Fig. 1A), nuclear chromatin is only partially condensed and nuclear rotation commences. Abundant cytoplasm surrounds the nucleus. Mitochondria are displaced to the posterior part of the nucleus, which corresponds to the future midpiece of the spermatozoon. During this phase it is possible to see the developing cytoplasmic canal and tubular-vesicular compartment (Fig. 1A). During the late spermatid stage (Fig.1B), nuclear rotation is completed. A narrow strip of cytoplasm, devoid of organelles, surrounds the nucleus at this time. The centrioles and mitochondria are located in the anterior part of the developing midpiece near the base of the nucleus, while the tubular-vesicular system is confined to the posterior part of the midpiece (Fig. 1B). Nuclear elongation only takes place during late spermiogenesis.

Mature spermatozoa (Figs. 2, 3) have elongate nuclei measuring approximately 3 µm in length and 1.3 µm in width with highly electron dense chromatin. Acrosomal vesicles are absent. The midpiece contains multiple elongate mitochondria with lamellar cristae (Figs. 3, 4B,C) and a tubular-vesicular compartment (Figs 3A, 4D). It is possible to see a long cytoplasmic canal in some sections (Figs. 3C, 4D). The two centrioles are parallel one to another, with the proximal centriole slightly anterior to the distal centriole. The anterior portion of the proximal centriole is located within a shallow nuclear fossa (Fig. 3B). The distal centriole is also longer than the proximal one. Electron-dense spurs are associated with the anterior and posterior ends of the distal centriole (Fig. 3B). Striated centriolar rootlets radiate both anteriorly and posteriorly from the distal centriole (Fig. 3B, C). The single flagellum is completely posterior to the nucleus and lacks axonemal fins. The axoneme in the proximal region of the flagellum is surrounded by

nine longitudinal accessory microtubules (Fig. 4C-F). The axoneme has the typical 9+2 arrangement of microtubules and there is no intratubular differentiation (Fig. 4E, F).

Atypical sperm cells were found in the testicular lumen together with normal spermatozoa (Fig.3A, 5). These atypical cells were seen in all specimens analyzed. These cells contain all of the unique structures found in normal spermatozoa, such as striated centriolar rootlets and accessory microtubules (Fig. 5). However, the nuclei of these cells are highly irregular in shape and contain uniformly granular chromatin that is much less electron-dense than that seen in normal spermatozoa (Fig. 5A, D). In addition, in some cases the spaces between the membranes of the mitochondrial cristae are wider than in normal spermatozoa.

DISCUSSION

Many externally fertilizing teleosts, including all externally fertilizing characid species studied to date, have spermatozoa with spherical to slightly ovoid nuclei referred to as aquasperm (Jamieson, 1991). On the other hand, the great majority of inseminating characids, including *Macropsobrycon uruguayanae*, have spermatozoa with moderately to extremely elongate nuclei with only seven inseminating species producing typical aquasperm (Burns and Weitzman, 2005; Malabarba et al., 2004). Several ideas have been put forth as possible selective advantages of nuclear elongation, which in turn results in elongation of the sperm head. More streamlined sperm heads may facilitate penetration of egg coats (Jamieson, 1991). At any given moment, thinner cells would be able to pass through the female gonopore in greater numbers (Burns and Weitzman, 2005), as well as permit more efficient movement through the tortuous pathways of the ovary (Gardiner, 1978). In addition, even in species that do not produce distinct sperm packets, more streamlined cells may increase the side-to-side alignment and clumping of cells, which in turn may reduce loss of spermatozoa to the water column during sperm transfer (Ginzburg, 1968; Burns and Weitzman, 2005). Finally, narrower sperm heads would encounter less resistance

moving in the forward direction, so some directionality may be maintained (Burns and Weitzman, 2005).

After the completion of nuclear rotation in *Macropsobrycon uruguayanae*, the nucleus elongates in the direction anterior to the centrioles. Therefore, flagellar movement in the mature spermatozoon would result in the cell being pushed from behind the nucleus. The spermatozoa of all other inseminating characids with elongate nuclei, for which published information is available, do not undergo nuclear rotation. In these species, during spermiogenesis the nucleus elongates mainly in the direction posterior to the centrioles (Burns et al., 1998). The species that exhibit this method of nuclear elongation include the following: in the subfamily Stevardiinae, *Diapoma speculiferum*, *Diapoma* sp., *Corynopoma riisei*, and *Pseudocorynopoma doriae* (Burns and Weitzman, 2005; Burns et al., 1998) and *Scopaeocharax rhinodus*, *Tyttocharax cochui* and *T. tambopatensis* (Pecio et al, 2005); in the subfamily Glandulocaudinae, *Mimagoniates barberi* and *M. microlepis* (Pecio and Rafiński, 1994, 1999; Burns et al., 1998); and two species currently *incertae sedis*, *Bryconadenos tanaothoros* (Weitzman et al., 2005) and *Brittanichthys axelrodi* (Javonillo et al., 2007).

Mature spermatozoa of *Macropsobrycon uruguayanae* have striated rootlets that radiate both anteriorly and posteriorly from the distal centriole. Striated rootlets, composed of the protein rootletin, have been shown to help ciliated cells withstand mechanical stresses (Yang et al., 2002, 2005). The location of the striated rootlets in *M. uruguayanae* suggests that they may support and stabilize the base of the flagellum, i.e. the distal centriole. Striated rootlets are also present in the inseminating characid, *Brittanichthys axelrodi*, a species currently listed as *incertae sedis*, where it is suggested that a robust anchorage of the flagellum may be advantageous for sperm cells that must travel through the viscous fluids and circuitous pathways of the ovary (Javonillo et al., 2007). Striated rootlets are also reported for species of externally fertilizing elopomorph teleosts (Jamieson, 1991), as well as many invertebrates (Justine, 1995; Gracenea et al., 1997); thus they are not restricted to the spermatozoa of inseminating species.

In *Macropsobrycon uruguayanae*, nine accessory microtubules, located peripheral to and between the microtubule doublets of the axoneme, are confined to the midpiece and anterior portion of the flagellum. Accessory microtubules have also been found in the spermatozoa of other inseminating characids, including the glandulocaudines *Mimagoniates barberi* and *M. microlepis* (Pecio and Rafiński, 1994, 1999; Burns et al., 1998), the stevardiines *Scopaeocharax rhinodus*, *Tyttocharax cochui* and *T. tambopatensis* (Pecio et al, 2005), and *Bryconadenos tanaothoros* currently *incertae sedis* (Weitzman et al., 2005). Given the relatively wide spacing between adjacent accessory microtubules in all of these species, it is unlikely that these organelles function directly in cell movement similar to the doublets of the axoneme. Instead, they may impart rigidity to the cell, given that more rigid cells produce less drag (Fauci, 1996), which could permit more efficient movement of cells through the viscous fluids of the ovarian cavity (Burns and Weitzman, 2005).

In all specimens of *Macropsobrycon uruguayanae* analyzed, two morphologically distinct types of sperm cells are found within the testis ducts. The main differences between these two cells types are the shape of the nucleus and degree of chromatin condensation. The cells that appear to be “normal” sperm (eusperm) have bullet-shaped nuclei containing condensed, highly electron-dense chromatin, a characteristic seen in mature spermatozoa of nearly all other characids studied to date. The atypical cells, on the other hand, have larger, irregularly shaped nuclei containing a much less electron-dense, granular chromatin unlike any thus far observed in mature spermatozoa of other characids. These atypical spermatozoa do not appear to be spermatids that have been released early, a phenomenon observed in teleosts with semicystic spermatogenesis (Lahnsteiner et al., 1990; Mattei et al., 1993; Hernández et al., 2005). The spermatids of *M. uruguayanae* (Fig. 1) have spherical nuclei with chromatin that condenses into more electron-dense aggregations, whereas the atypical spermatozoa have irregularly shaped nuclei containing a uniformly distributed light, granular chromatin unlike that seen in any spermatid stage. Unfortunately we were unable to locate spermatocysts containing

developing atypical spermatozoa, so we do not know if they are formed within separate spermatocysts or if they develop along with eusperm within the same spermatocyst.

Atypical sperm, often referred to as dimorphic sperm or parasperm, are relatively common in some invertebrate taxa (Jamieson, 1986; Oppliger et al., 1998; Ferraguti et al., 2002). Within the vertebrates, on the other hand, they have only been reported in certain cottoid fishes (Hayakawa and Munehara, 2004), unless the production of deformed sperm in mammals, including humans, can be included in this category (Baker and Bellis, 1988). The parasperm of cottoid fishes appear to result from an incomplete second meiotic division (Hayakawa and Munehara, 2004). Depending on the species, these cells have either two nuclei or a bi-lobed nucleus, and two or no flagella (Hayakawa et al., 2002a,b; Hayakawa and Munehara, 2004). Because of the abnormal morphology and diploid genome of the cottoid parasperm, these cells appear to be incapable of fertilizing eggs. However, experimental evidence indicates that parasperm may help reduce the lateral dispersion of semen and thus increase the number of eusperm that can reach an egg mass (Hayakawa et al., 2002b; Hayakawa and Munehara, 2004), as well as play a role in sperm competition in cottoids (Hayakawa et al., 2002a). The atypical sperm of *Macropsobrycon uruguayanae* bear little resemblance to the parasperm of cottoids. Their possible function in the reproductive biology of this species remains to be determined.

