Universidade Federal do Rio Grande do Sul

Centro de Biotecnologia

Programa de Pós-Graduação em Biologia Celular e Molecular

# Caracterização da biologia de populações de planárias do gênero *Girardia* nativas do Rio Grande do Sul.

Tanise Knakievicz

Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS como requisito parcial para a obtenção do grau do Doutor em Ciências

Orientador: Prof. Henrique Bunselmeyer Ferreira

Porto Alegre, 15 de junho de 2007.

Este trabalho foi realizado no Laboratório de Biologia Molecular de Cestódeos do Centro de Biotecnologia da UFRGS, sendo financiado pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e pela Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

"*A justificativa da ciência não está nas aplicações. Provavelmente haverá aplicações, mas o mecanismo intelectual da pesquisa, a motivação, não é a aplicação. A motivação é a compreensão*".

(Oriol Bohigas)

## Agradecimentos

Ao meu orientador, Dr. Henrique, exemplo de profissional, pela oportunidade, confiança, dedicação, apoio e direcionamento nos momentos em que precisei de auxílio durante a realização deste trabalho.

À minha Comissão de Acompanhamento, Dr. Augusto Schrank e Dra. Irene Schrank, pela dedicação e atenção dispensadas e pelas críticas e sugestões acrescentadas a este trabalho.

Ao Dr. Arnaldo Zaha pelo suporte infra-estrutural, e a todos a professores do PPGBCM pela oportunidade de aprendizado.

Aos Professores Alexandre Arenzon, Suzana Bencki, José Felipe Amato e Ivan Antônio Pinheiro.

À Rosane Fagundes, Renato Moreira Rosa e Isabel Vilella e Luiz Carlos Campos Daudt pela gentil colaboração.

A todos os colegas dos laboratórios 204, 206 e 210 e às alunas de iniciação científica Sabrina Moura Vieira, Angela Menegassi e Priscila Alves da Silveira.

Aos funcionários do Centro de Biotecnologia, em especial à Sílvia R. Centeno e ao Luciano Saucedo, pelo atendimento sempre amável e gentil.

Aos meus amigos Sergio Artur Luz Wagner, Rejane Gueno, Érica Hermel, Daniel Ruschel, Alexandre Chiele, especialmente a Graziela Beck Porto, amiga do coração de todas as horas, e ao Teonísio Ludke.

Aos amigos do CIEC-IIPC.

À minha família, José, Domercília, Telmo, Tanara, João Augusto, Laine e Bárbara, pelo amor incondicional que de tão intenso se fez sempre presente apesar da distância física continental.

# Sumário

## Lista de abreviaturas, símbolos e unidades

| | |
|---|---|
| CONAMA | Conselho Nacional do Meio Ambiente |
| Fc | Número de casulos/indivíduos/semana |
| Fr | Número de filhotes/indivíduos/semana |
| Fs | Número de fissiparidade/indivíduo/semana |
| CEM | Campo eletromagnético |
| C:E | Fotoperíodo claro:escuro de 12 h:12 h cada |
| C:C | Fotoperíodo sempre claro |
| E:E | Fotoperíodo sempre escuro |
| FC | Medida de intensidade luminosa em Food candle. |
| $LC_{50}$ | Concentração letal para metade dos organismos expostos |
| $mIC_{50}$ | Concentração de inibição de 50 da mobilidade |
| $rIC_{50}$ | Concentração de inibição de 50 da mobilidade |
| MN | Micronúcleo(s) |
| RNAi | RNA de interferência |
| BrdU | Bromodesoxiuridina - marcação de síntese de DNA |
| EST | *Expressed sequence tag* |
| miRNA | microRNA |

# Lista de tabelas

## Lista de figuras

## Resumo

As planárias são organismos de escolha para a realização de uma ampla gama de estudos básicos, abrangendo desde aspectos ecotoxicológicos até abordagens de biologia celular e molecular. Isso se deve, especialmente, à capacidade extraordinária de regeneração desses organismos e à presença de células-tronco somáticas (neoblastos) no adulto. Portanto, a disponibilização de espécies e linhagens de planárias com características fisiológicas bem definidas é fundamental. As espécies *Girardia tigrina* e *Girardia schubarti*, nativas do Rio Grande do Sul, foram estudadas quanto ao modo de reprodução, ciclo de vida, capacidade de regeneração e à suscetibilidade a agentes tóxicos e mutagênicos. Em ambas as espécies, o modo de reprodução correlaciona-se com a ploidia; indivíduos diplóides são geralmente sexuados, e os indivíduos triplóides e mixoplóides são exclusivamente fissiparitários. Planárias fissiparitárias geram um número menor de descentes e são mais longevas do que as sexuadas. A freqüência de reprodução sexuada e a longevidade em ambas as espécies sofrem influência das condições ambientais, mas alterações no ambiente não levam a trocas entre os modos de reprodução sexuada e assexuada. *G. tigrina* e *G. schubarti* apresentam capacidades similares de regeneração e suscetibilidades a agentes clastogênicos. Assim, ambas as espécies permitem avaliações da toxicidade aguda e/ou crônica de amostras de interesse através de bioensaios de mortalidade/mobilidade, de regeneração, de micronúcleos e de reprodução. Tais ensaios foram padronizados para a avaliação dos efeitos da exposição de *G. tigrina* a soluções de sulfato de cobre. Portanto, este trabalho disponibilizou um sistema padronizado de bioensaios rápidos, sensíveis e baratos para biomonitoramento ambiental dos ecossistemas de água doce. Além disso, os resultados obtidos forneceram subsídios para estudos dos mecanismos de manutenção de neoblastos, os quais são diretamente responsáveis pela regeneração, pela homeostase dos tecidos e pela reprodução.

## Abstract

Planarians are choice organisms used in a wide series of basic studies including both ecotoxicology aspects and cellular and molecular approach. It is especially due to its extraordinary regeneration capacity and to the presence of somatic stem cell (neoblasts) in adult organisms. Therefore, the availability of species and lineage with well known physiological characteristics is fundamental. The species *Girardia tigrina* e *Girardia schubarti*, native from Rio Grande do Sul State, Brazil, were studied as to the reproductive modes, life cycle, regenerative capacity and toxicity, and susceptibility to teratogenic and mutagenic agents. In both species, there was inter-relation between reproductive mode and ploidy. Diploid individuals were usually sexual and triploid, and mixoploid individuals were exclusively fissiparous. Fissiparous planarians produce smaller number of descendents, and show longer lifespan than sexual planarians. In both species the reproduction sexual frequency and the longevity undergo influence of the environmental conditions; nevertheless, environmental changes do not lead to the switching from sexual to asexual reproduction. *G. tigrina* and *G. schubarti* species show similar regeneration capacity and clastogenic agent susceptibility. Thus, both species allow assessment of acute and/or chronic toxicity of choice samples by lethality/mobility, regeneration, micronuclei, and reproduction bioassays. Such assays were standardized for assessment of effects of *G. tigrina* exposure to copper sulfate solutions. In addition, this work provided a rapid, sensitive and expensive standardized bioassay system for environmental biomonitoring of freshwater ecosystems, based on native organisms from Rio Grande do Sul State. Besides, the obtained results provide bases for further studies on the neoblast maintenance mechanisms, which are directly responsible for regeneration, homeostasis of all tissue and reproduction.

# 1. Introdução

## *1.1 Sistemática, habitat e nicho ecológico de G. tigrina e G. schubarti*

O filo Platyhelminthes apresenta uma grande diversidade de organismos, que gradualmente fazem uma transição evolutiva entre organismos de vida livre a parasitos. Ele é, portanto, um dos mais instrutivos em biologia funcional e evolutiva (RUPPERT *et al*., 2005). Esse filo inclui vermes chatos tanto de vida livre (Classe Turbellaria), quanto ectoparasitas (Classes Temnocephalidae) e parasitas (Classes Trematoda e Cestoda).

O nome planárias é genericamente aplicado aos membros da classe Turbellaria, que são predominantemente aquáticos, havendo poucos terrestres (SLUYS, 1989). As planárias de água doce (infraordem Paludicola) são habitantes de bentos de lagos, poças, riachos e nascentes.

As planárias paludicolas que ocorrem na América do Sul pertencem à família Dugesiidae e ao gênero *Girardia* (VRIES & SLUYS,1991). As espécies de planárias que já foram coletadas no Rio Grande do Sul são *Girardia* (*Cura*) *schubarti* (MARCUS, 1946), *Girardia tigrina* (GIRARD, 1850), *Girardia anderlani* (KAWAKATSU et al., 1983), *Girardia uroriograndeana* (KAWAKATSU *et al*., 1992), *Girardia arndti* (MARCUS, 1946) e *Girardia biapertura* (SLUYS *et al*., 1997). As duas primeiras espécies, objetos de estudo desta tese, são as relativamente mais abundandes e conhecidas.

As espécies *G. tigrina* e *G. schubarti* são organismos livre-nadantes e habitantes de ecossistemas paludícolas lênticos (lagos e lagoas) e lóticos (nascentes de rios e riachos), respectivamente (KNAKIEVICZ *et al*., 2007). Assim, as espécies de planárias possuem micro-habitats específicos, o que pode levar a diferenças em sua distribuição geográfica, como pode ser observado para a distribuição de *G. tigrina* e *G. schubarti* no Rio Grande do Sul (ver Figura 1).

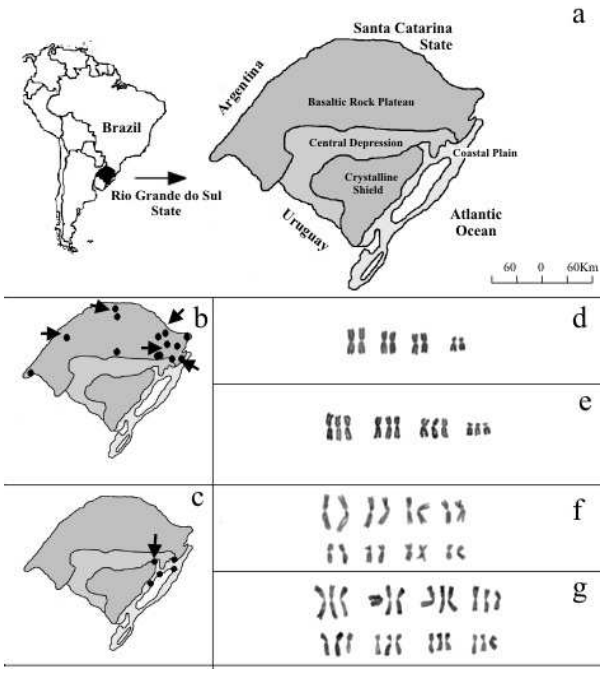



**Figura 1**. Distribuição geográfica e cariótipos de planárias paludícolas no Estado do Rio Grande do Sul, Brasil. Os pontos e setas indicam onde planárias diplóides e poliplóides (triplóides ou mixoplóides) foram encontradas, respectivamente. (**a**) Regiões morfogeológicas do Rio Grande do Sul. Ocorrência de planárias da espécie *G. schubari* (**b**) e *G.tigrina* (**c**). Cariótipos 2n e 3n da espécie *G. schubarti* (**d,e,**); da espécie *G. tigrina* (**f,g**) respectivamente (modificado de KNAKIEVICZ *et al*., 2007).

Planárias são organismos de ciclo de vida simples, os quais sofrem influências das condições de alimentação, do tamanho populacional (KOSTELECKY *et al*., 1989), das estações do ano (GAMO & NOREÑA-JANSSEM, 1998) e da assimilação de poluentes (INDEHERBERG *et al*., 1999). As planárias são predadores de pequenos invertebrados e larvas de insetos, detritívoros e servem de alimento a invertebrados e vertebrados predadores; portanto, ocupam diversos níveis da teia alimentar. Devido a essas características, planárias são bons candidatos a organismo-teste nativo alternativo para o uso no monitoramento da poluição aquática.

### *1.2 Características anatômicas e fisiológicas gerais das planárias*

Planárias são animais pequenos, achatados dorso-ventralmente, bilateralmente simétricos e acelomados, que têm o corpo coberto por uma epiderme unilaminar, ciliada e glandular (RUPPERT *et al*., 2005). Não apresentam sistema circulatório, mas possuem protonefrídios para a secreção e musculatura bem desenvolvida. O sistema nervoso central (SNC) é composto por um cérebro bilobular na região anterior do animal e por dois cordões

ventrais longitudinais (AGATA, *et al*., 1998), com várias funções e domínios estruturais definidos molecularmente (MINETA *et al*., 2003, CEBRIÀ *et al*., 2002) que correspondem aos fotorreceptores (ocelos), estatocistos (receptores de gravidade) e quimiorreceptores (aurículas, usadas na localização de alimentos e de parceiros sexuais). O cérebro participa do controle da reprodução sexuada (FAIRWEATHER & SKUCE, 1995), da reprodução assexuada (MORITA & BEST, 1984) e da regeneração (MARTELLY & FRANQUINET, 1984).