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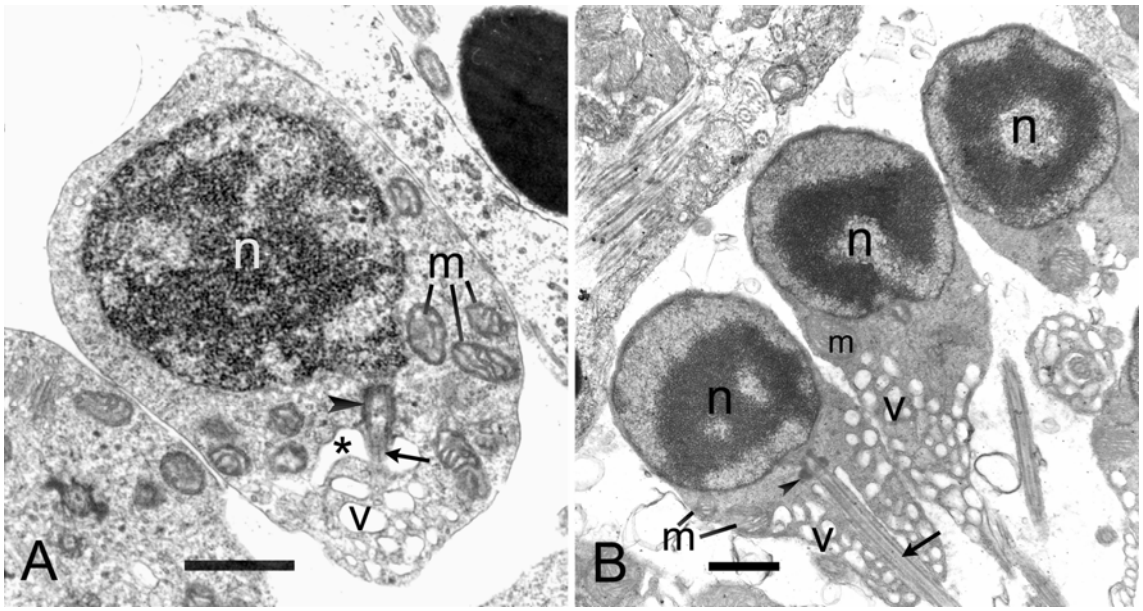

Fig. 1. TEMs through spermatids of *Macropsobrycon uruguayanae*. A: Longitudinal section through early spermatid undergoing nuclear rotation; developing tubular-vesicular compartment is seen. B: Longitudinal sections through three later spermatids showing further developed tubular-vesicular system; nuclear rotation is complete but nuclear elongation has not yet begun. arrow, flagellum; arrowhead, centriolar spur; asterisk, cytoplasmic canal; m, mitochondrion; n, nucleus; v, tubular-vesicular compartment;. Scale bars: 0.5 µm.

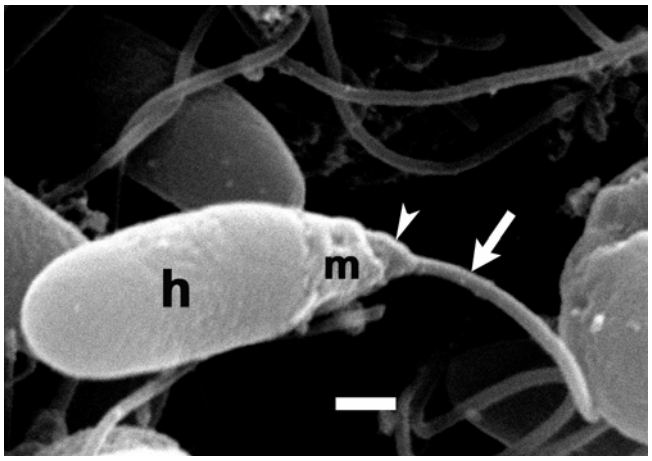

Fig. 2. SEM of mature spermatozoon of *Macropsobrycon uruguayanae.* arrow, flagellum; arrowhead, tubular-vesicular compartment region; h, sperm head; m, midpiece. Scale bar: 0.5µm.

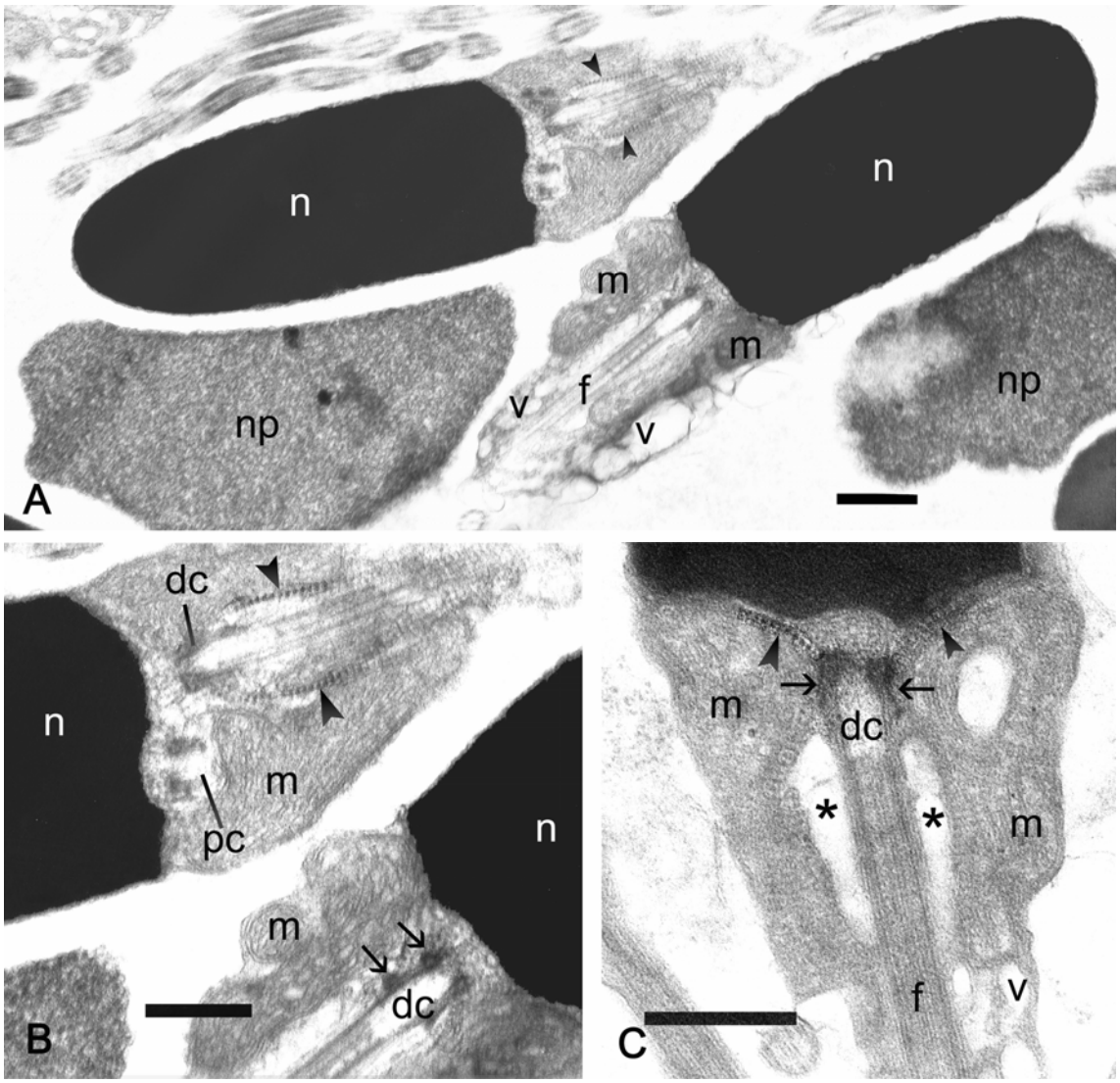

Fig. 3. TEMs of longitudinal sections through mature spermatozoa of *Macropsobrycon uruguayanae*. A: two spermatozoa showing elongate nuclei with highly condensed chromatin; B: midpiece region showing centriolar arrangement and striated rootlets. C: midpiece region showing electron-dense centriolar spurs and striated rootlets radiating anteriorly and posteriorly from distal centriole. arrow, centriolar spur; arrowhead, striated rootlet; asterisk, cytoplasmic canal; dc, distal centriole; f, flagellum; m, mitochondrion; n, nucleus of spermatozoon; np, nucleus of parasperm; pc, proximal centriole; v, tubular-vesicular compartment. Scale bars: 0.5 µm.

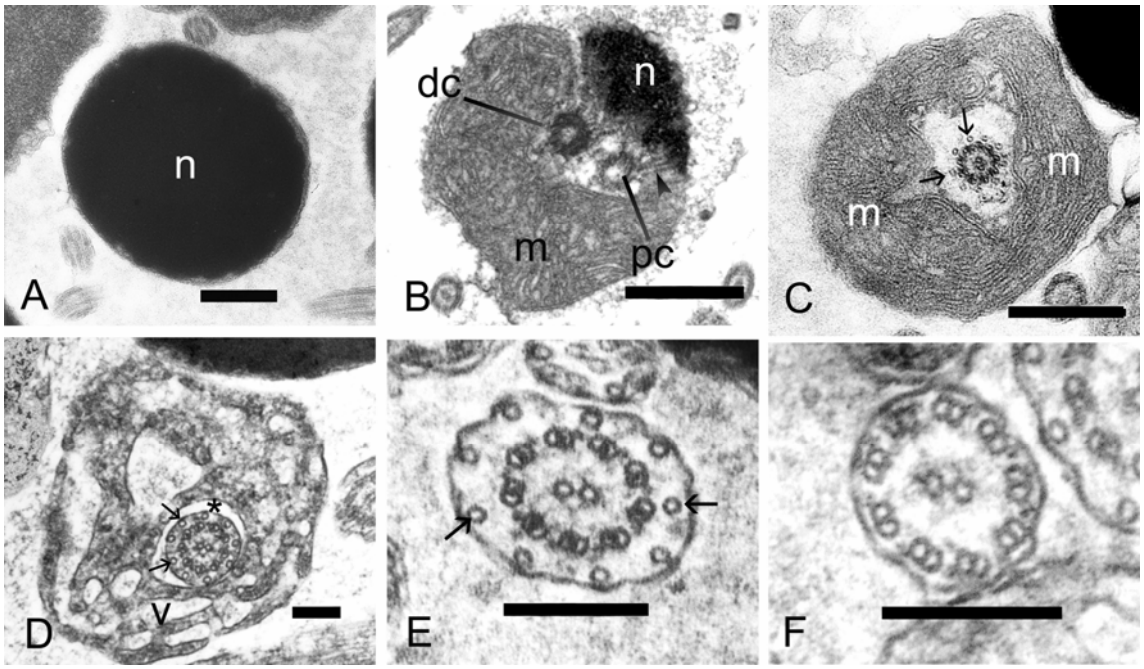

Fig. 4. TEMs of successively more posterior transverse sections through mature spermatozoa of *Macropsobrycon uruguayanae*. A: section through nucleus. B: section through proximal portion of midpiece showing mitochondria and centriolar arrangement. C: section through more distal portion of midpiece showing mitochondria and accessory microtubules. D: section through tubular-vesicular compartment showing accessory microtubules. E: section through proximal flagellum showing accessory microtubules. F: section through distal flagellum. arrow, accessory microtubule; asterisk, cytoplasmic canal; dc, distal centriole; pc, proximal centriole; m, mitochondrion; n, nucleus. Scale bars: 0.5 µm.

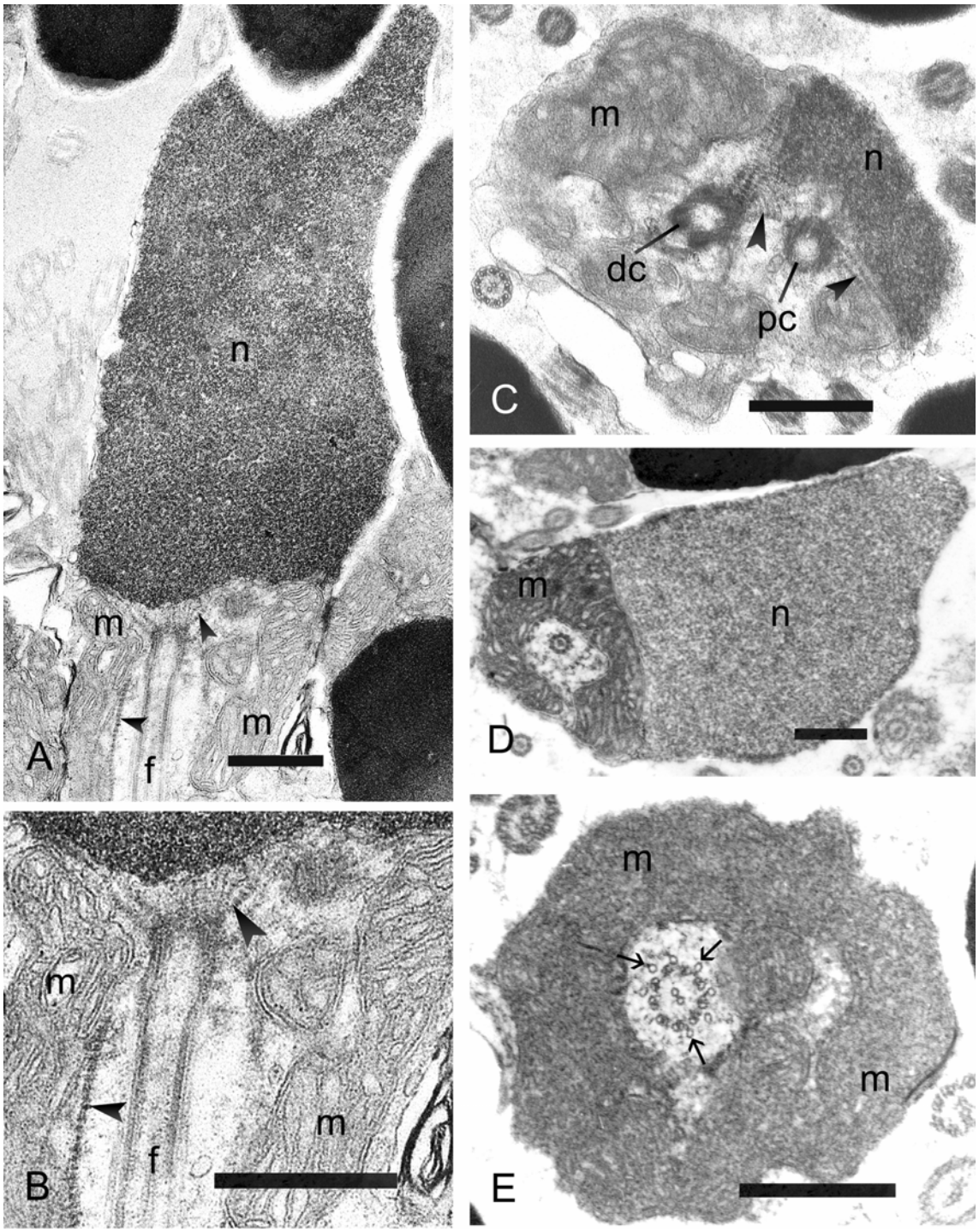

Fig. 5. TEMs of sections through atypical sperm of *Macropsobrycon uruguayanae*. A: longitudinal section showing irregular shape of nucleus containing chromatin that is much less condensed that that seen in spermatozoa. B: higher magnification showing striated rootlets and mitochondria. C-E: transverse sections showing most of the structures also found in eusperm. arrow, accessory microtubule; arrowhead, striated

rootlet; dc, distal centriole; f, flagellum; m, mitochondria; n, nucleus; pc, proximal centriole. Scale bars: 0.5 µm

Capítulo 3:

Gill-derived glands morphology in Cheirodontinae (Teleostei: Characidae)

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ABSTRACT

The gill gland of 17 species of Cheirodontinae is described. The gill gland is located in the anterior region of the gill cavity on either side of the midline of all analyzed mature males of the Cheirodontinae. The gill gland is small in externally fertilizing species of the Cheirodontinae, reaching up to 10 gill filaments. In the inseminating species of the tribe Compsurini, the gland occupies a large extension or almost the entire gill arch. In some portions of the gland of *A. hemigrammus, C. heterura, K. theloura, M. uruguayanae* and *S. hastatus*, the lamellae are not retained, remaining only secretor gill gland cells in these regions. *Kolpotocheirodon theloura* showed noncellular material within the gill gland chambers and they were turgid. There is no relation between the presence of a gill gland and insemination since both externally fertilizing and inseminating characids have or not gill glands. The function of the gill gland is not known yet, but presence of the gill gland in mature males and absence in females suggest that the secretion produced may be used to attract the females during the reproductive period or competition between males. The gill glands of cheirodontines and other characids are compared.

INTRODUCTION

Cheirodontinae is composed by two tribes, Cheirodontini and Compsurini, plus some genera considered *incertae sedis* in the subfamily by Malabarba (1998). These *incertae sedis* genera were hypothesized to belong to a single tribe, the Odontostilbini, in the unpublished work of Bührnheim (2006). The Cheirodontini and the *incertae sedis* genera include are all externally fertilizing species and Compsurini the inseminating species (species that transfer sperm of the mature testes for the ovaries of females, although the actual moment of fertilizing is not known, Burns et al., 1997, 1998; Burns and Weitzman, 2005).

The gills of teleosts are formed by four gill arches (holobranches) on both sides of the body. Each gill arch is formed by two rows of filaments (Roberts, 1981), denominated hemibranches, one more internal and another more external. These primary filaments are sustained by cartilage and they have fine parallel secondary lamellae on both sides. The secondary lamellae are constituted by several capillary inserted by sustentation cells, the pillar cells, and a layer of epithelial cells which cover the structure. Gaseous exchanges occur in those lamellae, when water flows among them in a direction, and blood in the opposite direction (Schmidt-Nielsen, 1996). The gill arch is covered by typical epidermic tissue, thicker in the origin of the primary lamellae and usually with mucous cells (Roberts, 1981).

Burns and Weitzman (1996) described a gill-derived gland, termed gill gland in *Corynopoma riisei* Gill 1858, a stevardiine. The union and functional modification of the most ventral filaments of the first right and left gill arches form the gill gland. The gill gland filaments are united externally by stratified epithelial tissue, maintaining individuality internally. Secretor cells are found between lamellae that compose the gland. The union between each pair of primary filaments forms chambers, through where the secretion is led to the exterior (Burns and Weitzman, 1996, Bushmann et al., 2002). These chemical signals may help during reproduction. The gill gland was found in

some Characidae subfamilies, such as Stevardiinae (Burns and Weitzman, 1996; Bushmann et al., 2002); Cheirodontinae (Oliveira, 2000, 2003 - unpublished data; Azevedo, 2004 - unpublished data; Bührnheim, 2006 - unpublished data); Aphyocharacinae (Gonçalves et al., 2005), and some species *incertae sedis* in Characidae (Weitzman et al., 2005). The purpose of the present study is to determine the distribution of gill glands in the Cheirodontinae by histological and scanning electron microscopy, to describe the structure gill glands and to compare the gland between inseminating and externally fertilizing species of the Cheirodontinae and other Characidae.

MATERIAL AND METHODS

At least 1 species of each cheirodontine genera was analyzed, totalizing 17 species, plus 4 comparative species of other characid genera. Species of *Pseudocheirodon* Meek and Hildebrand, 1916 and Spintherobolus Eigenmann, 1911 were not analyzed due to the lack of mature specimens. Gills of at least 3 mature males and 2 mature females of each species were selected. Histological sections were made in the gonads to confirm degree of sexual maturity. The specimens were obtained from museum collections; therefore the whole fishes had been fixed in formalin and stored in 70% ethanol. The first and second gill arches in both sides of body/head were removed, the right arches were used in histology and the left arches were used in Scanning Electron Microscopy (SEM). The first left gill arches of 5 mature males of *Compsura heterura* were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 and kept under refrigeration until the start of sample processing for Transmission Electron Microscopy (TEM) analysis.

Inseminanting species analyzed: *Acinocheirodon melanogramma* Malabarba and Weiztman, 1999 (MCP 19142, males: 32.21, 34.97, 32.3 mm SL, females: 33.57, 38.3

mm SL); *Compsura heterura* Eigenmann, 1915 (UFRGS 8978, males: 29.7, 28.1, 29.1 mm SL, females: 28.1, 28.7 mm SL); *Kolpotocheirodon theloura* Malabarba and Weitzman, 2000 (MNRJ 18081, males: 25.96, 25.48, 26.30, 26.11, 25.12, 25.03 mm SL, females: 24.13, 24.84 mm SL); *Macropsobrycon uruguayanae* Eigenmann, 1915 (UFRGS 8792, males: 35.25, 32.89, 32.77 mm SL, females: 29.44, 38.85 mm SL); *Odontostilbe dialeptura* (Fink and Weitzman, 1974) (USNM 209511, males: 32.61, 34.92, 32.17, 35.05 mm SL, females: 32.64, 32.51 mm SL); *Saccoderma hastatus* (Eignmann, 1913) (ANSP 139487 males: 29.22, 30.13, 29.14, 29.64, 30.5, 30.02, 31.25, 29.90 mm SL, females: 29.47, 29.51, 29.02 mm SL, CAS 70918 males: 26.25, 25.11 mm SL, females 26.11, MCP 16169 males: 24.44, 24.60, females: 26.05 mm SL).