Nas planárias, a cavidade digestiva é geralmente de fundo cego, sendo a boca, equipada com uma faringe protraível, usada tanto para ingestão quanto para egestão (RUPPERT *et al*., 2005). Esses organismos são principalmente carnívoros e predam invertebrados (protozoários, rotíferos, larvas de insetos, crustáceos, lesmas e anelídeos). Enzimas proteolíticas auxiliam na perfuração do corpo da presa, e o conteúdo parcialmente digerido e liquefeito é bombeado para o interior do trato digestivo pela peristalse faríngea. As planárias liberam o excesso de água e, provavelmente, outros resíduos metabólicos usando os protonefrídios, que estão espalhados por todo o corpo.

### 1.2.1 Reprodução e desenvolvimento

Muitas espécies de planárias consistem de raças distintas, cuja definição está tipicamente associada ao nível de ploidia e ao modo de reprodução (D´SOUZA *et al*., 2004). Por exemplo, a espécie *G. tigrina* consiste de indivíduos diplóides (2n = 16) e triplóides (3n = 24), enquanto *G. schubarti* pode consistir de indivíduos simpátricos diplóides (2n = 8), triplóides (3n = 12) e mixoplóides (2n = 8 e 3n = 12) (ver Figura 1). Geralmente, indivíduos diplóides são sexuados e os poliplóides (triplóides e mixoplóides) são assexuados (STORHAS *et al*., 2000).

Quando sexuadas, as planárias são hermafroditas e reproduzem-se por cópula simultânea e fecundação interna (RUPPERT *et al*., 2005). Companheiros de cópula trocam quantidades similares de esperma, e esse fato pode ajudar a explicar o estabelecimento e manutenção do hermafrodismo, pois machos ou fêmeas puras poderiam ser desvantajosos ou deficientes em uma população onde os acasalamentos dependem da reciprocidade (VREYS & MICHIELS, 1998). O desenvolvimento dos ovos fecundados é direto, e estes ovos, juntamente com gotículas de vitelo, são envolvidos por uma cápsula (RUPPERT *et al*., 2005). Por serem animais pequenos, produzem poucos casulos (cápsulas de ovos), que contêm também poucos filhotes (de 3 a 15) e são cuidadosamente fixados em rochas. Há uma

correlação positiva entre o tamanho dos casulos e o número de filhotes, mas a correlação é negativa entre o número e o tamanho dos filhotes em uma mesma cápsula (PREZA, 1995).

Quando assexuadas, as planárias podem ser partenogênicas ou fissiparitárias (RUPPERT *et al*., 2005). Indivíduos partenogênicos geram filhotes a partir de óvulos não fecundados; no entanto, são dependentes da cópula, pois os óvulos partenogenéticos necessitam dos espermatozóides para a indução do desenvolvimento embrionário (D´SOUZA *et al*., 2004). Planárias fissiparitárias, em geral, dividem-se em duas partes e então regeneram as partes faltantes após a separação, em um processo conhecido como arquitomia. O plano de fissão, com freqüência, se forma atrás da faringe, e a separação parece depender da locomoção: a extremidade posterior do verme prende-se ao substrato, ao passo que a metade anterior continua a se mover para a frente, até que as regiões se separem. São conhecidos muitos fatores que controlam a freqüência de fissão, incluindo a temperatura da água, as condições de alimentação e o ritmo circadiano (HORI & KISHIDA, 1998; ITOH *et al*., 1999). Nos metazoas, a correlação entre a ocorrência de fissiparidade e de regeneração pode sugerir que esses processos surgiram juntos, ou seja, a regeneração poderia ser uma co-opção dos mecanismos de fissiparidade (SÁNCHEZ ALVARADO, 2000).

### 1.2.2 Regeneração

A regeneração é um dos mais fascinantes e interessantes problemas da biologia, pois está correlacionada com o desenvolvimento, isto é, aos processos de proliferação celular, morfogênese e organogênese (SÁNCHEZ ALVARADO, 2000). Quase todos os filos possuem uma ou várias espécies capazes de regenerar. A ampla variedade e as distâncias evolutivas que existem entre os animais capazes de realizar a regeneração de partes perdidas do corpo e, em alguns casos, de regenerar todo o organismo a partir de distintas partes de seu corpo, são realmente surpreendentes.

As estratégias que cada animal utiliza para a regeneração são questões de grande interesse da pesquisa contemporânea. A regeneração nos Metazoa pode ser classificada em morfalaxia e epimorfose. No primeiro caso, a diferenciação de novas estruturas no regenerante surge na ausência de proliferação celular, como, por exemplo, em hidras (HOLSTEIN *et al*., 1991). No segundo caso, há exigência de proliferação celular. A regeneração epimórfica está dividida em duas categorias: a regeneração não-baseada no blastema (regeneração de anfíbios e do fígado e ossos humanos) e a regeneração baseada

no blastema (regeneração de planárias) (SÁNCHEZ ALVARADO, 2000).

A regeneração blastemal envolve a formação de uma estrutura especializada, conhecida como blastema de regeneração, pela interação dorso-ventral de tecidos durante o fechamento da ferida por contrações musculares (KATO *et al*., 1999). Essa estrutura é similar em forma e organização aos brotos embriológicos dos membros durante a embriogênese de invertebrados e vertebrados (SÁNCHEZ ALVARADO, 2000). Dependendo do organismo, o blastema de regeneração pode formar-se horas após a amputação ou ferimento. As partes perdidas são regeneradas a partir da diferenciação do blastema, como ocorre em planárias, moluscos, equinodermos e urocordatos e na regeneração de membros ou caudas de vertebrados. Essa similaridade observada entre blastemas de diversos metazoários pode ser explicada pela origem ancestral comum dos processos de regeneração.

As planárias apresentam capacidade de regeneração sem paralelo, exibindo uma plasticidade excepcional no desenvolvimento, que permite que se regenerem completamente quando cortadas transversal ou longitudinalmente, ou em pequenos fragmentos, que podem ter apenas 1/279 do tamanho de seu corpo (MORGAN, 1898). Quando uma planária é cortada, a região do corte é rapidamente fechada por contrações musculares (SCHÜMANN & PETER, 1998). Como conseqüência, a parte dorsal adere-se à parte ventral induzindo a expressão de genes específicos que sinalizarão a formação do blastema (epimorfose) (INOUE *et al*., 2004). No blastema não ocorrem divisões celulares, somente migração da progênie dos neoblastos do mesênquima (REDDIEN *et al*., 2005). No blastema, então, essas células diferenciam-se em células específicas, utilizando-se das informações posicionais relativas do fragmento de origem (REDDIEN & SÁNCHEZ ALVARADO, 2004). As estruturas interpoladas formadas na região blastema/toco também coordenam a produção de sinais moleculares para o rearranjo dos padrões do corpo (morfalaxia), através da repadronização da expressão dos genes Hox ao longo do eixo dorso-ventral e do eixo antero-posterior no novo organismo (INOUE *et al*., 2004). Além do fechamento da ferida, a participação ativa do sistema nervoso é importante para a formação bem sucedida do blastema (AGATA, 2002).

Recentemente, a investigação dos processos moleculares de diferenciação em planárias tem sido intensificada (ver, por exemplo, GONZALEZ-ESTEVEZ *et al*., 2003; REDDIEN *et al*., 2005a; GUO *et al*., 2006), mas há ainda muitas questões não esclarecidas.

### 1.2.3 Neoblastos

Uma característica fundamental das planárias é a presença de uma população relativamente grande (20 a 30% do número total de células) de células-tronco conhecidas como neoblastos (BAGUÑÀ *et al*., 1989). Os neoblastos são células pequenas e indiferenciadas, com pouco citoplasma e núcleo proeminente (HORI & KISHIDA, 1998; SCHUMANN & PETER, 2001). A importância dos neoblastos na biologia dos turbelários é amplamente reconhecida. Eles são responsáveis pelo restabelecimento de todos os tipos celulares durante o desenvolvimento e, nos adultos, são as únicas células mitoticamente ativas, originando todos os tipos de células diferenciadas durante a reposição celular e a regeneração (GSCHEWENTNER *et al*., 2001; REDDIEN *et al*., 2004), incluindo a linhagem germinativa (SATO *et al*., 2006).

No adulto, os neoblastos parecem estar dividindo-se regularmente e não permanecem quiescentes por mais de três dias (SÁNCHEZ ALVARADO, 2000). Entretanto, após alimentação ou corte, há um rápido aumento na atividade mitótica dos neoblastos (BAGUÑÁ, 1974). Apesar do estado dinâmico da renovação celular de todos os tecidos, os neoblastos localizam-se exclusivamente no mesênquima, um compartimento bem definido espacialmente (SALVETTI *et al*., 2000). Após proliferação, migram para outras regiões (por exemplo, a região anterior aos fotorreceptores, originalmente sem neoblastos), onde se diferenciaram para substituir células mortas (SÁNCHEZ ALVARADO, 2000; REDDIEN *et al*., 2005b). Durante a regeneração, a proliferação celular está restrita ao compartimento mesenquimal, ocorrendo no blastema somente diferenciação celular (SALVETTI *et al*., 2000).

Essas caraterísticas contrastam diretamente com a falta de flexibilidade dos tecidos de *Caenorhabditis elegans* e de *Drosophila melanogaster*, organismos-modelo para estudos de desenvolvimento, que carecem de células-tronco somáticas. Assim, as planárias oferecem uma excelente oportunidade para estudo tanto da biologia do desenvolvimento quanto, mais específicamente, das células-tronco somáticas (SÁNCHEZ ALVARADO & KANG, 2005). O estudo de células-tronco de planárias pode também servir como um modelo para as investigações sobre células-tronco de organismos mais complexos, incluindo seres humanos. Além disso, estudos genômicos em planárias podem contribuir para a biomedicina, devido à dificuldade de manipulação experimental dos platielmintos parasitas, através da identificação de genes específicos desse grupo, e assim colaborar na identificação de moléculas canditadas à interação terapêutica (SANCHEZ ALVARADO *et al*., 2002).

## *1.3 Monitoramento Ambiental*

O impacto ambiental pode ser definido como qualquer alteração das propriedades físicas, químicas ou biológicas do meio ambiente resultante de atividades humanas que, direta ou indiretamente, afetem a saúde, a segurança e o bem-estar das populações humanas; das atividades sociais e econômicas; da biota; das condições estéticas e sanitárias do ambiente e da qualidade dos recursos ambientais (Resolução do CONAMA n.º 01 de 23/01/86). A avaliação preliminar de riscos ecológicos é realizada através do monitoramento ambiental preventivo dos ecossistemas em risco. Em função da grande diversidade de impactos ambientais sobre os ecossistemas aquáticos, o controle ambiental de riscos ecológicos deve envolver uma abordagem integrada, através do monitoramento da qualidade física, química e biológica da água, bem como a avaliação da qualidade estrutural de habitats (GOULART & CALLISTO, 2003). Para atender a essa demanda, o uso de organismos bioindicadores é recomendado. Bioindicador é todo e qualquer organismo, ou um conjunto de organismos, que permite caracterizar o estado de um ecossistema e evidenciar tão precocemente quanto possível as modificações naturais ou provocadas neste (NASCIMENTO, *et al*., 2006).

Nos ambientes aquáticos, vertebrados e invertebrados são diretamente expostos ao polutentes (VARGAS *et al*., 2001). Portanto, para o biomonitoramento desses ambientes, recomenda-se a utilização de espécies nativas, em um número mínimo de três: um representante dos produtores; um representante dos consumidores primários e/ou secundário; e um representante dos consumidores secundários e/ou terciários (ZAGATTO & BERTOLETTI, 2006). O estudo ecológico de invertebrados bentônicos nativos, como bioindicadores de qualidade de água, é amplamente utilizado na Austrália, nos Estados Unidos, no Canadá e em diversos países da Europa. No entanto, a utilização desses organismos em estudos de impacto ambiental ainda é incipiente no Brasil (menos de 20 anos).

### 1.3.1 Testes de toxicidade ambiental

Os ensaios de toxicidade desenvolvidos em laboratórios são úteis e necessários para a caracterização de amostras ambientais, embora diversos fatores possam afetar os resultados. Por exemplo, pode haver divergências relacionadas aos procedimentos experimentais, aos organismos-teste utilizados ou a fatores ambientais. Assim, a utilização de

métodos padronizados é recomendada para minimizar as variabilidades, melhorar a precisão e a reprodutibilidade dos testes e permitir que os resultados gerados em diferentes laboratórios possam ser comparados entre si (ARAGÃO & ARAÚJO, 2006). Além disso, a escolha dos organismos e a interpretação dos resultados devem ser feitas criteriosamente. Por exemplo, os resultados de ensaios de toxicidade com organismos aquáticos não podem ser extrapolados para organismos de lodo, de solo ou marinhos. Diversos ensaios de toxicidade já foram desenvolvidos e padronizados, pois é necessária a disponibilização de uma grande variedade de teste para que sejam atendidas as demandas específicas de cada ambiente a ser monitorado. (ZAGATTO & BERTOLETTI, 2006).