Externally fertilizing species analyzed: *Aphyocheirodon hemigrammus* Eigenmann, 1915 (NRM 17307 male: 29.0 mm SL), *Cheirodon ibicuhiensis* Eigenmann, 1915 (UFRGS 9042 males: 34.1, 32.4, 31.5 mm SL, UFRGS 9041 females: 38.5, 43,1 mm SL); *Cheirodontops geayi* Schultz, 1944 (MCNG 14197, males: 30.04, 30.12, 27.71, 29.91, 29.19 mm SL, females: 29.94, 31.31 mm SL); *Heterocheirodon jacuiensis* Malabarba and Bertaco, 1999 (MCP 14283, males: 42.09, 40.05, 39.66 mm SL, females: 43.04, 38.92, 39.62 mm SL), *Heterocheirodon yatai* (Casciotta, Miquelarena and Protogino, 1992) MCP 14283, males: 32.88, 32.70, 34.85, 31,57 mm SL, females: 35.25, 34.48, 33.69 mm SL), *Nanocheirodon insignis* (Steindachner, 1880) (USNM 121518 males: 25.62; 22.53; 21.04; 20.3 mm SL; female: 21.10 mm SL); *Odontostilbe fugitiva* Cope, 1870 (MCP 35775 males: 36.0, 33.8 34.27, 35.02, 34.5 mm SL); *Odontostilbe pequira* (Steindachner, 1882) (UFRGS 8980, males: 37.2, 38.7, 36.7 mm SL, females: 41.1, 40.9 mm SL); *Prodontocharax melanotus* Pearson, 1924 (USNM 326941, male: 38.1 mm SL); *Serrapinnus heterodon* (Eigenmann, 1915) (UFRGS 8793, males: 29.7, 27.8, 29.4, 30.8, 29.97, 29.93 mm SL, females: 29.0, 28.7 mm SL) and *Serrapinnus piaba* (Lütken, 1875) (UFRGS 8794, males: 28.5, 28.23, 28.42 mm SL, females: 31.23, 28.97 mm SL).

Examined specimens belong to ANSP - Academy of Natural Sciences, Philadelphia, USA; CAS – California Academy of Sciences, San Francisco, USA; MCP - Museu de Ciências e Tecnologia, Porto Alegre, Brazil; MCNG – Museo de Ciencias Naturales, Guanare, Venezuela; MNRJ - Museu Nacional, Rio de Janeiro, Brazil; NRM - Swedish Museum of Natural History, Stockholm, Sweden; UFRGS - Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; and USNM - National Museum of Natural History, Washington D.C., USA.

Histology

The first and second right gill arches were decalcified in ethylenediamine tetracetic acid (EDTA) 10% during 5 days, dehydrated in ethanol series, infiltrated and embedded in glycol methacrylate. The gills were cut in sagital position with thickness varying from 3 to 5µm and stained with Hematoxilin-Eosin (HE) and Toluidin Blue. Some slides were stained with periodic acid of Schiff (PAS).

SEM

The first and second left gill arches were cleaned with ultrasound equipment during 6 minutes, dehydrated in a graded ethanol series, critical point dried in liquid CO_2, glued on stub with carbon double-stick tape and sputter-coated with carbon and gold. Images were visualized and photographed in a Philips XL 30 scanning electron microscopy from Pontifícia Universidade Católica do Rio Grande do Sul, Brazil or in a Jeol 6060 scanning electron microscopy from Universidade Federal do Rio Grande do Sul, Brazil.

TEM

At field, the fishes were sacrificed by a cut through the spinal cord. The first left gill arches of 5 mature males of *Compsura heterura* were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 and kept under

refrigeration until the start of sample processing. The gills were post-fixed in 1% osmium tetroxide in phosphate buffer, dehydrated in acetone series, infiltrated and embedded in Araldite-Epon (Embed-812). Ultrathin sections were stained with a saturated of uranyl acetate at 50% alcohol and lead citrate. The sections were viewed in a Philips CM100 transmission electron microscope from Universidade Estadual Paulista- Campus Botucatu, Brazil.

RESULTS

The gill glands were located in the anterior region of the gill cavity on either side of the midline (Fig. 1) of all analyzed mature males of Cheirodontinae (Figs. 2, 3). They were absent in females. The specimens of Spintherobolus broccae are immature. The development of the gill glands begins with the multiplication of the epithelial cells that cover the anteriormost portion of the first gill arch, extending then for the following filaments. Unmodified filaments are located soon after of the gill glands (Figs. 2- 5), when they do not occupy the whole gill arch. They are covered by stratified epithelial tissue externally, and internally the gill filaments form chambers. There is no connection between chambers (Figs. 4, 5). In some sectioning was viewed skeletal muscle associated with hyaline cartilage of gill filaments (Fig.4f, i). The secretion supposedly produced leaves freely through the existent space in the distal extremity of the filaments that are not united. Secretor cells fill out the secondary lamellae that form the gill glands (Figs. 4, 5).

The pseudostratified epithelial tissue is formed by tall columnar cells. They are located between secondary lamellae, when these are present, or covering the chambers formed among the gill filaments. In TEM is possible to see several dark vesicles filling almost the whole cell (Fig. 6). The nucleus, endoplasmatic reticule and Golgi complex are in the base of cells.

In spite of gill glands structures are similar among species, some differences were observed in the gland size and in the degree of gill modification. The gill glands are

larger in inseminating Cheirodontinae than in externally fertilizing ones (Table 1; Figs. 2-5), except in *A. melanogramma* (Figs. 2A, 4A) that showed small glands with filaments. The gill glands of *C. heterura* reach 15 filaments*, K. theloura* reach 18 filaments*, M. uruguayanae* reach 25 filaments, "*O." dialeptura* reach 28 filaments and *S. hastatus* reach 28 filaments in the first gill arch. In externally fertilizing species, the gill glands reach 4-10 filaments (*A. hemigrammus* – 9 filaments, *C. ibicuhiensis* – 9*, C. geayi* – 10*, H. jacuiensis* –7*, H. yatai* – 7*, N. insignis* – 7*, O. fugitiva* – 6*, O. pequira* – 10*, P. melanotus*- 4*, S. heterodon* – 8 and *S. piaba* – 7). The gill glands are small also in *A. anisitsi* and *B. iheringi,* being composed by 5 and 6 filaments respectively and they are absent in the comparative species, *Bryconops melanurus* and *Charax stenopterus*

Kolpotocheirodon theloura showed filaments united through their distal tips, while in other cheirodontine species the distal tips are free. The same species has a turgid gill glad viewed externally, and internally it is possible to observe large chambers with noncellular stained material within (Fig. 4E).

Aphyocheirodon hemigrammus, C. heterura, K. theloura, M. uruguayanae and *S. hastatus* did not retain lamellae in some portions of the gland, remaining only secretor gill gland cells in these regions (Fig. 4F).

Compsura heterura have glands in both hemibranches of the first gill arch and both have the same extension (Fig.7A). In *K. theloura,* the glands of the external hemibranch seem to embrace the internal filaments (Fig. 7B). All other species have the gill gland only in external hemibranch of the first gill arch.

The gill glands also were present in the second gill arch of *C. heterura* and *S. hastatus*. However these structures occupy half-length of the filaments and are smaller than of those of the first gill arch, reaching 5 and 4-13 filaments in each species respectively. In *S. hastatus*, the glands may be in the external or internal hemibranch of the second gill arch (Fig.7C, D).

DISCUSSION

The gill gland of all Characidae species in which this organ has been described possess the same structure. They are formed externally by the growth of pseudostratified epithelial tissue over and around the most anterior gill filaments of gill arch, and internally by chambers covered with a columnar or cuboidal epithelium between secondary lamellae (Burns and Weitzman, 1996; Bushmann et al., 2002). This similar structural pattern of the gill gland suggests it is homologous among the species of Characidae.

In Cheirodontinae, the gland was observed in *C. ibicuhiensis* (Oliveira, 2000 – unplublished data), *C. heterura, O. pequira* (Oliveira, 2003 – unplublished data) and in all mature males of the Cheirodontinae examined by Bührnheim (*Acinocheirodon melanogramma*, *Amblystilbe alleni*, *Aphyocheirodon hemigrammus*, *Cheirodon ibicuhiensis, C. interruptus*, *Cheirodontops geayi*, *Compsura heterura*, *Kolpotocheirodon theloura, Macropsobrycon uruguayanae*, *Odontostilbe fugitiva, O. pequira, O. euspilurus* Fowler 1945*, O. paraguayensis* Eigenmann & Kennedy 1903, *O. ecuadorensis, O. nareuda, O. parecis, O.microcephala, O. splendida, O. pao,* plus 8 new species of Odontostilbe, *Prodontocharax melanotus*, *Pseudocheirodon terrabae, P. arnoldi*, *Saccoderma hastatus, Serrapinnus heterodon*, *S. microdon*, *S. micropterus*), excepting in *Amblystilbe alleni* and *Spintherobolus* sp. n, whose specimens were immature.

The gill glands were described by Bushmann et al. (2002) in 12 inseminating species of the Stervardiinae (*Acrobrycon* Eigenmann & Pearson 1924*, Argopleura* Eigenmann 1913*, Chrysobrycon* Weitzman & Menezes 1998*, Corynopoma* Gill 1858*, Gephyrocharax* Eigenmann 1912*, Hysteronotus* Eigenmann 1911*, Phenacobrycon* Eigenmann 1922*, Planaltina* Böhlke 1954*, Pterobrycon* Eigenmann 1913*, Scopaeocharax* Weitzman & Fink 1985*, Tyttocharax* Fowler 1913 and *Xenurobrycon* Myers & Miranda Ribeiro 1945), but were absent in the inseminating species of 3

stervardiine genera (*Diapoma* Cope 1894*, Pseudocorynopoma* Perugia 1891 and *Ptychocharax* Weitzman, Fink, Machado-Allison & Royero L. 1994), and in all inseminating species of the Glandulocaudinae (Bushmann et al., 2002; Burns, personal communication).