### 1.3.2 O uso de planárias no monitoramento de impactos ambientais

As planárias são amplamente distribuídas em nascentes de rios, riachos, lagos e lagoas (KNAKIEVICZ *et al*., 2007), biomas extremamente vulneráveis à degradação antrópica (SALA *et al*., 2000), as quais são sensíveis tanto à poluição orgânica (BEST & MORITA, 1991) quanto ás modificações estruturais dos ambientes (GAMO & NOREÑA-JANSSEM, 1998). Elas têm sido usadas como organismos-teste para detecção de poluição ambiental deste 1940 (ERICHSEN JONES, 1940), e já foi demonstrado, em diversas espécies de planárias de diferentes partes do mundo, que são orgnismos sensíveis a inúmeros poluentes ambientais, apresentando potencial para a detecção dos efeitos associados à exposição a agentes tóxicos, teratogênicos e genotóxicos (BEST, & MORITA, 1991; CALEVRO *et al*, 1998, 1999; PRÁ *et al*., 2005). Assim, dentre os invertebrados bentônicos aquáticos, as planárias permitem a avaliação do efeito dos poluentes através da análise de diferentes bioensaios, os quais detectam efeito em distintos níveis da organização biológica, tais como molecular, celular, morfológico e fisiológico ou compartamental (BEST & MORITA, 1991, CALEVRO *et al*., 1998, 1999, GUECHEVA *et al*., 2001), permitindo a obtenção de informações complementares a partir de diferentes biomarcadores sobre a toxicidade das amostras avaliadas.

Dessa forma, as informações acima sugerem que as planárias são organismos promissores para uso tanto como organismos-teste quanto como organismos bioindicadores da qualidade dos ambientes aquáticos. Assim, planárias podem ser potencialmente úteis na ecotoxicologia, que busca integrar o monitoramento da toxicidade de poluentes com a conservação, visando garantir a manutenção integral da saúde dos ecossistemas.

## 2. Objetivos

O filo Platyhelminthes apresenta uma composição filogenética bastante interessante para estudos de evolução comparada, entre outros, mas ainda carece de espécies-modelo mais bem caracterizadas que viabilizem o estudo do desenvolvimento. Planárias surgem como os platelmintos de vida livre de escolha para a realização de uma ampla gama de estudos básicos, abrangendo desde aspectos ecológicos até abordagens de biologia celular e molecular. Esses organismos são particularmente atrativos para a pesquisa científica, pois apresentam grande capacidade de regeneração, a qual envolve processos similares aos observados na embriogênese de vertebrados, tais como proliferação, diferenciação e migração celulares, morfogênese e organogênese. Além disso, devido a sua sensibilidade a poluentes ambientais, presta-se muito bem a estudos ecotoxicológicos. Contudo, para a utilização desses organismos como sistemas-modelo, tanto para estudos básicos de desenvolvimento quanto para estudos aplicados de ecotoxicologia, há necessidade da disponibilização de espécies e linhagens com prévia caracterização fisiológica.

O uso de planárias em ecotoxicologia, como organismos bioindicadores, demanda conhecimentos prévios sobre o seu comportamento na natureza e no laboratório (STOHLER *et al*., 2004), bem como a identificação de marcadores de resposta biológica, isto é, biomarcadores que demonstram desvios da normalidade frente a poluentes ambientais ou compostos específicos (NASCIMENTO *et al*., 2006). Uma vez identificados, os biomarcadores podem ser ferramentas úteis para a caracterização de fenótipos, etapa fundamental para a seleção de indivíduos e populações com diferentes características fisiológicas, e para o estabelecimento de linhagens bem caracterizadas para estudos mais refinados, em nível celular e/ou molecular, por exemplo. O estabelecimento de linhagens de planárias também pode contribuir para o aprimoramento de testes ecotoxicológicos, uma vez que tais linhagens são candidatas promissoras ao biomonitoramento da poluição ambiental.

Considerando os aspectos mencionados acima, o presente trabalho teve por objetivo principal caracterizar populações nativas de planárias *G. tigrina* e *G. schubarti* do Rio Grande do Sul, cultivadas em laboratório, e padronizar com elas alguns testes ecotoxicológicos para

detecção de poluentes aquáticos.

O estudo foi subdividido em duas áreas, correspondendo a primeira delas aos resultados descritos nos Capítulos I e IV e a segunda correspondendo aos resultados descritos nos Capítulos II e III. Os objetivos específicos de cada área do estudo estão listados abaixo:

1 – Caracterização de ciclos de vida, modos de reprodução e de processos de regeneração (Capítulos I e IV)

- Caracterizar o modo de reprodução de *G. tigrina* e *G. schubarti*, verificando as influências da ploidia, da densidade populacional, de alterações físico-químicas da água de cultivo e da alimentação.
- Avaliar a capacidade regenerativa de *G. tigrina* e *G. schubarti* em diferentes estágios de desenvolvimento e em diferentes condições ambientais.
- Avaliar a longevidade das populações de *G. tigrina* diplóide (sexuada) e de *G. schubarti*.mixoplóide (assexuada).

2 – Caracterizar a suscetibilidade de *G. tigrina* e *G. schubarti* a agentes tóxicos (Capítulos II e III).

- Identificar biomarcadores para exposições agudas ou crônicas a compostos tóxicos, teratogênicos e/ou mutagênicos.
- Padronizar e validar o ensaio de micronúcleos em neoblastos de *G. tigrina* diplóide e *G. schubarti* mixoplóide
- Padronizar bioensaios para a avaliação dos efeitos de toxicidade aguda de poluentes aquáticos em diferentes níveis da organização biológica (molecular, celular e sistêmico) de *G. tigrina*.

# 3. Resultados

## *3.1 Capítulo I - Reproductive modes and life cycles of freshwater planarians (Platyhelminthes, Tricladida, Paludicula) from southern Brazil*

Tanise Knakievicz, Sabrina Moura Vieira, Bernardo Erdtmann, Henrique Bunselmeyer Ferreira.

Artigo publicado em *Invertebrate Biology* 125: 212-221, 2006.

Invertebrate Biology 125(3): 212–221.
© 2006, The Authors
Journal compilation © 2006, The American Microscopical Society, Inc.
DOI: 10.1111/j.1744-7410.2006.00054.x

# Reproductive modes and life cycles of freshwater planarians (Platyhelminthes, Tricladida, Paludicula) from southern Brazil

Tanise Knakievicz,[1] Sabrina Moura Vieira,[2] Bernardo Erdtmann,[2] and Henrique Bunselmeyer Ferreira[1,3,a]

[1] Laboratorio de Biologia Molecular de Cestodeos, and Programa de Pos-Gradua@ao em Biologia Celular e Molecular, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[2] Departamento de Genetica, Instituto de Biociencias, UFRGS, Porto Alegre, RS, Brazil
[3] Departamento de Biologia Molecular e Biotecnologia, Instituto de Biociencias, UFRGS, Porto Alegre, RS, Brazil

Abstract. Life cycles, ploidy levels, reproductive modes, and regeneration capacities of laboratory populations of the southern Brazil freshwater planarians Girardia tigrina and Girardia schubarti were studied. The mating behavior and life cycle of different populations from both species were analyzed regarding their karyotype, body size, modes of reproduction, and regeneration potential. Reproduction was evaluated according to fecundity, fertility, and fissiparity indices. In both species we observed that diploid planarians are ≈25% larger than triploid or mixoploid ones and that sexually reproducing populations have more offspring than asexual ones. Cocoon incubation time was shorter for G. tigrina, and G. schubarti showed a higher frequency of spontaneous malformations. G. tigrina was both more fecund (produced more cocoons) and more fertile (produced more hatchlings) than G. schubarti. The effects of alternative food sources (liver or egg yolk), demographic density, and water–salt concentration were evaluated in different populations of both species. All evaluated environmental factors had effects on fecundity and fertility indices and were more prominent for G. schubarti than for G. tigrina. Our data suggest that the populations of G. schubarti were more sensitive to environmental factors, and thus may be useful as bioindicators.

Additional key words: Girardia schubarti, G. tigrina, ploidy, fissiparity

Planarians (Platyhelminthes, Turbellaria, Tricladida) have a wide distribution in unpolluted streams, lakes, and estuaries, and their natural sensitivity to environmental variations, associated with their easy experimental manipulation, make them potential candidates as bioindicator organisms. Thus, the study of local planarians is important to define species and/or populations adequate for environmental monitoring. In addition, the knowledge of the biology of different planarian species may bring new insights to comparative studies of reproductive mechanisms and regeneration among metazoans.

Different freshwater planarian species and populations present a variety of modes of reproduction. Some are exclusively asexual (fissiparous or parthenogenetic), seasonally sexual, or exclusively sexual, and the reproductive mode is typically associated with ploidy levels (Beukeboon & Vrijenhoek 1998; D'Souza et al. 2004). In some planarian species there is an alternation between sexual and asexual reproduction, and its occurrence may compensate, at least in part, for some disadvantages presented by each reproductive mode (Benazzi 1981; Hase et al. 2003), as has been argued for other metazoans (Doncaster et al. 2000, 2003).

The positive correlation between the occurrence of regeneration and asexual reproduction in metazoans may result from the co-option of asexual reproductive mechanisms for regenerating events (Alvarado 2000; Peter et al. 2001). The study of biological and environmental factors involved in planarian reproduction and regeneration may shed some light on the

[a] Author for correspondence.
E-mail: henrique@cbiot.ufrgs.br

cellular and molecular mechanisms involved, and the possible correlations between them. It has been recognized in short-term cultures that turbellarians that employ different reproductive modes (sexual, asexual by parthenogenesis, or asexual by fissiparity) may help in understanding the evolution of reproductive strategies and the function of sexuality (Benazzi 1981; Rieger 1998; Kobayashi et al. 1999a,b).

The southern Brazilian planarians Girardia tigrina GIRARD 1850 and Girardia schubarti MARCUS 1946 have been used in morphological, karyological, and taxonomic studies (see, e.g., Kawakatsu et al. 1981, 1982, 1985; Vreys et al. 2002) and as test organisms in toxicity assays (Rivera & Perich 1994; Guecheva et al. 2001, 2003). However, little is known about their mating behavior, life cycles, and ecology. Species of Girardia present both sexual and asexual reproduction and their reproductive capacities may vary as a function of ploidy in different populations (Kawakatsu et al. 1981, 1984). Therefore, G. tigrina and G. schubarti are good biological systems to study the possible relationships between reproduction mode and ploidy and between asexual reproduction and regeneration. With this aim, we have evaluated the life cycles, ploidy levels, reproduction modes, and regeneration capacities of laboratory populations of G. tigrina and G. schubarti in vitro. In addition, the possible origin and selective advantage of polyploidy and the coexistence of sexual and asexual individuals within planarian populations are discussed.

## Methods

### Field collection and laboratory cultures

Specimens of Girardia tigrina and Girardia schubarti were collected from lotic and lentic freshwater environments, respectively, at different sites in Rio Grande do Sul (RS), the southernmost Brazilian state. Individuals of G. tigrina were collected in Dois Irmaos (population 1, GtPop1) and Salvador do Sul (population 2, GtPop2) in 1993; individuals of G. schubarti were collected from lentic freshwater environments at the National Forest of Sao Francisco de Paula (population 1, GsPop1) in 1994–2000; in Salvador do Sul (population 2, GsPop2) in 1983; and in Santo Angelo (population 3, GsPop3) in 2000. Individuals collected at each site were considered a population and were kept in standard laboratory conditions since their collection.

At least 40 specimens from each collected sample were morphologically analyzed and karyotyped for species identification and ploidy level determination, as previously described (Kawakatsu et al. 1981, 1984), and the length and width of adults were measured. In the laboratory, planarians were cultivated in atoxic plastic containers with reconstituted water (Cabridenc 1979) and fed weekly with small chunks of bovine liver, with the water immediately changed after feeding. Populations were kept at the established ideal temperatures that supported the highest survival and reproduction rates for each species, i.e., 18–22°C for G. tigrina (Gee et al. 1998) and 13–15°C for G. schubarti (Knakievicz T & Ferreira HB, unpubl. data).

### Karyology

Regenerating blastemas, obtained as described in Baguna et al. (1989), were used for karyotyping. The regenerating tissue (blastema) was dissected and treated with colchicine (0.2% for 3 h). The colchicine-treated blastema was minced and further disaggregated by pipetting several times, and incubated for 20 min in a KCl-hypotonic solution (0.075 mol $L^{-1}$). Free cells and tissue fragments were sedimented by centrifugation and resuspended in 5 mL of cold Carnoy's solution. Slides were prepared using the air-drying method and stained with 10% Giemsa (Lamatsch et al. 1998) for standard analysis in a Zeiss (Oborkochen, Germany) Axiophot optical microscope. Up to 100 mitotic cells were analyzed to define the ploidy level of 2n or 3n individuals. For mixoploid (2n/3n) populations, at least 100 cells from at least 13 individuals were analyzed to establish the 2n:3n ratio.