The gill glands were viewed in mature inseminating males of genera considered *incertae sedis* in Characidae and belonging to Clade A of Malabarba and Weitzman (2003), as *Attonitus bounites* Vari & Ortega 2000*, Attonitus irisae* Vari & Ortega 2000*, Bryconadenos tanaothoros* Weitzman, Menezes, Evers & Burns 2005 (Weitzman et al., 2005)*, Creagrutus melasma* Vari, Harold & Taphorn 1994 and *Monotocheirodon* Eigenmann & Pearson 1924 (Burns, personal communication) and in one undescribed new genus and species of inseminating characid (Malabarba et al., in prep.).

These glands are also present in mature males with externally fertilizing species of the Clade A of Malabarba and Weitzman (2003), as observed in the genera *Bryconamericus* Eigenmann 1907*, Caiapobrycon* Malabarba & Vari 2000*, Creagrutus* Günther 1864*, Cyanocharax* Malabarba & Weitzman 2003*, Hemibrycon* Günther 1864*, Hypobrycon* Malabarba & Malabarba 1994*, Knodus* Eigenmann 1911*, Piabina* Reinhardt 1867*, Rhinobrycon* Myers 1944 and *Rhinopetitia* Géry 1964 (Burns, personal communication) as well as *Phenacogaster franciscoensis* Eigenmann 1911*,* a Characinae and *Aphyocharacidium bolivianum* Géry 1973*, incertae sedis* in Characidae (Bührnheim, 2006 – unplublished data). In Aphyocharacinae, the gland was found in *A. anisitsi* by Gonçalves et al. (2005).

Through of the list of species with gill gland, we can observe that externally fertilizing and inseminating Characidae have or not gill glands, therefore there is no relation between the presence of a gill gland and insemination.

Although the gill gland is viewed in many Characidae species, little is know about number of gill filaments that compose the gill gland and details about its internal morphology. In spite of the gill glands possess similar structure and to be located in the same area of the first gill arch, some differences among cheirodontine species and

between Cheirodontinae and other Characidae were observed, in the extension of the gland, the format of the cells, the presence of the gland in both hemibranchs of the first arch, the presence of the gland in the second gill arch, the turgidity and the presence or of the secondary lamellae.

The gill gland is small in externally fertilizing species of the Cheirodontinae, being composed by 10 gill filaments. Nevertheless, the gland occupies a large extension or almost the entire gill arch in the inseminating species of the tribe Compsurini, such as in *M. uruguayanae, O. dialeptura* and *S. hastatus*. *Acinocheirodon melanogramma* is the exception among compsurins and show a small gland. Considering the non basal position of *Acinocheirodon* in both phylogenies proposed in Malabarba (1998) and Oliveira et al. (this volume), the possession of a large gill gland may constitute a synapomorphy for the Compsurini, and the small gland a reversal in *A. melanogramma*, because the specimens analysed had mature testis. Although there is no relation between the presence or absence of a gill gland and insemination in Characidae, the increasing size of the gill gland seems to be advantageous for the inseminating cheirodontines that possess a clearly larger organ than those of the externally fertilized species of this subfamily.

The presence of the glands in both hemibranches of first arch is a characteristic showed only by *C. heterura*. The presence of glands in the second gill arch is a characteristic shared by *C. heterura* and *S. hastatus*. These species formed one natural group in the analyses of Malabarba (1998) and Bührnheim (2006), and gland morphology seems to corroborate their hypotheses. Nevertheless, Oliveira et al. (2007-this volume) presents a hypothesis based on spermatozoa ultrastructure characters that groups *S. hastatus* in a clade with *A. melanogramma*, "*O*"*. mitoptera* and "*O*"*. dialeptura*, instead of *Compsura.* Therefore, additional characters are necessary to solve these relationships.

The gill glands of most species of Compsurini reach larger sizes than those of the Stevardiinae. Stervardiinae also show varying degrees of gill gland extension

(Bushmann et al., 2002). The glands are formed by 4-13 gill filaments. It is bigger in *Hysteronotus megalostomus* Eigenmann 1911 (11 gill filaments) and in Stevardiini species (= Corynopomini to past authors). In this tribe the gill gland is composed by 7-9 gill filaments in *Gephyrocharax melanocheir* Eigenmann 1912*,* by 11-12 gill filaments in *Pterobrycon myrnae* Bussing 1974 and by 8-13 gill filaments in *Corynopoma riisei* (Bushmann et al., 2002). Other Stevardiinae species analyzed by Bushmann et al. (2002) had less than 9 gill filaments in the gill gland. *Bryconadenos tanaothoros* had a gill gland composed by 5 filaments and unmodified gills were observed at the extreme left and right of gill glands (Weitzman et al., 2005). In *A. bounties,* the glands were formed by 14 gill filaments and in *A. irisae* by 3 gill filaments*,* but the glands appeared to be in the initial stages of formation (Weitzman et al., 2005*).*

Kolpotocheirodon theloura showed noncellular material within the gill gland chambers and they were turgid. The state of expansion and secretion of glands varies greatly (Burns and Weitzman, 1996) and it is related with maturation of testis. The immature does not have glands, early stage of sexual maturation males have initial glands and mature males have glands developed (Burns and Weitzman, 1996; Oliveira, 2003; Azevedo, 2004; Gonçalves et al., 2005). Some specimens of *C. riisei* had gill glands with regions more expanded and turgid, one specimen had a light yellow viscous secretion in the enlarged chambers, and a third specimen showed involuted glands (Burns and Weitzman, 1996). However, none reduction was observed by Oliveira (2003) in the gill glands of *C. heterura* after the reproductive period.

In some species occur reduction or disappearance of secondary lamellae, as observed in *C. heterura, K. theloura, M. uruguayanae* and *S. hastatus.* The union of the filaments, the covering with a stratified epithelium, and the loss of the secondary lamellae, decrease or impede gaseous exchanges in the portion of the gill glands (Burns and Weitzman, 1996).

The function of the gill gland is not known yet, but the development of the gill gland in males occurs along with testis maturation (Gonçalves et al., 2005). The

presence of the gill gland in mature males and absence in females suggest that the secretion produced may be used to attract the females during the reproductive period (Burns and Weitzman, 1996; Bushmann et al., 2002) or intermale aggression (Bushmann et al., 2002). The comparatively larger size of the gill gland in inseminating versus externally fertilized species of the Cheirodontinae demonstrates these secretions are particularly important in the reproductive success of inseminating cheirodontines.

Bührnheim (2006) viewed that the concavity of the posteriormost branchiostegal ray forms a hole ventrally on head exactly near the most ventral portion of the gill gland of the first gill arch of the most of Cheirodontinae. This author suggests that the osseous modification could be related to the gill gland, functioning as an opening to facilitate excretion. Posterior studies about gill gland physiology are necessary for understand its function and working.

ACKNOWLEDGMENTS

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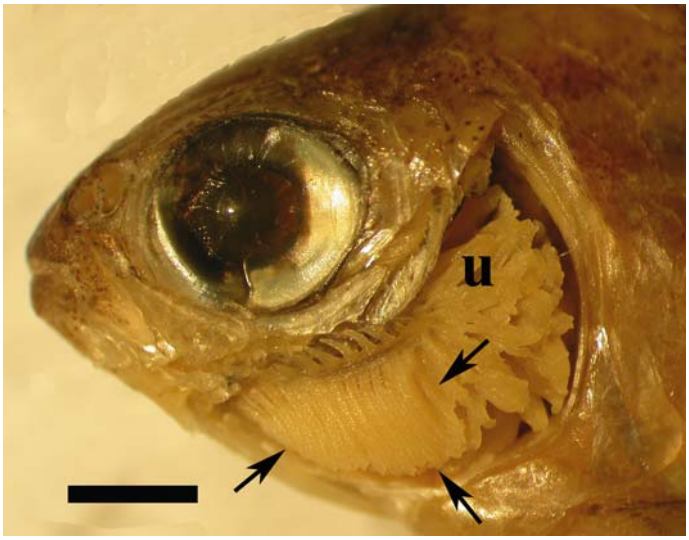

Fig. 1. Entire head of a mature male of *Compsura heterura* (30.4 mm SL) with operculum removed, showing the location of the gill gland. Arrows indicate the modified portion of the gill arch containing glandular tissue. “u” indicated unmodified gill filaments. Scale bar: 0.5 mm.

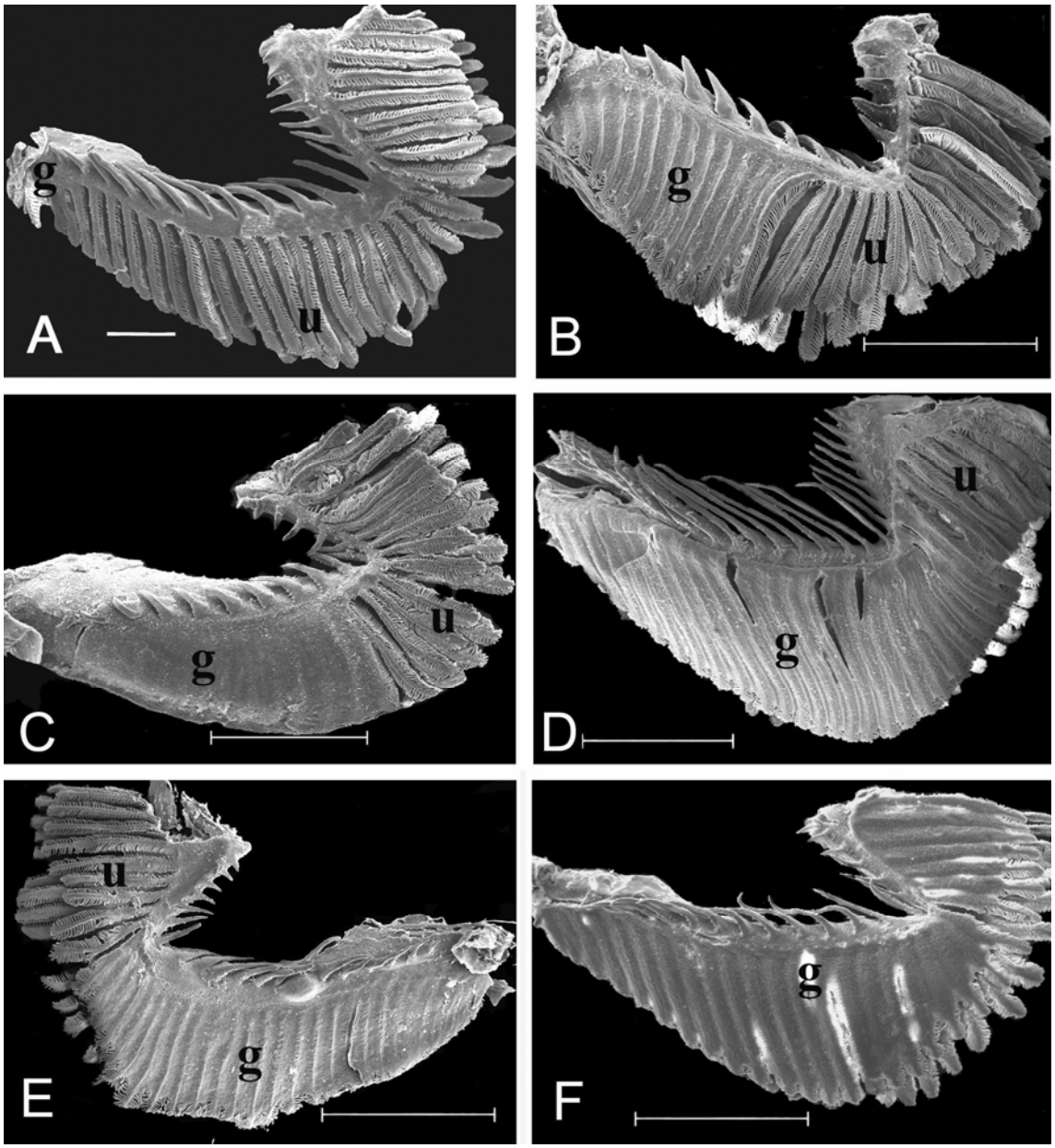

Fig. 2. Scanning electron micrograph of first left (A-D, F) and right (E) gill arches of a male of inseminating cheirodontines, *Acinocheirodon melanogramma* (A), *Compsura heterura* (B), *Kolpotocheirodon theloura* (C), *Macropsobrycon uruguayanae* (D), *Odontostilbe dialeptura* (E), and *Saccoderma hastatus* (F). The gill glands (g) occupy a large extension of first gill arches. Unmodified gill filaments (u) are located soon after of the gill glands. Scale bars: 1mm.

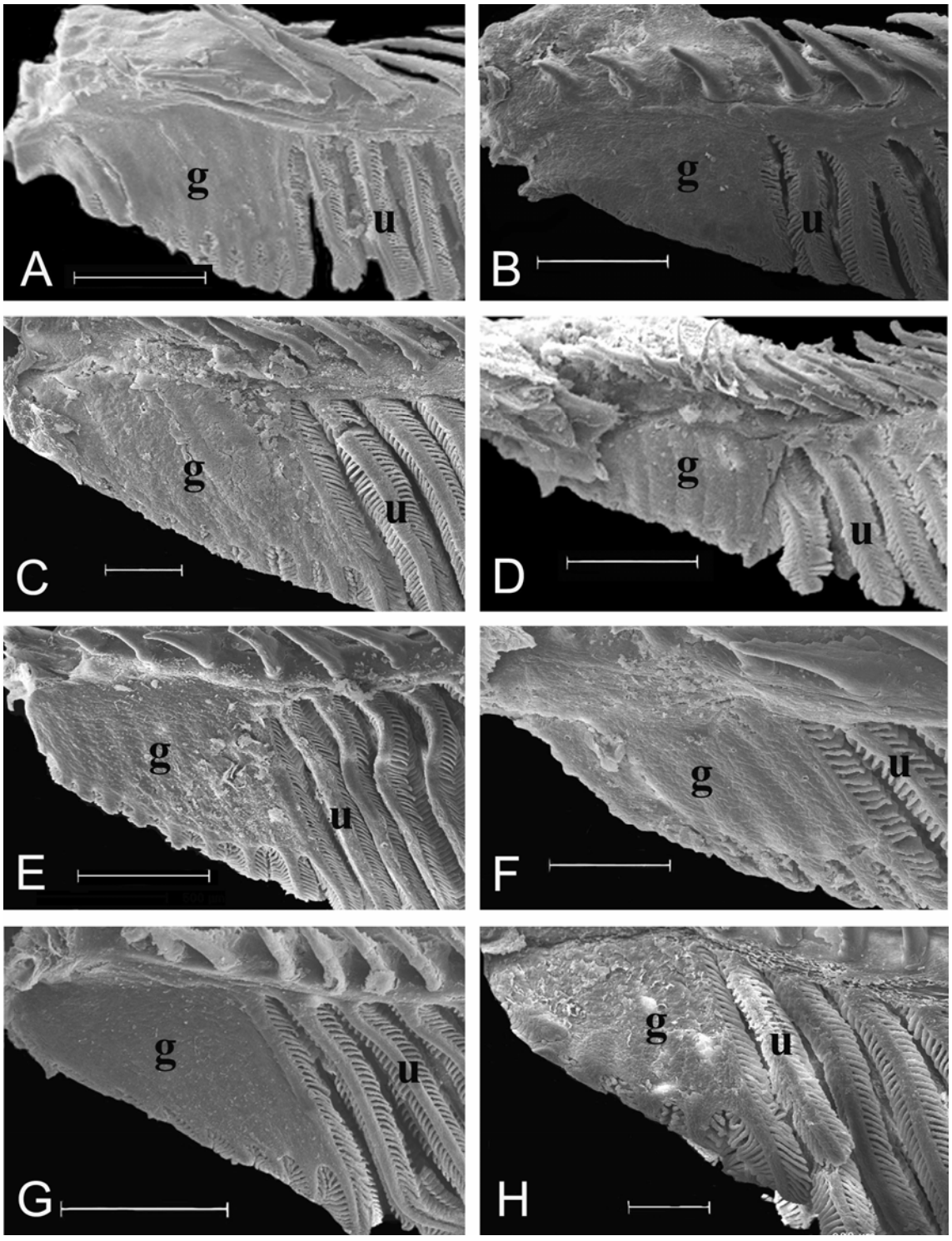

Fig. 3. Scanning electron micrograph of first left gill arch of a male of externally fertilizing cheirodontines, *Aphyocheirodon hemigrammus* (A), *Cheirodon ibicuhiensis* (B), *Cheirodontops geayi* (C), *Heterocheirodon yatai* (D), *Odontostilbe fugitiva* (E),

Nanocheirodon insignis (F), *Prodontocharax melanotus* (G), *Serrapinnus heterodon* (H). The gill glands (g) occupy a small extension of first gill arches. Unmodified gill filaments (u) are located soon after of the gill glands. Scale bars: 200 µm (A, B, C, D, H), 500 µm (E, G).

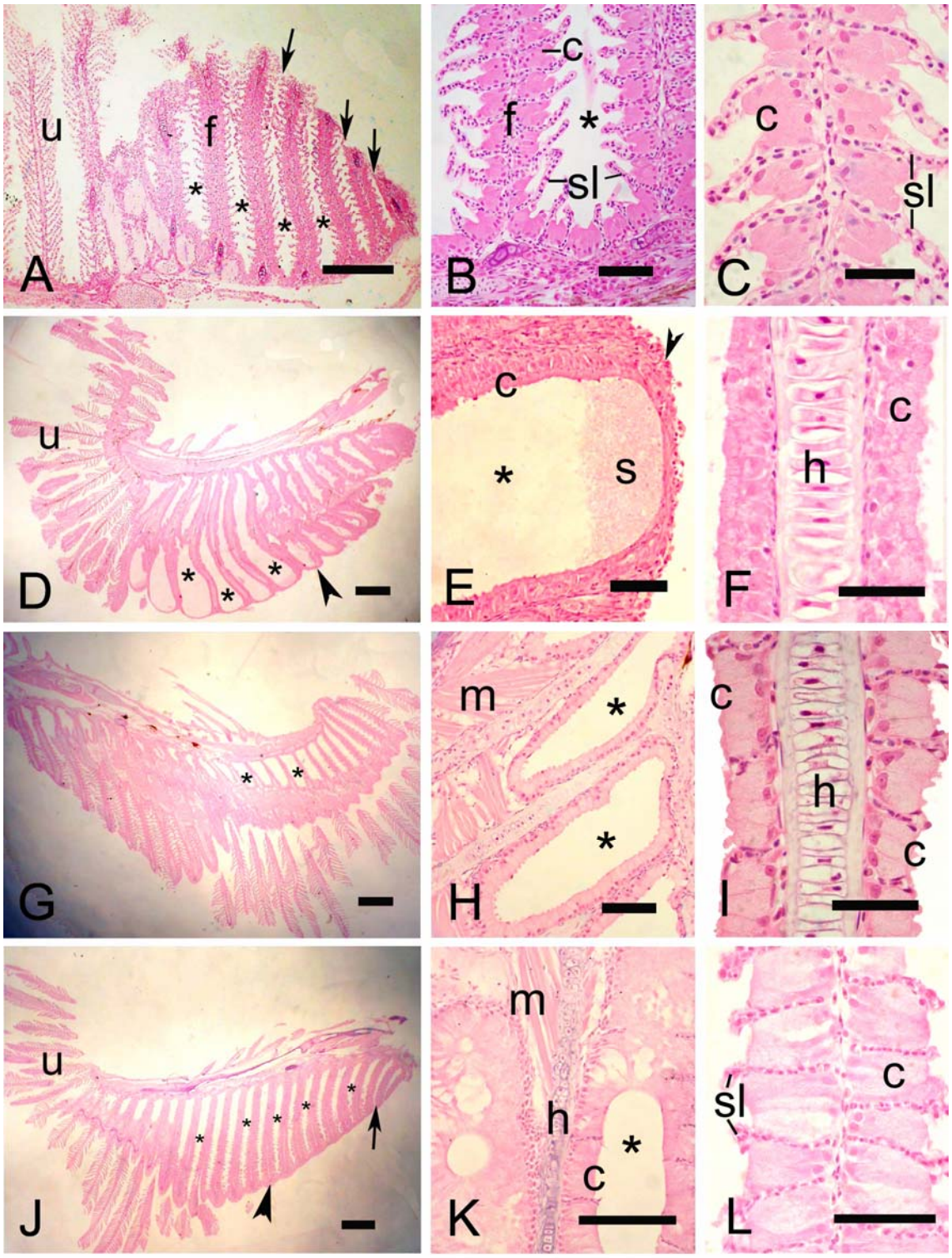

Fig. 4. Sagital histological sections through first gill arches of male of inseminating cheirodontines, *Acinocheirodon melanogramma* (A, B, C), *Kolpotocheirodon theloura* (D, E, F), *Macropsobrycon uruguayanae* (G, H, I), *Saccoderma hastatus* (J, K, L). asterisks, lumina of the gill gland chambers; c, columnar cells; f, gill filaments; sl, gill secondary