### Evaluation of reproductive modes

The reproductive modes in G. tigrina and G. schubarti were evaluated by monitoring populations of randomly selected individuals and pairs. Cocoons and hatchlings produced by sexual or parthenogenetic reproduction in each population or pair were counted weekly and transferred to isolated containers. It was assumed, according to Hoshi et al. (2003) and D'Souza et al. (2004), that 2n planarians produce cocoons only by sexual reproduction and do not present parthenogenesis. Fissiparous individuals or populations were defined as those populations that reproduce only by fissiparity. Fecundity was measured as the number of cocoons produced by sexual or parthenogenetically reproducing animals. Cocoons that did not hatch within 3 months were considered unviable. Fertility was measured as the number of hatchlings per individual per week. Fecundity (Fc) and fertility (Fr) indexes for each species were calculated as

$$Fc = \frac{CC}{NT} \quad (1)$$

Table 1. Ploidy level and size body in populations of Girardia tigrina (Gt) and G. schubarti (Gs). NA, not applicable; SD, standard deviation.

| Population | Ploidy (sample size[1]) | Sizes (mean±SD)[2] | | 2n:3n:2n/3n ratio for mixed populations | 2n:3n cell ratio in 2n/3n specimens[3] (sample size[4]) |
|---|---|---|---|---|---|
| | | Length (cm) | Width (cm) | | |
| GtPop1 | 2n ($n_{ind}$ = 25) | 1.12±0.15[a] | 0.16±0.03[a] | NA | NA |
| GtPop2 | 3n ($n_{ind}$ = 13) | 0.81±0.12[b] | 0.11±0.02[b] | NA | NA |
| GsPop1 | 2n ($n_{ind}$ = 49) | 2.09±0.44[c] | 0.22±0.06[c] | 1.00:0.12:0.25 | NA |
| | 3n ($n_{ind}$ = 6) | 1.53±0.21[d] | 0.15±0.03[d] | — | NA |
| | 2n/3n ($n_{ind}$ = 12) | 1.39±0.56[d] | 0.17±0.05[d] | — | 5.55:1[e] ($n_{cel}$ = 292) |
| GsPop2 | 3n ($n_{ind}$ = 6) | 1.54±0.25[d] | 0.13±0.05[d] | 0:1.00:4.67 | NA |
| | 2n/3n ($n_{ind}$ = 28) | 1.55±0.23[d] | 0.14±0.04[d] | — | 1:2.21[f] ($n_{cel}$ = 1024) |
| GsPop3 | 3n ($n_{ind}$ = 8) | 1.24±0.19[d] | 0.11±0.06[d] | 0:1.00:0.62 | NA |
| | 2n/3n ($n_{ind}$ = 5) | 1.55±0.19[d] | 0.15±0.05[d] | — | 1:2.47[f] ($n_{cel}$ = 118) |

[1]Sample size ($n_{ind}$) refers to the number of animals analyzed for each population.
[2]Means indicated by different letters differ significantly (a, b refer to comparisons between populations of G. tigrina, t-test, $t_{0.05,36}$ = 6.408, $p < 0.001$; c, d refer to comparisons among populations of G. schubarti; ANOVA, $F_{6,114}$ = 13.94, $p < 0.001$, Tukey test).
[3]2n:3n cell ratios indicated by different letters (e, f) do not differ significantly (ANOVA, $F_{2,42}$ = 14.03, $p < 0.001$).
[4]Sample size ($n_{cel}$) refers to the total number of mitotic cells analyzed, obtained from a minimum number of five individuals of a given mixoploid (2n/3n) subpopulation.

where CC is the number of cocoons produced by individuals or pairs in a given week and N1 is the number of individuals within a sampled population or twice the number of analyzed pairs in the experiment, and

$$Fr = \frac{HC}{N1} \quad (2)$$

where HC is the number of hatchlings produced by individuals or pairs in a given week and N1 is the number of individuals within a sampled population or twice the number of analyzed pairs in the experiment.

To evaluate fissiparous asexual reproduction, the number of fissiparous events in 2n and polyploid (3n or 2n/3n) randomly sampled pairs of G. tigrina and G. schubarti, cultivated for 25 weeks, was assessed. The fission rate (Fs) per individual per week was calculated as

$$Fs = \frac{FE}{N3} \quad (3)$$

where FE is the number of offspring produced by fissiparous individuals or pairs in a given week and N3 is the total number of individuals or twice the number of analyzed pairs in the experiment.

## Assessment of regenerative capacity

The regenerative capacity of different populations of G. tigrina and G. schubarti was scored as the time for complete head regeneration in decapitated planarians. Samples of 20 adult individuals of average size (defined for each population in Table 1) were decapitated. For decapitation, each individual was immobilized over a block of ice and a single cut was made, as precisely as possible, immediately behind the auricles. Decapitated individuals were observed daily for 15 d, until complete head regeneration (regeneration of eyes and auricles). Before and during the experiments, the planarians were kept in standard culture conditions as described above.

## Analysis of environmental effects

The effects of population density, diet, and salinity upon fecundity and fertility parameters of different populations of G. tigrina and G. schubarti were analyzed. Population density experiments were carried out with samples of 12, 25, 50, or 100 individuals from each population and were observed for 26 weeks. Diet experiments were carried out with four samples (for each, n = 60) of each analyzed population. Samples were fed with either bovine liver or chicken-egg yolk for 12 weeks. Salinity experiments were carried out with four samples (for each, n = 60) from each population. Each sample was cultured in water under standard salt conditions (1 x salt concentration: 0.228 mmol $L^{-1}$ $NaHCO_3$, 0.013 mmol $L^{-1}$ KCl, 0.060 mmol $L^{-1}$ $MgSO_4$, 0.090 mmol $L^{-1}$ $CaSO_4$; Cabridenc 1979) or in 0.5 x or 3 x salt concentration, and observed for 24 weeks. Aeration experiments were carried out

with one sample (n = 60) of each analyzed population. Samples were maintained in standard culture conditions either with or without aeration.

For each experimental condition, the fecundity (Fc), fertility (Fr), and fissiparity (Fs) parameters were calculated as above.

### Statistical analysis

All statistical analyses were performed using SPSS® for Windows® (SPSS Inc., Chicago, IL), and each experimental population or pair was treated as a separated data point. Body size and 2n:3n ratio com- parison analyses were performed using the independ- ent-samples t-test (for data with normal distribution). Multiple comparisons (between body size and 2n:3n ratio, and between 2n:3n cell ratios in mixoploid individuals) were performed using one-way ANOVA followed by the Tukey test. Comparison analyses of
the population density, salt concentration, and aeration effects were performed using Friedman's test followed by the Nemenyi test (Zar 1999); for these tests, data were collected weekly for each sample and, therefore, n corresponds to the total number of weeks of the experiment.

## Results

### Karyotyping and body sizes

Each population of Girardia tigrina (GtPop1 and 2) and Girardia schubarti (GsPop1–3) was karyotyped and measured, and the data are summarized in Table 1. For G. tigrina (n = 8), the GtPop1 population was 2n and GtPop2 was 3n. For G. schubarti (n = 4), population 1 (GsPop1) presented 2n, 3n, and 2n/3n individuals, in the proportions specified in Table 1 along with the 2n:3n cell ratio for mixoploid specimens. Noticeably, the 2n:3n cell ratio for GsPop1 2n/3n hatchlings (10:1), determined from the analysis of 193 mitotic cells from four individuals, is different from that observed for 2n/3n individuals from their parental population (5.5:1), which may indicate that this ratio does vary either between individuals or from generation to generation. In addition, the 2n:3n cell ratio from GsPop1 2n/3n individuals differs significantly from that of GsPop2 and GsPop3 2n/3n individuals (ANOVA, $F_{2, 42} = 14.03$, $p < 0.001$).

For both species, 2n individuals were larger than 3n individuals. For populations of G. tigrina, 2n individuals are, on average, 28% larger than 3n ones (t-test, $t_{0.05, 36} = 6.408$, $p < 0.001$ for width and length). In G. schubarti, 3n and 2n/3n populations are not significantly different in size when compared with each other (GsPop1 x GsPop2, GsPop1 x GsPop3, and GsPop2 x GsPop3; ANOVA, $F_{5, 65} = 0.115$, $p = 0.885$). However, 2n individuals of G. schubarti (found only in GsPop1) are on average 25% larger than both 3n and 2n/3n individuals, regardless of the population(s) considered (ANOVA, $F_{6, 114} = 13.94$, $p < 0.001$).

### Characterization of reproductive modes

In order to determine the reproductive mode of the populations, parameters of fecundity (Fc), fertility (Fr), and fissiparity (Fs) were assessed for 16 weeks for both selected couples (Table 2) and populations. In G. tigrina, diploid couples (from GtPop1) presented only sexual reproduction and triploid ones (from GtPop2) reproduced only by fission, reproductive behaviors also observed in the corresponding populations. In G. schubarti, populations GsPop2 and GsPop3, which are devoid of 2n individuals, showed only fission reproduction, both for couples and for

Table 2. Parameters of fecundity (Fc), fertility (Fr), and fissiparity (Fs) for couples from populations of Girardia tigrina (Gt) and G. schubarti (Gs).

| Population (ploidy) | Number of couples | Fc[1,4] (mean±SD) | Fr[2,4] (mean±SD) | Fs[3,4] (mean±SD) |
|---|---|---|---|---|
| GtPop1 (2n) | 10 (sexual) | 0.861±0.282[a] | 2.866±1.161[c] | 0 |
| GtPop2 (3n) | 9 (fissiparous) | 0 | 0 | 0.205±0.062[e] |
| GsPop1 (2n, 3n, and 2n/3n) | 37 (sexual/parthenogenetic) | 0.098±0.066[b] | 0.385±0.156[d] | 0 |
| | 14 (fissiparous) | 0 | 0 | 0.096±0.033[f] |
| GsPop2 (3n and 2n/3n) | 20 (fissiparous) | 0 | 0 | 0.235±0.067[e] |
| GsPop3 (3n and 2n/3n) | 10 (fissiparous) | 0 | 0 | 0.168±0.094[e] |

[1]Fc means indicated by different letters (a, b) differ significantly (t-test, $t_{0.05, 35} = 4.147$, $p < 0.001$).
[2]Fr means indicated by different letters (c, d) differ significantly (t-test, $t_{0.05, 35} = 5.300$, $p < 0.001$).
[3]Fs means indicated by different letters (e, f) do not differ significantly (ANOVA, $F_{3, 53} = 8.067$, $p < 0.001$, Tukey test).
[4]$a_{Bonf} = 0.006$.

populations. However, for the 51 couples formed from GsPop1 hatchlings, which included only 2n (80%) and 2n/3n (20%) individuals (3n hatchlings were not found among GsPop1 hatchlings), two distinct classes were observed, one only with fissiparous couples (n = 14, 27.5%) and another with both sexual and parthenogenetic reproducing ones (n = 37, 72.5%), considering that the production of both 2n and 2n/3n offspring by 2n parents is evidence of parthenogenetic reproduction within a population (Schley et al. 2004). As the switching between reproduction modes was not observed for any of these couples, they may represent reproductively distinct subpopulations of the GsPop1 population, in which sexual/parthenogenetic and fissiparous reproduction modes are simultaneously present.

Sexual offspring of G. tigrina are 7× larger (Fc = 2.866 for GtPop1) than those of G. schubarti (Fc = 0.385 for GsPop1; Table 2). Comparing different populations within each species, the sexual offspring of G. tigrina is 14× larger than that generated by fissiparity (GtPop1 Fc/GtPop2 Fs = 13.978), while in G. schubarti the sexual/parthenogenetic offspring are only 2× larger (GsPop1 Fc/GsPop1 Fs = 4.011; GsPop1 Fc/GsPop2 Fs = 0.689; GsPop1 Fc/GsPop3 Fs = 2.291). The GtPop2 population of G. tigrina and the GsPop2 and GsPop3 populations of G. schubarti did not differ significantly in their fissiparity rates (Table 2). Therefore, the prevalence of fissiparous offspring hardly differs between these two species (GtPop2 Fs/GsPop2 Fs = 0.872; GtPop2 Fs/GsPop3 Fs = 1.220).

Attempts to induce the switching from fissiparous to sexual reproductive modes were performed as previously described by Hauser (1987) and Kobayashi et al. (1999a), feeding 10 pairs of mixoploid (2n/3n) individuals of G. schubarti (from GsPop2) with minced 2n G. tigrina individuals. After 6 months of daily monitoring, no cocoon production was observed.

## Fecundity and offspring viability

Cocoon production and offspring viability were assessed for the sexual population of G. tigrina (GtPop1) and for the sexual/parthenogenetic population of G. schubarti (GsPop1). The number of cocoons produced per individual was 10× higher in G. tigrina (Fc = 0.861) than in G. schubarti (Fc = 0.098) (Table 2). In G. tigrina, cocoon incubation time was shorter, with most of them hatching within 2 (64.9%) or 3 weeks (33.0%) after laying. In G. schubarti, most of the cocoons hatched within 3 (52%) or 4 weeks (31.9%) after laying.

Viability was comparatively evaluated for both cocoons and hatchlings. In G. tigrina, 100% of the cocoons were viable and 80% of their hatchlings remained alive after 4 weeks (n = 1233). The viability of sexual/parthenogenetic offspring in G. schubarti was lower, with 77% of the cocoons viable and 72% of their hatchlings remaining alive after 4 weeks (n = 71).

## Teratogenesis

The occurrence of malformations was analyzed in the sexual offspring of G. tigrina and in the sexual/parthenogenetic offspring of G. schubarti (GtPop1 and GsPop1 populations, respectively). Morphological malformations in hatchlings, such as double heads, deformed tails, and conjoined individuals, occurred with frequencies of 0.17% in G. tigrina (n = 3083) and 0.72% in G. schubarti (n = 354).

## Regeneration potential

To verify possible differences in regeneration capacity between populations of G. tigrina and G. schubarti, we observed the time necessary for regeneration of decapitated individuals of the GtPop1 population of G. tigrina and the GsPop1, GsPop2, and GsPop3 populations of G. schubarti. Sampled individuals (n = 20) of each population were decapitated and allowed to regenerate in standard culture conditions, being observed every 24 h. The GtPop1, GsPop2, and GsPop3 individuals took an average of 4 d for full-head regeneration. GsPop1 individuals, on the other hand, took twice this time (an average of 8 d).

## Environmental influence on reproduction

To evaluate the influence of different environmental factors on reproductive parameters of sexual (G. tigrina) and sexual/parthenogenetic (G. schubarti) populations, sexually mature individuals, of known age, from GtPop1 and GsPop1 populations (12 and 24 weeks after hatching, respectively) were submitted to different culture conditions in population density, salinity, aeration, and diet (egg yolk or bovine liver). An increase in population density had different effects on G. tigrina and G. shubarti populations (Table 3), significantly reducing fecundity and fertility in G. schubarti, with a density of 100 individuals $L^{-1}$ being 100% lethal within 4 weeks with no reproduction. In G. tigrina, on the other hand, no clear correlation between fecundity/fertility rates and population density was observed.

Table 3. Effects of population density on sexual reproduction parameters of Girardia tigrina (Gt) and G. schubarti (Gs).

| Population | Individuals $L^{-1}$ | Fc (mean±SD) | Fr (mean±SD) |
|---|---|---|---|
| GtPop1[1] | 12 | 0.337±0.280[a] | 1.155±1.154[b] |
| | 25 | 0.249±0.273[a] | 0.741±0.835[b] |
| | 50 | 0.456±0.407[a] | 1.082±1.147[b] |
| | 100 | 0.206±0.207[a] | 0.472±0.460[b] |
| GsPop1[2] | 12 | 0.135±0.160[c] | 0.604±0.653[e] |
| | 25 | 0.066±0.084[c,d] | 0.242±0.245[f] |
| | 50 | 0.032±0.055[d] | 0.166±0.210[f] |
| | 100[3] | — | — |

[1]Fc and Fr means in G. tigrina indicated by the same letters (a and b, respectively) did not differ significantly (Friedman test; Fc, $w^2 = 9.305$, $p = 0.025$, $n = 26$; Fr, $w^2 = 11.251$, $p = 0.030$, $n = 26$).
[2]Fc and Fr means in G. schubarti indicated by different letters (c, d and e, f, respectively) differed significantly (Friedman test; Fc, $w^2 = 8.578$, $p < 0.014$, $n = 26$; Fr, $w^2 = 9.500$, $p < 0.009$, $n = 26$; Nemenyi test, $a = 0.05$).
[3]In this sample, all individuals died within 4 weeks without reproducing.

The influence of water aeration and salt concentration was also more noticeable for G. schubarti than for G. tigrina (Table 4). Culture aeration slightly but significantly reduced fecundity and fertility in G. schubarti (Friedman test; Fc, $w^2 = 26.24$, $p < 0.001$, $n = 24$; Fr, $w^2 = 29.60$, $p < 0.001$, $n = 24$), while extreme salt concentrations (1/2 or 3x) drastically reduced or even eliminated sexual/parthenogenetic reproduction. However, both water aeration and a 3x salt concentration determined the occurrence of fissiparity among GsPop1 individuals, which was not observed in the standard control culture. This low fission rate was similar to that observed for GsPop1 fissiparous couples (see Table 2).

Finally, the effects of an alternative diet (egg yolk) instead of the standard bovine liver diet were assessed for both species. The egg yolk diet was 100% lethal within 9 weeks in G. schubarti. This lethal effect was milder in G. tigrina, with 50% of the individual dying after 12 weeks. No reproduction, either sexual/parthenogenetic or fissiparous, was observed for G. schubarti, while in G. tigrina sexual reproduction was presented (Fc = 0.014±0.047), although severely impaired (see Table 4).

## Discussion

Girardia tigrina and Girardia schubarti are planarian species that present populations with inter- and intraspecific variation in several respects, from genetics to reproductive behavior and regeneration capacity. G. tigrina is a widespread, native American triclad (Gee et al. 1998; Tamura et al. 1998) with sexual, fissiparous, and parthenogenetic populations (Vreys et al. 2002) that may also differ in their ploidy (Benazzi & Benazzi-Lentati 1976). G. schubarti is a species whose occurrence is described only in Brazil (Benazzi & Benazzi-Lentati 1976), and its populations may also vary in ploidy and reproductive behavior (Kawakatsu et al. 1984).

Both species occur in the Brazilian southern state of RS, but with distinct biogeographic distributions. While individuals of G. tigrina are typically found attached to aquatic plants in ponds and lakes of the sedimentary central depression and coastal plain (200 m above sea level on average), individuals of G. schubarti are found in freshwater lentic streams of

Table 4. Effects of aeration and salt concentration on reproductive parameters in Girardia tigrina and G. schubarti.

| Population | Culture condition | Fc (mean±SD) | Fr (mean±SD) | Fs (mean±SD) |
|---|---|---|---|---|
| GtPop1[1] | Standard | 0.880±0.620[a] | 1.766±1.510[b] | 0 |
| | Standard with aeration | 0.808±0.743[a] | 1.643±2.148[b] | 0 |
| | 0.5 x salt concentration | 0.929±0.637[a] | 1.903±1.952[b] | 0 |
| | 3 x salt concentration | 0.750±0.554[a] | 1.714±1.330[b] | 0 |
| GsPop1[2] | Standard | 0.038±0.077[c] | 0.045±0.125[f] | 0[h] |
| | Standard with aeration | 0.027±0.041[d] | 0.097±0.182[g] | 0.034±0.059[i] |
| | 0.5 x salt concentration | 0[e] | 0[f] | 0[h] |
| | 3 x salt concentration | 0.007±0.024[d,e] | 0.018±0.078[f] | 0.012±0.027[h] |

[1]Fc and Fr means in G. tigrina indicated by the same letters (a and b, respectively) did not differ significantly (Friedman test: Fc, $w^2 = 2.805$, $p < 0.421$, $n = 35$; $w^2 = 3.170$, $p < .365$, $n = 35$).
[2]Fc, Fr, and Fs means in G. schubarti indicated by different letters (c, d, e; f, g; and h, i; respectively) differed significantly (Friedman test: Fc, $w^2 = 26.24$, $p < 0.001$, $n = 24$; Fr, $w^2 = 29.60$, $p < 0.001$, $n = 24$; and Fs, $w^2 = 32.34$, $p < 0.001$, $n = 24$; Nemenyi test, $a = 0.05$).

basaltic elevated plains (700 m above sea level on average), usually beneath stones. In this work, we have analyzed two and three laboratory populations of G. tigrina and G. schubarti, respectively, with emphasis on basic biological aspects such as their ploidy, reproductive behavior, and relative sensitivity to some environmental conditions. The populations used in our study were collected at different times and kept in stable laboratory conditions for long and different periods of time (5–22 years). A founder effect and potential bottlenecks (Stohler et al. 2004) may have determined that their current behavior does not exactly reflect that of natural populations. In addition, some of the observed differences between species or populations may be due at least in part to differential laboratory adaptation. However, it is important to note that data for the GsPop3 population refer to freshly collected planarians (in the year 2000) and that their current (in the year 2005) reproductive behavior and regeneration capacity have not been significantly altered (Knakievicz T & Ferreira HB, unpubl. data).

Ploidy variation may be an important determinant of morphological and physiological plasticity (Gregory 2002) and may favor speciation events at least in some cases (Gregory 2003). Ploidy variations were observed between the populations of G. tigrina and G. schubarti analyzed. However, in G. tigrina, the analyzed populations were exclusively 2n (GtPop1) or 3n (GtPop2), while in populations of G. schubarti the ploidy level (considering both the proportion of 2n, 3n, and/or 2n/3n individuals within a population and the 2n:3n rate for a given mixoploid individual) apparently presents fluctuations in response to different factors that remain to be elucidated. The identification of such factors, which may range from genetic to environmental, will be important to understand how different degrees of ploidic plasticity are maintained among populations of G. tigrina and G. schubarti.

Changes in genome size are considered by some authors as major determinants of the enormous morphological diversity among invertebrates and, at least for turbellarian flatworms and copepod crustaceans, a significant positive relationship between genome size and body size has been observed (Gregory et al. 2000). For G. tigrina and G. schubarti, however, this relationship seems to be inverse, as individuals with 3n cells (either triploids or mixoploids) tend to be smaller than diploid ones and planarians with 16 chromosomes (2n individuals of G. tigrina) are smaller than those with just eight (2n individuals of G. schubarti).

The origin of triploid cells or individuals may be explained by events involving either mitotic (Oki et al. 1981; D'Souza et al. 2004) or meiotic (Thorne & Sheldon 1991) non-disjunctions. Within mixoploid individuals of G. schubarti, the observation of variable 2n:3n cell rates, which can be even altered in response to environmental conditions including food abundance (Knakievicz T & Ferreira HB, unpubl. data), is suggestive of mitotic non-disjunctions leading to 3n cells generation. On the other hand, we have never observed the sexual generation of triploid individuals from sexually reproducing couples in either G. tigrina or G. schubarti, which suggests that events of meiotic non-disjunction are rare. Triploid planarians could also be generated by successive events of fissiparity involving a lineage of 2n/3n individuals with decreasing 2n:3n ratios. Within such a lineage, individuals with a high proportion of 3n cells might be able to generate 3n individuals within a few generations of fissiparity, a hypothesis that could be investigated using G. schubarti as a model.

It is interesting to speculate on the advantage of simultaneously maintaining 2n, 3n, and 2n/3n individuals within a natural population, as observed for native American populations of G. schubarti (this work), some European populations of G. tigrina (Ribas et al. 1989), and some Japanese populations of Dugesia japonica ICHIKANA & KAWAKATSU 1964 and D. ryukyuensis KANAKATSU 1976 (Tamura et al. 1995). This may be related to different degrees of adaptation of each karyotype (for 2n or 3n individuals) or particular 2n:3n cell rates (for mixoploid individuals) to their environment. For natural populations of G. schubarti, including those sampled to generate GsPop1, 2, and 3 populations and followed over several years (Knakievicz T & Erdtmann B, unpubl. data), it was observed that mixoploid individuals are more frequent in warmer seasons and usually die in temperatures ≤16°C. Therefore, it seems that these 2n/3n specimens can constantly reemerge within local populations, for example after a cold season.

Among metazoans, sexual reproduction is dominant, while asexual reproduction is rare (Welch & Meselson 2000). Between these two extremes, there are species that present both reproductive modes, alternatively using one or the other as a function of developmental or environmental factors (Normark et al. 2003). For both G. tigrina and G. schubarti, the reproductive mode shows correlation with the ploidy level, with sexual reproduction apparently being exclusive for diploid individuals, as observed for other planarians (Hoshi et al. 2003; D'Souza et al. 2004). This is especially clear for G. tigrina, with the 2n GtPop1 population being sexual while the 3n GtPop2 population is fissiparous. For G. schubarti, the GsPop2 and GsPop3 populations, both only with

triploid and mixoploid individuals, are fissiparous, while GsPop1 presented both sexual/parthenogenetic and fission reproduction, possibly associated to 2n and 2n/3n or 3n subpopulations, respectively.

Despite the positive correlation between a 2n karyotype and sexuality observed for the populations of G. tigrina and G. schubarti analyzed, it is possible that ploidy is not the sole determinant of the reproductive mode for these species; they may also be influenced by other, more specific genetic factors, such as those under investigation for D. ryukyuensis (Kobayashi et al. 1999a,b; Kobayashi & Hoshi 2002).

The acquisition of asexuality by sexual populations, or vice versa, has been demonstrated for planarians (Weinzierl et al. 1999; D'Souza et al. 2004). However, the mechanisms of switching reproductive modes are so far unknown. In the GsPop1 population of G. schubarti, the possible generation of 2n/3n fissiparous individuals from cocoons produced by 2n sexual parents would represent a switch in reproductive mode. On the other hand, the offspring of fissiparous couples maintained the asexual reproductive mode either under the standard culture conditions or with a ''sexual-inducing diet,'' suggesting that a switch to a sexual reproductive mode is rare and/or depends on other factors, either genetic or environmental.

In the GsPop1 population of G. schubarti, a stable coexistence of sexual and asexual (fissiparous and, possibly, parthenogenetic) individuals has been observed. The observation that 2n individuals produce 2n and 2n/3n offspring is suggestive of the possible simultaneous occurrence of sexual outcrossing and parthenogenesis. The parthenogenesis may be sperm dependent, as observed for planarians of Schmidtea polychroa SCHMIDT 1861 (D'Souza et al. 2004), which poses both evolutionary and ecological questions (Beukeboon & Vrijenhoek 1998). Ecologically, the maintenance of sperm-dependent parthenogenetic individuals (pseudogamy) in the presence of sexual individuals presents aspects of both parasitism and competition (Schley et al. 2004). It leads to sperm waste by sexual individuals, and the otherwise similar pseudogamous and sexual forms compete intensively for resources.