lamellae; s, secretion; u, unmodified gill filaments; arrow, ventral chamber opening; arrowhead, gill gland covering; m, skeletal muscle; h, hyaline cartilage of gill filaments. Scale bar: 150 µm (A, D, G, J), 50 µm (B, E, H, K), 25 µm (C, F, I, L).

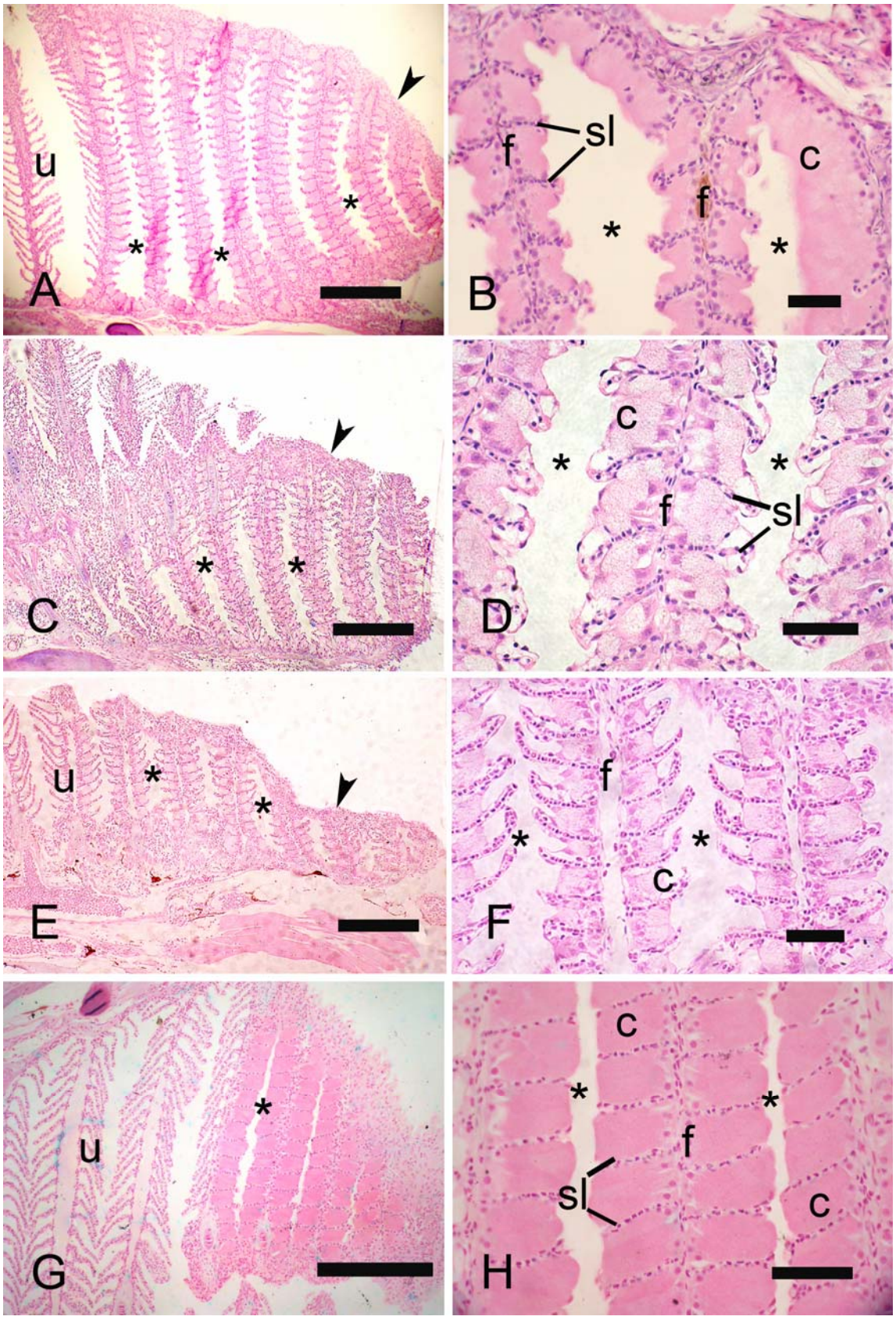

Fig. 5. Sagital histological sections through first gill arches of male of externally fertilizing cheirodontines, *Aphyocheirodon hemigrammus* (A, B), *Heterocheirodon yatai* (C, D),

Prodontocharax melanotus (E, F), *Serrapinnus heterodon* (G, H). asterisks, lumina of the gill gland chambers; f, gill filaments; sl, gill secondary lamellae; u, unmodified gill filaments; arrowhead, ventral chamber opening; arrow, gill gland covering. Scale bar: 150 µm (A, C, E, G), 25 µm (B, D, F, H).

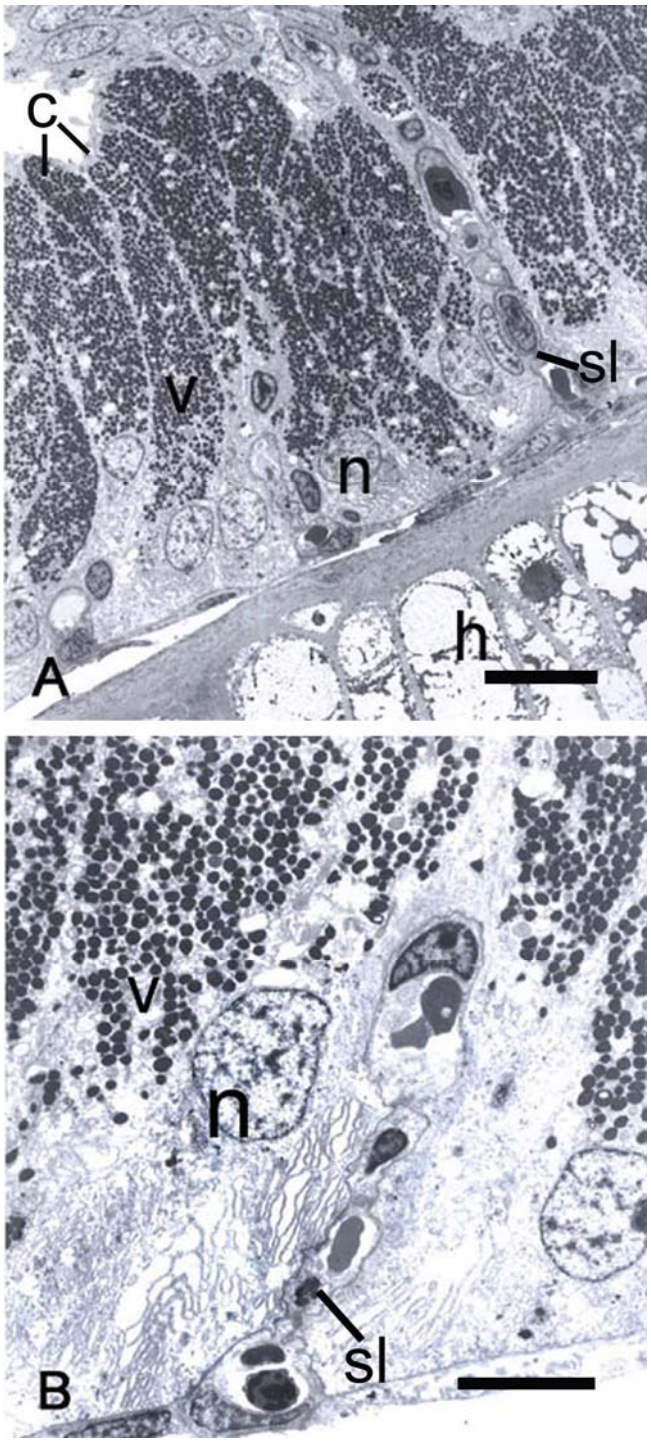

Fig. 6. Transmission electron micrograph of sagital sections through gill glands of *Compsura heterura*. Tall columnar cells (c) are found between secondary lamellae (sl). Several dark vesicles (v) occupy almost whole cell and nuclei (n) are in the base of cells. h, hyaline cartilage. Scale bar: 8µm (A) 5µm (B).