Evolutionarily, most models of the origin of sex consider obligate sexual and obligate asexual organisms (D'Souza et al. 2004). However, some studies show that a stable, mixed mode of reproduction (occasional sex) is viable, and different theories have been proposed to explain its maintenance, including in planarians (Michiels et al. 2001; D'Souza et al. 2004). These theories are based on the disadvantage of asexual individuals due to the rapid adaptation of parasites to common clones, on the loss of fitness of asexual clonal genomes due to the accumulation of deleterious mutations, on a trade-off between growth capacity and interspecific competitive impacts, or on a superior intrinsic capacity of pseudogamous triploids to exploit a given resource in relation to sexual diploids. Therefore, along with S. polychroa, G. schubarti would be a good model organism to experimentally address the validity of these theories.

Individuals of G. tigrina are more prolific than those of G. schubarti. In sexual reproduction, individuals of G. tigrina are **B15x** more prolific, considering either number of cocoons (fecundity) or number of hatchlings (fertility) produced. In addition, both cocoons and hatchlings of G. tigrina presented a higher viability than those of G. schubarti. However, capacity for asexual reproduction was similar in G. tigrina (0.205 fission per planarian per week) and asexual populations of G. schubarti (0.235 fission per planarian per week for GsPop2 and 0.168 for GsPop3).

Asexual reproduction is often associated with regeneration potential (Alvarado 2000; Reuter & Kreshchenko 2004), but the GtPop1 population of G. tigrina, with no fissiparity, presented the same regeneration capacity as GsPop2 and GsPop3 populations of G. schubarti. Only the GsPop1 population of G. schubarti differed significantly in regeneration capacity, compared with both populations of G. tigrina and to other populations of G. schubarti, approximately doubling the time for full-head regeneration. The reasons for the lower regeneration capacity of GsPop1 remain to be investigated, because it is apparently correlated with ploidy or fissiparity indices (Table 2).

It has been stated that environmental factors may influence the dynamics of planarian reproduction (Reynoldson et al. 1965; Reynoldson 1981). Sexual reproduction parameters in G. tigrina were not significantly affected by changes in population density, salt concentration, or aeration. Sexual/parthenogenetic and fissiparous reproductive parameters in G. schubarti, however, were clearly altered by these factors. Higher population densities progressively decreased sexual/parthenogenetic reproductive efficiency, with a lethal effect in a density of 100 individuals $L^{-1}$. This negative crowding effect on the numbers of deposited cocoons was also observed in Dugesia dorotocephala WOODWORTH 1897 (Kostelecky et al. 1989). Interestingly, high salt (3x ) and aeration both induced the occurrence of fission reproduction in G. schubarti, which was not observed in the control cultures. This suggests that, at least to a limited degree, these environmental

factors could determine a reproductive mode switch in G. schubarti, stimulating a fissiparous reproductive mode that, in our standard culture conditions, would be partially or totally inactive.

An alternative egg-yolk diet negatively affected both G. tigrina and G. schubarti, but to a different extent. While in G. schubarti this diet was 100% lethal and did not allow reproduction, individuals of G. tigrina fed with egg yolk still presented a low fecundity rate (2.81%) and a survival rate of B50% after 12 weeks, as was observed in D. dorotocephala (Kostelecky et al. 1989). Future toxicological studies may identify the specific factors in this diet that cause these affects.

Acknowledgments. This work was supported by Funda@ao de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and by Conselho Nacional de Desenvolvimento Cientıfico e Tecnologico (CNPq). T.K. is a recipient of a CNPq predoctoral fellowship. S.M.V. was a recipient of an FAPERGS scholarship. We thank Dr. G. Chemale and Dr. C.V. Bizarro for the critical reading of this manuscript.

## References

Alvarado AS 2000. Regeneration in the metazoans: why does it happen? BioEssays 22: 578–590.

Baguna J, Salo E, & Auladell C 1989. Regeneration and pattern formation in planarians. III. Evidence that neoblasts are totipotent stem cells and the source of blastema cells. Development 107: 77–86.

Benazzi M 1981. Reproductive biology of Dugesia sanchezi, a fresh-water planarian from Chile. Hydrobiologia 84: 163–165.

Benazzi MG & Benazzi-Lentati G 1976. Platyhelminthes. Animal Cytogenetics, Series vol. 1, John B, ed., Gebruder Borntraeger, Berlin.

Beukeboon LW & Vrijenhoek RC 1998. Evolutionary genetics and ecology of sperm-dependent parthenogenesis. J. Evol. Biol. 11: 755–782.

Cabridenc R 1979. Dureza (teores em Sais Minerais). In: Les Bioassais en Ecolotoxicologie. Institut National de Recherche Chimique Appliquee (IRCHA). IV, Sao Paulo. p. 3.

Doncaster CP, Graeme EP, & Cox SJ 2003. Dynamics of regional coexistence for more or less equal competitors. J. Anim. Ecol. 72: 116–126.

Doncaster CP, Pound GE, & Cox SJ 2000. The ecological cost of sex. Nature 404: 281–285.

D'Souza TG, Storhas M, Schuenburg H, Beukeboom LW, & Michiels NK 2004. Ocasional sex in an 'asexual' polyploid hermaphrodite. Proc. Biol. Sci. 271: 1001–1007.

Gee H, Pickavance JR, & Young JO 1998. A comparative study of the population biology of the American immigrant triclad Dugesia tigrina (Girard) in two British lakes. Hydrobiologia 361: 135–143.

Gregory TR 2002. Genome size and developmental complexity. Genetica 115: 131–146.

HRF 2003. Variation across amphibian species in the size of the nuclear genome supports a pluralistic, hierarchical approach to the C-value enigma. Biol. J. Linn. Soc. 79: 329–339.

Gregory TR, Hebert PN, & Kolasa J 2000. Evolutionary implications of the relationship between genome size and body size in fatworms and copepods. Heredity 84: 201–208.

Guecheva TN, Henriques JAP, & Erdtmann B 2001. Genotoxic effects of copper sulphate in freshwater planarian in vivo. Studied with the single-cell gel test (comet assay). Mutat. Res. 497: 19–27.

Guecheva TN, Erdtmann B, Benfato MS, & Henriques JAP 2003. Stress protein response and catalase activity in freshwater planarian Dugesia (Girardia) schubarti exposed to copper. Ecotoxicol. Environ. Safety 56: 351–357.

Hase S, Kobayashi K, Koyanagi R, Hoshi M, & Matsumoto M 2003. Transcriptional pattern of a novel gene expressed specifically after the point-of-no-return during sexualization in planaria. Dev. Genes Evol. 212: 585–592.

Hauser J 1987. Sexualization of Dugesia anderlani by feeding. Acta Biol. Leopoldensia 9: 111–128.

Hoshi M, Kobayashi K, Arioka S, Hase S, & Matasumoto M 2003. Switch from asexual to sexual reproduction in the planarian Dugesia ryukyuensis. Integr. Comp. Biol. 43: 242–246.

Kawakatsu M, Oki I, Tamura S, Yamayoshi T, Hauser J, & Friedrich SMG 1981. Morphological, karyological and taxonomic studies of freshwater planarians from South Brazil. II Dugesia tigrina (Girard. 1850) (Turbellaria. Tricladida. Paludicola). Bull. Fuji Women's Coll. 19: 113–136.

Kawakatsu M, Hauser J, Friedrich SMG, & Lima OS. 1982. Morphological, karyological and taxonomic studies of freshwater planarians from South Brazil. III. Dugesia tigrina (Girard. 1850) Dugesia schubarti (Marcus 1946) from the vicinity of Sao Carlos, estado de Sao Paulo (Turbellaria. Tricladida. Paludicola). Bull. Fuji Women's Coll. 20: 73–90.

Kawakatsu M, Oki I, Tamura S, Yamayoshi T, Hauser J, & Friedrich SMG 1984. Morphological, karyological and taxonomic studies of freshwater planarians from South Brazil. VI. Dugesia schubarti (Marcus 1946) from the vicinity of Sao Leopoldo, estado de Rio Grande do Sul (Turbellaria. Tricladida. Paludicola). Bull. Fuji Women's Coll. 22: 45–62.

Kawakatsu M, Hauser J, & Friedrich SMG 1985. Morphological, karyological and taxonomic studies of freshwater planarians from South Brazil. VII. Supplementary notes on Dugesia schubarti. Marcus. 1946 from the vicinity of Sao Leopoldo, estado de Rio Grande do Sul. Annot. Zool. Japon. 23: 101–109.

Kobayashi K & Hoshi M 2002. Switching from asexual to sexual reproduction in the planarian Dugesia ryukyuensis: change of the fissiparous capacity along with the sexualizing process. Zool. Sci. 19: 661–666.

Kobayashi K, Koyanagi R, Matsumoto M, Cabrera JP, & Hoshi M 1999a. Switching from asexual to sexual reproduction in the planarian Dugesia ryukyuensis: bioassay system and basic description of sexualizing process. Zool. Sci. (Tokyo) 16: 291–298.

Kobayashi K, Koyanagi R, Matsumoto M, & Hoshi M 1999b. Switching from asexual to sexual reproduction in the planarian Dugesia ryukyuensis. Invertebr. Reprod. Dev. 36: 153–158.

Kostelecky J, Elliott B, & Schaeffer DJ 1989. Planarians in toxicology. I. Physiology of sexual-only Dugesia dorotocephala: effects of diet and population density on adult weight and cocoon production. Ecotoxicol. Environ. Safety 18: 286–295.

Lamatsch DK, Sharbel TF, Martin R, & Bock C 1998. A drop technique for flatworm chromosome preparation for light microscopy and high-resolution scanning electron microscopy. Chromosome Res. 6: 654–656.

Marcus E 1946. Sobre Turbellaria brasileiros. Boletins da Faculdade de Filosofia, Ciencias e Letras de Sao Paulo. Zoologia 11: 5–252.

Michiels NK, Beukeboom LW, Pongratz N, & Zeitlinger J 2001. Parthenogenetic flatworms have more symbionts than their coexisting, sexual conspecifics but does this support the Red Queen? J. Evol. Biol. 14: 110–119.

Normark BB., Judson OP., & Moran NA 2003. Genomic signatures of ancient asexual lineages. Biol. J. Linn. Soc. 79: 69–84.

Oki I, Tamura S, Yamayoshi T, & Kawakatsu M 1981. Karyological and taxonomic studies of Dugesia japonica Ichikawa et Kawakatsu in the Far East. Hydrobiologia 84: 53–68.

Peter R, Ladurner P, & Rieger RM 2001. The role of stem cell strategies in coping with environmental stress and choosing between alternative reproductive modes: turbellaria rely on a single cell type to maintain individual life and propagate species. Marine Ecol. 22: 35–38.

Reuter M & Kreshchenko N 2004. Flatworm asexual multiplication implicates stem cells and regeneration. Can. J. Zool. 82: 334–356.

Reynoldson TB 1981. The ecology of the Turbellaria with especial reference to the freshwater triclads. Hydrobiologia 84: 87–90.

Reynoldson TB, Young JO, & Taylor M 1965. The effect of temperature on the life-cycle of four species of lake-dwelling triclads. J. Anim. Escol. 34: 23–43.

Ribas M, Pala M, & Baguna J 1989. Morphological and biochemical variation in populations of Dugesia (G.) tigrina (Turbellaria, Tricladida, Paludicola) from the western Mediterranean: biogeographical and taxonomic implications. J. Zool. London 218: 609–626.

Rieger RM 1998. 100 years of research on 'Turbellaria'. Hydrobiologia 383: 1–27.

Rivera VR & Perich MJ 1994. Effects of water quality on survival and reproduction of four species of planarian (Turbellaria: Tricladida). Invertebr. Reprod. Dev. 25: 1–7.

Schley D, Doncaster CP, & Sluckin T 2004. Population models of sperm-dependent parthenogenesis. J. Theor. Biol. 229: 559–572.

Stohler RA, Curtis J, & Minchella DJ 2004. A comparison of microsatellite polymorphism and heterozygosity among field and laboratory populations of Schistosoma mansoni. Int. J. Parasitol. 34: 595–601.

Tamura S, Oki I, & Kawakatsu M 1995. A review of chromosomal variation in Dugesia japonica and D. ryukyuensis in the Far East. Hydrobiologia 305: 79–84.

Tamura S, Yamamoto K, Takai M, Oki I, & Kawakatsu M 1998. Karyology and biogeography of Dugesia japonica and Dugesia ryukyuensis in Kyushu, Southern Japan. Hydrobiologia 383: 321–327.

Thorne MH & Sheldon BL 1991. Cytological evidence of maternal meiotic errors in a line of chickens with a high incidence of triploidy. Cytogenet. Cell Genet. 57: 206–210.

Vreys C, Craim J, Hamilton S, Williamson S, & Sfeffanie N 2002. Evidence for unconditional sperm transfer and sperm-dependent parthenogenesis in a hermaphroditic flatworm (Girardia tigrina) with fissipary. J. Zool. (Lond) 257: 43–52.

Weinzierl RP, Schmidt P, & Michiels NK 1999. High fecundity and low fertility in parthenogenetic planarians. Invertebr. Biol. 118: 87–94.

Welch DM & Meselson M 2000. Evidence for the evolution of bdelloid rotifers without sexual reproduction or genetic exchange. Science 288: 1211–1215.

Zar JH. 1999. Biostatistical Analysis. Prentice-Hall, New Jersey. 267 pp.

## 3.2 *Capítulo II* - Planarian neoblast micronucleus assay for mutagenicity evaluation

Tanise Knakievicz, Priscila Alves da Silveira & Henrique Bunselmeyer Ferreira.

Artigo enviado para publicação em Mutation Research-Genetic Toxicology and Environmental Mutagenesis

Elsevier Editorial System(tm) for Genetic Toxicology and Environmental Muta

Manuscript Draft

Manuscript Number:

Title: Planarian neoblast micronucleus assay for mutagenicity evaluation

Article Type: Research Paper

Keywords: micronuclei; neoblast; planarian; biomarker; mutagenesis; regeneration.

Corresponding Author: Dr. Henrique Bunselmeyer Ferreira, PhD

Corresponding Author's Institution:

First Author: Tanise Knakievicz, MSc.

Order of Authors: Tanise Knakievicz, MSc.; Priscila Alves Silveira; Henrique Bunselmeyer Ferreira, PhD

Abstract: Girardia tigrina and Girardia schubarti planarians were used to develop a neoblast-based micronucleus (MN) assay. Intact or regenerating planarians were exposed to γ-rays and MMS, and neoblast MN frequency was assessed. Exposure to the clastogens had no detectable effect on MN frequency of intact planarian neoblasts. For regenerating individuals, on the other hand, with active neoblast proliferation induced by decapitation, γ-rays doses and MMS concentrations as low as 0.5 Gy and 0.8 mM, respectively, induced a significant increase in MN frequencies. A dose-response correlation was observed when planarians were exposed to γ-rays, while, for those exposed to MMS, this correlation was limited to the lower concentrations (up to 1.6 mM), due to a MMS cytotoxic effect. After regeneration completion, MN frequencies returned to those of unexposed controls within 24 h, indicating that the neoblast MN assay for regenerating G. tigrina or G. schubarti is adequate to assess mutagenic hazard caused by acute exposure to genotoxic agents. Therefore, the standardized assay represent an interesting alternative for monitoring damages caused to freshwater organisms by potentially genotoxic environmental pollutants, with a sensitivity equivalent to that of the more expensive and laborious MN assays involving mammalian cells.

**Title**: Planarian neoblast micronucleus assay for mutagenicity evaluation

**Authors:** Tanise Knakievicz[1,2] Priscila Alves da Silveira[1] & Henrique Bunselmeyer Ferreira[1,2,3].

**Address:**

[1] Laboratório de Biologia Molecular de Cestódeos, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.

[2] Programa de Pós-Graduação em Biologia Celular e Molecular, Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil.

[3] Departamento de Biologia Molecular e Biotecnologia, Instituto de Biociências, UFRGS, Porto Alegre, RS, Brazil.

**Number of tables:** 04

**Keywords:** micronuclei, neoblast, planarian, biomarker, mutagenesis, regeneration.

**Short running title:** Planarian MN assay.

**Corresponding author:**
Dr. Henrique Bunselmeyer Ferreira
Centro de Biotecnologia. Av. Bento Gonçalves, 9500 - Prédio 43-421, sala 210
Caixa Postal 15005
91501-970 Porto Alegre, RS, Brazil.
Tel: +55 51 3316-7768/7769 Fax: +55 51 3316-7309 E-mail:
henrique@cbiot.ufrgs.br**Abstract**

*Girardia tigrina* and *Girardia schubarti* planarians were used to develop a neoblast-based micronucleus (MN) assay. Intact or regenerating planarians were exposed to γ-rays and MMS, and neoblast MN frequency was assessed. Exposure to the clastogens had no detectable effect on MN frequency of intact planarian neoblasts. For regenerating individuals, on the other hand, with active neoblast proliferation induced by decapitation,
γ-rays doses and MMS concentrations as low as 0.5 Gy and 0.8 mM, respectively, induced a significant increase in MN frequencies. A dose-response correlation was observed when planarians were exposed to γ-rays, while, for those exposed to MMS, this correlation was limited to the lower concentrations (up to 1.6 μM), due to a MMS cytotoxic effect. After regeneration completion, MN frequencies returned to those of unexposed controls within
24 h, indicating that the neoblast MN assay for regenerating *G. tigrina* or *G. schubarti* is adequate to assess mutagenic hazard caused by acute exposure to genotoxic agents. Therefore, the standardized assay represents an interesting alternative for monitoring damages caused to freshwater organisms by potentially genotoxic environmental pollutants, with a sensitivity equivalent to that of the more expensive and laborious MN assays involving mammalian cells.**1. Introduction**

Freshwater planarians are potentially useful test organisms for aquatic environmental pollutants, because they are commonly present in the aquatic fauna of unpolluted streams and susceptible to environmental changes. Besides, they are easy and inexpensive to culture in laboratory and offer several biomarkers that can be used to assess the effects of potentially harmful substances. Planarians have been used to evaluate the toxic [1,2,3], genotoxic [4,5], teratogenic [6,7,8,9] effects of metals, and also to evaluate the activity of anti-oxidant enzymes [10].

Neoblasts are morphologically distinct of differentiated cells [11], for being spherical and having round nuclei and relatively little cytoplasm [12]. They are the only mitotically active planarian cells, which divide to replace cells lost in the course of physiological cell turnover or following amputation [13,14]. Approximately 16-20% of neoblasts are in G2 [15] and 99% are labeled with BrdU after 3 days of continuous labeling [14], indicating that nearly all of them undergo DNA synthesis within this period. During regeneration, first there is formation of the blastema through local neoblast proliferation [16], which is followed by differentiation for the restitution of the lost body part.

Mitotic anomalies, chromosomal aberrations and decrease in mitotic activity have been described for neoblasts treated with potassium dichromate and cadmium sulfate [17,18], which suggests that these cells are appropriate for the analysis of the mutagenic potential of substances of interest in micronuclei (MN) assays. MN assays have been shown to detect clastogens with the same accuracy as chromosomal aberrations (CA) assays, and, sometimes, with higher sensitivity [19]. The MN assay can be a robust and dependable test system for mutagenicity assessment, at least for cell types or populations with well defined cell cycles, as in the case of planarian neoblasts.

Here, we describe the standardization of a planarian neoblast MN assay for mutagenicity evaluation. We have used neoblasts obtained from both intact and regenerating planarians of two species, *Girardia tigrina* and *Girardia schubarti*, previously exposed to gamma radiation (γ-rays) and methyl methanesulphonate (MMS), which are known clastogens. The standardized assay allowed the detection of mutagenic effects, in the form of quantifiable micronuclei generation, after exposure to doses as low as 0.5 Gy or 0.8 μM MMS. The number of detectable micronuclei was proportional to the radiation dose and to the MMS concentration in the ranges of 0.5 - 1.25 Gy and 0.8 - 1.6

μM, respectively. The applicability of the assay as an additional or alternative test for toxicological studies is discussed.

## 2. Materials and methods

### *2.1. Animals*

Sexual diploid (2n) *Girardia tigrina* and asexual mixoploid (2n/3n) *Girardia schubarti* populations from lotics and lentics aquatic environments, respectively, were captured in the Rio Grande do Sul State (Brazil). In the laboratory, the planarian samples were cultivated in containers with reconstituted water [20] at 20 ± 1$^{o}$C and fed weekly with bovine liver, as previously described [21].

Samples of regenerating planarians were produced by decapitation of healthy adult individuals, as described by Knakievicz *et al*. [21]. Briefly, each individual was immobilized over a block of ice and a single cut was made immediately behind the auricles. After decapitation, planarians were immediately submitted to treatment conditions.

### *2.2. MMS toxicity tests*

In toxicity tests, intact and regenerating planarians (two independent samples of 10 animals for each treatment or control) were cultured in water in which MMS (CAS no. 66-27-3, Sigma, USA) was dissolved to final concentrations ranging from 0.8 to 64 μM for 24 h in the dark. After exposure, planarians were transferred to culture water without MMS and followed for 96 h, with daily visual inspection for recording of the mortality rate, for intact animals, or the regeneration stage, for regenerating animals. The mortality rate was used for calculation of the lethal concentration for 50% of the intact planarians ($LC_{50}$),

using the Trimmed Spearman-Karber Methods (version 1.5) [22], and the regeneration state was used for the calculation of the inhibition concentration that reduces 50% of the eyes or auricles regeneration ($IC_{50}$) of decapitated planarians, using the Inhibition Concentration (ICp) Approach (version 2.0) software [23]. During and after exposure planarians were not fed and culture water was changed daily.

*2.3. Exposure to clastogens*

In each experiment, two independent samples (n = 10 each) of intact and regenerating *G. tigrina* and *G. schubarti* planarians were exposed to sublethal gamma radiation (γ-rays) doses, according to previously established parameters [24], or MMS concentrations, established according to the toxicity tests (see above). A $^{137}Cs$ γ-ray source (Hospital de Clínicas, Porto Alegre, Brazil) was used, and after exposure (from 9,70 to 24,06 seconds according to the dose), planarians were rinsed in reconstituted water and kept in culture for 0 to 144 h, as indicated for each experiment, prior to use in the MN assays. Exposure to MMS was performed culturing planarians for 24 h in the dark, in water in which MMS was dissolved. After exposure to MMS, planarians were rinsed in reconstituted water and kept in culture for additional 48 h prior to use in the MN assays. During and after exposure to γ-rays or MMS, planarians were not fed and culture water
was changed daily. Animals from negative control samples (two independent samples of 10 animals per experiment) were submitted to the same culture conditions, but without any exposure to either γ-rays or MMS.

*2.4. MN assays*

MN assays were performed essentially as previously described [25]. For cell isolation, planarian samples were minced in MTTP solution [12] (Nutricell, Brazil) and cell suspensions were collected with a Pasteur pipette. To each 500 µL of cell suspension, 500 µL 1% sodium citrate (ISOFAR, Brazil) was added and the sample was incubated for 5 min at room temperature, prior to fixation with the addition of 200 µL of 4% (v/v) formalin (Merck, Germany). Fixed samples were stored at 4ºC until preparation of slides. At least two slides were prepared from each planarian sample (n = 10) submitted to a given treatment or control condition.

For MN analysis, fixed cell samples were spread on microscope slides pre-coated with 0.05 % gelatin (Sigma, USA), and gradually dehydrated through an ethanol series. Cell slides were then stained with Feulgen reagent [26], fucsin (Merck, Germany), and 0.005% (w/v) fast green (Sigma, USA). The stained slides were analyzed in a Zeiss Axiophot optical microscope. Neoblasts were morphologically identified, according to their characteristic size, shape, and nucleocytoplasmic ratio [11, 12]. Each neoblast containing one or more micronuclei was scored as a MN positive cell. For any given experimental or control condition, two independent experiments were performed, each of them with two replicas (two samples of 10 planarians submitted to a given experimental or control condition). At least two slides from each replica were analysed, and, for each slide, two independent countings of at least 1000 neoblasts each were done. In all experiments, slides were coded to avoid any counting bias. The scored frequency for a given slide was the mean of the two countings and the scored frequency for a replica was the mean of the scores of two slides. The scored frequency of an experiment was the mean of two replicas and the presented MN frequency for a given experimental condition correspond to the mean of the two independent experiments.

*2.5. Statistical analysis*

MN data of exposed planarians were compared to the correspondent non-exposed controls using the Kruskal-Wallis test using SPSS® for Windows™ (http://www.spss.com), followed by the Dunn test [27]. A value of $P < 0.05$ was considered for statistical significance.

**3. Results**

Gamma ray sublethal exposure levels have been previously established for *G. schubarti* and *G. tigrina* [24] and, based on that, planarians were exposed to doses ranging from 0.50 to 1.25 Gy. In our experimental conditions, exposure to such levels of radiation has not determined any lethal effects for *G. tigrina* or *G. schubarti* (data not shown).

Toxicity tests were carried out in order to establish the MMS sublethal concentrations, exposing intact planarians for 24 h to MMS concentrations ranging from 0.8 µM to 6.4 µM. For *G. tigrina*, MMS $LC_{50}$ of 2.0 µM, in 24 h, and 1.4 µM, in 96 h, were calculated. For *G. schubarti*, the $LC_{50}$ was above 6.4 µM in 24 h and was not calculated, while a $LC_{50}$ of 3.7 µM MMS, in 96 h, was established. The MMS effects on regenerating planarians were also evaluated, for calculation of the $IC_{50}$, since exposure to MMS caused significant blastema growth retardation (data not shown), with consequent and differential retardation in the formation of both eyes and auricles. For *G. tigrina*, $IC_{50}$ in 96 h were 0.7 and 1.2 µM for eyes and auricles, respectively, while, for *G. schubarti*, they were 2.7 µM and 2.3 µM, respectively. Based on the calculated $LC_{50}$, sublethal MMS concentrations between 0.8 µM and 3.2 µM in exposure times of 24 h were used in the MN

assays with both intact and regenerating animals. Such exposure levels showed no detectable effects on regeneration rates in comparison to that of the controls within a 24 h period (data not shown).