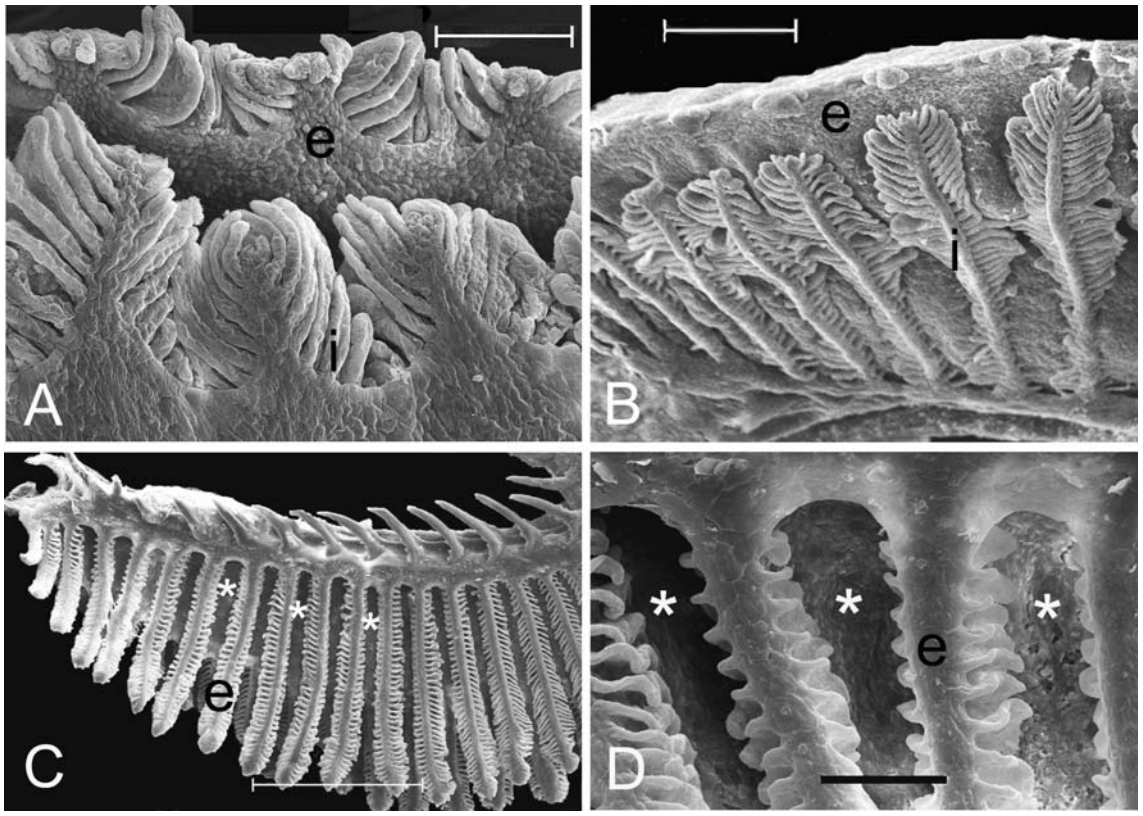

Fig. 7. Scanning electron micrograph of gill arches. A: Lateral view of first gill arch of *Compsura heterura*, showing that the gill glands occupy both hemibranches and that both have the same extension. Scale bar: 100 µm. B: Internal view of first gill arch of *Kolpotocheirodon theloura*, showing that the glands of external hemibranch seems embrace the internal filaments. Scale bar: 200 µm. C: Lateral view, showing that the gill glands also are present in the second gill arch of *S. hastatus.* In *S. hastatus*, the glands may be in the external (e) or internal hemibranch (i and asterisks) of the second gill arches. Scale bar: 500 µm. D: Detail of picture C. Scale bar: 100 µm.

CONCLUSÕES

- O uso de caracteres de ultraestrutura de espermatozóides mostrou-se informativo, contribuindo para o reconhecimento de Compsurini como um grupo monofilético e permitindo o estabelecimento relações parciais entre as espécies da tribo.

- Os espermatozóides de espécies de fertilização externa mostram ter estruturas conservativas enquanto que os espermatozóides de espécies inseminadoras apresentam mudanças estruturais, principalmente no núcleo espermático.

- Compsurinis apresentam três formas de espermatozóides: aquasperma, forma de projétil e alongado.

- O aquasperma de Cheirodontini e Odontostilbini difere de outras espécies de caracídeos de fertilização externa por apresentar vesículas numerosas e pequenas na região basal da peça intermediária.

- *Kolpotocheirodon theloura* apresenta um aquasperma derivado que difere das espécies de Cheirodontini e Odontostilbini por apresentar peça intermediária mais alongada que se estreita progressivamente no sentido distal, e vesículas mais largas, com formato irregular e em menor número. Cheirodontini e Odontostilbini possuem um aquasperma com peças intermediárias mais curtas e que terminam abruptamente, e vesículas menores, com formato regular e em maior número.

- *Compsura heterura*, *M. uruguayanae*, *A. melanogramma*, *S. hastatus*, "*Odontostilbe*" *mitoptera* and "*O*." *dialeptura* formam um grupo monofilético sustentado por três caracteres, forma do núcleo, relação comprimento e largura do núcleo e posição do complexo centriolar em relação à fossa nuclear.

- *Acinocheirodon melanogramma, "O". dialeptura, "O". mitoptera* and *S. hastatus*

constituem um grupo monofilético de Compsurini sustentado por quatro caracteres, forma do núcleo, a ausência de rotação nuclear, a posição dos centríolos, e a posição das mitocôndrias.

- A rotação nuclear ocorre em todos Cheirodontini e Odontostilbini analisados e em *C. heterura*, *K. theloura* e *M. uruguayanae,* estando ausente no clado formado por *Acinocheirodon melanogramma, "O". dialeptura, "O". mitoptera* and *S. hastatus .*

- *Macropsobrycon uruguayanae* possui espermatozóides normais e espermatozóides atípicos denominados paraespermatozóides, encontrados no lúmen testicular.

- Os espermatozóides de *M. uruguayanae* apresentam duas autapomorfias, como microtúbulos acessórios ao redor do axonema e estrias centriolares denominadas rootlets.

- A glândula branquial possui a mesma estrutura em todos os caracídeos analisados até o momento, diferindo em sua extensão, presença ou não no segundo arco branquial e presença ou não nas duas hemibranquias.

- A glândula branquial é foi encontrada em todos machos maduros, estando ausente em fêmeas.

- Queirodontíneos de fertilização externa possuem glândula branquial menor ocupando no máximo 10 filamentos branquiais e as espécies inseminadoras apresentam uma extensa glândula (até 28 filamentos branquiais), com exceção de *Acinocheiron melanogramma.*

- As glândulas branquiais de compsurinis alcançam maiores extensões do que as glândulas branquiais de Stervardiinae.

- A presença da glândula branquial nas duas hemibranquias é uma característica de

somente *C. heterura* e a presença da glândula no segundo arco branquial é uma característica dividida por *C. heterura* and *S. hastatus*.

- A glândula branquial está presente tanto em espécies inseminadoras quanto em espécies de fertilização externa, não existindo relação entre a presença de glândula branquial e inseminação.

- Em *C. heterura, K. theloura, M. uruguayanae* e *S. hastatus* observou-se à redução ou desaparecimento das lamelas secundárias.

- A função da glândula não é conhecida, mas devido a sua presença em somente machos maduros e em maturação, esta estrutura poderia ser usada na produção e liberação de secreção para a atração de fêmeas durante o período reprodutivo. Estudos posteriores sobre a fisiologia da glândula são necessários para conhecer a função e funcionamento da glândula.

Anexo 1

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. . . studies by Wittmann et al. (1993) reveal . . .

. . . an earlier report (Whittier, 1992) . . .

. . . earlier reports (Quinn and Baumel, 1993; Lindholm and Bass, 1993) . . .

Unpublished data should be referred to as personal communication. An example would be... studies by Mary Packard (personal communication)...In this case mention the full address in the acknowledgments, not in the literature. When references are made to more than one paper by the author published in the same year, they should be designated in the text as (Condon et al., 1990a,b) and in the literature list as follows:

Condon K, Silberstein L, Blau HM, Thompson WJ. 1990a. Development of muscle fiber types in the prenatal rat hindlimb. Dev Biol 138:256–274.

Condon K, Silberstein L, Blau HM, Thompson WJ. 1990b. Differentiation of fiber types in aneural musculature of the prenatal rat hindlimb. Dev Biol 138:275–295.

The literature list must be arranged alphabetically by author's surname in the following style:

Author's name (or names), year of publication, complete title, volume, and inclusive pages as follows:

Journal article

King VM, Armstrong DM, Apps R, Trott JR. 1998. Numerical aspects of pontine, lateral reticular, and inferior olivary projections to two paravermal cortical zones of the cat cerebellum. J Comp Neurol 390:537-551.

Book

Voet D, Voet JG. 1990. Biochemistry. New York: John Wiley & Sons. 1223 p.

Book chapter

Gilmor ML, Rouse ST, Heilman CJ, Nash NR, Levey AI. 1998. Receptor fusion proteins and analysis. In: Ariano MA, editor. Receptor localization. New York: Wiley-Liss. p 75-90.

References to papers by two authors follow those by the senior author. If there is more than one paper with a second author, these are listed alphabetically. References by three or more authors

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[3] See footnote 2, page . . .

Footnotes to a table should be typed directly beneath the table and numbered 1,2,3, etc. They should not be numbered in sequences with the footnotes in the text.

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Metric system. The metric system should be used for all measurements, weight, etc. Temperatures should be expressed in degrees Celsius (centigrade). Metric abbreviations, as listed below, should be expressed in lower-case without periods.

Length		Volume	
km	kilometer	km^3	cubic
			kilometer
m	meter	m^3	cubic
			meter
cm	centimeter	cm^3	cubic
			centimeter
mm	millimeter	mm^3	cubic
			millimeter
µm	micrometer	$µm^3$	cubic
	(micron)		
nm	nanometer		
pm	picometer		
Å	Angstrom unit		

	(10 Å = 1 nm)		micrometer
		nm^3	cubic
			nanometer
Area		kl	kiloliter
km^2	square	l	liter, always
	kilometer		spell out
m^2	square	ml	milliliter
	meter	µl	microliter
cm^2	square	nl	nanoliter
	centimeter	pl	picoliter
mm^2	square	kg	kilogram
	millimeter	gm	gram
$µm^2$	square	mg	milligram
	micrometer	µg	microgram
mm^2	square	ng	nanogram
	nanometer	pg	picogram

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To achieve greatest fidelity and rendition of detail, it is preferable that the printer work directly from original drawings or high-quality photographic prints (but not photocopies made on an office duplicating machine)or disks. All illustrations must be submitted in complete and finished form with adequate labeling.

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