The MN frequencies in response to exposure to sublethal γ-ray doses or MMS concentrations was then assessed in both intact and regenerating *G. tigrina* and *G. shubarti* planarians. After exposure to both clastogenic agents, MN frequencies were assessed in comparison to spontaneous MN frequencies from non exposed planarian controls. Four types of MN were observed in the analysed neoblasts (data not shown), essentially corresponding to those previously described by Nishikawa et al. [28], and no discrimination between them was done for the scoring of the overall MN frequency.

Tables 1 and 2 present the MN frequencies in intact and regenerating *G. tigrina* and *G. schubarti* planarians, respectively, after exposure to a physical clastogenic agent, γ-rays, in doses between 0.5 and 1.25 Gy. For both species, the MN frequency did not increase significantly ($P$ = 0.358 and $P$ = 0.475, respectively) for intact planarians in relation to corresponding non exposed controls. However, regenerating individuals from both species showed significantly increased MN frequencies ($P$ = 0.023 and $P$ = 0.016, respectively) in response to γ-ray exposure, with positive dose-response correlations. Comparison of the
MN frequencies in regenerating individuals between species showed that *G. tigrina* and *G. schubarti* do not differ significantly ($P$ = 0.127), suggesting similar sensitivities to the clastogenic effect of γ-rays.

The effect of MMS, a chemical clastogenic agent, on MN frequencies was then tested in *G. tigrina* and *G. schubarti* planarians. Similarly to the animals exposed to γ-rays, intact planarians exposed 24 h to MMS concentrations between 0.8 and 3.2 μM did not show significant increase in MN frequency in relation to not exposed controls (data not

shown). On the other hand, exposure of regenerating planarians to MMS concentrations in the range of 0.8 and 1.6 μM, for *G. tigrina*, and 1.2 and 1.6 μM, for *G. schubarti* resulted in an increase in MN frequency in comparison to non exposed controls (Table 3). Exposure to MMS concentrations above these ranges led to MN frequencies not significantly higher than those of the non exposed controls for both species. No significant differences in MN frequency in response to MMS was observed between *G. tigrina* and *G. schubarti* regenerants exposed to the same MMS concentrations ($\chi^2 = 3.10$, $P = 0{,}078$).

Upon exposure to clastogens (either γ-rays or MMS), it was observed that, in a period of up to 72 to 96 h, there was an increase in MN frequency, but, after that, MN progressively decreased, returning to the basal MN frequency of not exposed controls (data not shown). To better assess this phenomenon, the MN frequency variation for regenerating *G. tigrina* and *G. schubarti* was determined as a function of time after exposure to a 1 Gy dose of γ-rays (Table 4). As expected (see Tables 1 and 2), exposure to the 1 Gy γ-ray dose led to a significant increase in MN frequency within 96 h, but, after 120 h, the MN frequencies did not significantly (Table 4) differ from those of controls (scored immediately after exposure) for both species. This MN frequency reduction after 120 h of exposure may be attributed to differentiation and/or apoptosis of the generated micronucleated neoblasts.

**4. Discussion**

MN assays for the determination of micronucleated cell frequency within samples of proliferating cells provide a simple and fast method to assessing mutagenic hazard. MN assays are conventionally based on the formation of micronucleated polychromatic erythrocytes (MNPCE) in rodent bone marrow

[http://www.cfsan.fda.gov/~redbook/redivc1d.html], and they have been widely used to evaluate the genotoxicity of different chemical and physical agents [29,30,31]. MN assays allow consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes, which may vary among species, among tissues and among genetic endpoints [32,33]. Therefore, several alternative MN assay systems, involving different organisms, from invertebrate and vertebrate animals to plants, have been developed [34,35,36]. A wide variety of organisms amenable to MN assays is important to allow a more comprehensive analysis of potential damages caused to the biota of a given environment by genotoxic pollutants.

The use of MN assays for aquatic invertebrates is relatively new, and the range of species for which they were standardized is restricted to mollusks and crustaceans [37,38,39]. In this context, planarians are potentially interesting organisms to be used in MN assays, as representatives of the invertebrate fauna of lentic and lotic environments whose value as bioindicators has been demonstrated [4,5,10,21].

Planarian neoblasts present a good potential to be used in MN assays, as they are the only proliferating cell population of these organisms and are easily distinguishable from differentiated cells in light microscopy. For standardization of a planarian neoblast MN assay, we have investigated the effects of two well-known clastogens, one physical (γ-rays) and one chemical (MMS), on neoblast MN frequency of two species, *G. tigrina* and *G. schubarti*. The sublethal doses established for both agents [24, this work] and used in the experiments were within ranges commonly used for validation of mammalian MN assays [28,40]. High doses (as high as 30-60 Gy) of ionizing radiation are known to quickly and specifically eliminate planarian neoblasts [41,42]. However, the γ-ray doses used in this work (0.5 to 1.25 Gy) were well below these levels, and within a range in which a dose-

response correlation was evident in the MN assays. For MMS, on the other hand, a dose- response correlation was observed only for the lower MMS concentrations (0.8-1.6 μM). Exposure to higher (2.4-3.2 μM) concentrations led to an apparent decrease in MN frequency, which is suggestive of a MMS cytotoxic effect for neoblasts. This was corroborated by the fact that regeneration in individuals exposed to higher MMS concentrations was impaired, as demonstrated by the calculated $IC_{50}$ for both species.

For both *G. tigrina* and *G. schubarti*, the increase in MN frequency upon exposure to γ-rays or MMS was restricted to neoblasts of regenerating individuals, probably due to the fact that active neoblast division (above basal levels) depends on induction, that occurs upon decapitation and is kept in high rates until complete reconstitution of lost structures, such as eyes and auricles [15,16]. In intact planarians, neoblasts divide [14,16], but in rates below the minimum necessary to generate micronucleus in a detectable level for our assay conditions.

In our experimental conditions, full head (eyes and auricles) regeneration of decapitated *G. tigrina* and *G. schubarti* took 96 h [21]. After regeneration, neoblast MN frequency of animals exposed to γ-rays progressively decreased, returning to basal levels (that of unexposed controls) within 24 h after regeneration completion. This indicated that, as expected, neoblast proliferation returned to the lower rates typical of intact planarians [16], and that the micronucleated neoblasts generated in higher rates during regeneration either differentiated [42] or died [17,18].

Regarding sensitivity, our MN assays in regenerating planarians were able to detect increase in MN frequency in response to exposures to γ-rays doses as low as 0.5 Gy and 1.0 Gy or to MMS concentrations as low as 0.8 μM and 1.2 μM, for *G. tigrina* and *G. schubarti*, respectively. Such sensitivity, was comparable to that of the more laborious

regenerating planarian neoblast CA assay [24] and similar than that of mammalian MN assays based on MNPCE [30] or cultured B lymphocytes [31,43].

In conclusion, the high proliferative neoblasts of regenerating planarians can be regarded as adequate for MN assays. Their proliferation is easily induced by decapitation, and MN frequency variation upon exposure to genotoxic agents can be assessed within 48 h to 72 h, depending on the species. Therefore a MN assay for regenerating *G. tigrina* or *G. schubarti* would be useful for monitoring damages caused by acute exposure to aquatic environmental pollutants with mutagenic potential. They represent a complement or an alternative for the more expensive and time-consuming MN assays involving mammalian cells.

**Acknowledgements**

This work was supported by Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). T. K. is a recipient of a CNPq predoctoral fellowship. P.A.S. is a recipient of a CNPq scholarship. We thank Dr. T. R. O. Freitas for the use of the Zeiss Axiophot optical microscope, and Dr. C. V. Bizarro and Dr. I. S. Schrank for the critical reading of this manuscript.

**References**

[1] D.L.C. Preza, D.H. Smith. Use of newborn *Girardia tigrina* (Girard, 1950) in acute toxicity tests, Ecotoxicol. Environ. Saf. 50 (1995) 1-3.

[2] M. Kalafatić, I. Tomaskovic. Toxic effects of aluminium in neutral acidic media on the planarian *Polycelis felina*, Biologia 54 (1999) 713-718.

[3] D. Franjevic, A. Krajna, M. Kalafatić, N. Ljubesic. Toxic effects of copper upon the planarian *Polycelis felina* (Daly), Period. Biol. 102 (2000) 283-287.

[4] T. Guecheva, J.A.P. Henriques, B. Erdtmann. Genotoxic effects of copper sulphate in freshwater planarian in vivo, studied with the single-cell gel test (comet assay), Mutation Res. 497 (2001) 19-27.

[5] D. Prá, A. H. Lau, T. Knakievicz, F. R. Carneiro, B. Erdtmann. Environmental genotoxicity assessment of an urban stream using freshwater planarians. Mutation Res. 585 (2005) 79–85.

[6] D. Franjevic, A. Krajna, M. Kalafatić, N. Ljubesic. The effects of zinc the survival and regeneration of planarian *Polycelis felina*, Biologia 55 (2000) 689-694.

[7] J.B. Best, M. Morita. Planarians as a model system for in vitro teratogenesis studies, Teratog., Carcinog. Mutagen. 2 (1982) 277-291.

[8] F. Calevro, S. Campini, C. Filippi, R. Batistoni, P. Deri, S. Bucci, M. Ragghianti, G. Mancino. Bioassays for testing effects of Al, Cr and Cd using development in the amphibian *Pleurodeles waltl* and regeneration in the planarian *Dugesia etrusca*, AEHM 2 (1999) 281-288.

[9] F. Calevro, C. Filippi, P. Deri, C. Albertosi, R. Batistoni. Toxic effects of Aluminum, Chromium and Cadmium in intact and regenerating freshwater planarians, Chemosphere 37 (1998) 651-659.

[10] T.N. Guecheva, B. Erdtmann, M.S. Benfato & J.A.P. Henriques. Stress protein response and catalase activity in freshwater planarian *Dugesia* (*Girardia*) *schubarti* exposed to copper. Ecotoxicol. Environ. Saf. 56 (2003) 351–357.

[11] I. Hori, Y. Kishida, 1998. A fine structural study of regeneration after fission in the planarian *Dugesia japonica*, Hydrobiologia 383 (1998) 131-136.

[12] W. Schürmann, R. Peter. Planarian cell culture: a comparative review of methods and an improved protocol for primary cultures of neoblasts, Belg. J. Zool. 131 (2001) 123-130.

[13] J. Baguñà, E. Saló, C. Auladell. Regeneration and pattern formation in planarian. III. Evidence that neoblasts are totipotent stem cells ans the source of blastema cells, Development 107 (1989) 77-86.

[14] P.A. Newmark, A.S. Alvarado. Bromodeoxyuridine specifically labels the regenerative stem cells of planarians, Dev. Biol. 220 (2000) 142-153.

[15] E. Saló, J Baguñà. Regeneration and patter formation in planarian. I. The pattern of mitosis in anterior and posterior regeneration in *Dugesia* (*G.*) *tigrina*, and a new proposal for blastema formation, J. Embryol. Exp. Morphol. 83 (1984) 63-80.

[16] J. Baguñà. Mitosis in the intact and regenerating planarian *Dugesia mediterranea* n.sp., II. Mitotic studies during regeneration, and a possible mechanism of blastema formation, J. Exp. Zool. 195 (1976) 65-80.

[17] M. Kalafatić, S. Tabarsak. Effects of chromium upon neoblast division in the regenerates of *Polycelis felina*, Biologia 53 (1998) 321-325.

[18] M. Kalafatić, N. Kopjar, V. Besendorfer. The impairments of neoblast division in regenerating planarian *Polycelis felina* (Daly) caused by in vitro treatment with cadmium sulfate, Toxicol. in Vitro 18 (2004) 99-107.

[19] B. Miller, F. Pötter-Lacher, A. Seelbach, H. Stopper, D. Utesch, S. Madle. Evaluation of the in vitro chromosomal aberration assay: position of the GUM working group on the in vitro micronucleus test, Mutation Res. 410 (1998) 81-116.

[20] R. Cabridenc, Dureza (Teores em Sais Minerais), in: Les bioassais en Ecolotoxicologie, IRCHA, IV, São Paulo, 1979, p. 3.

[21] T. Knakievicz, B. Erdtmann, S.M. Vieira, H.B. Ferreira. Reproduction modes and life cycle of freshwater planarians (Plathyhelminthes, Tricladida, Paludicula) from Southern Brazil, Invertebr. Biol. 125 (2006) 212-221.

[22] M.A. Hamilton, R.C.Russo, R.V. Thurston. Trimmed Spearman-Karber method for estimating median lethal concentrations, Environ. Sci. Tech. 11(1977) 714–719.

[23] T.J. Norberg-King. A Linear Interpolation Method for Sublethal Toxicity: The Inhibition Concentration (ICp) Approach, Technical Report 03-93; National Effluent Toxicity Assessment Center, Environment Research Laboratory, U.S. Environmental Protection Agency: Duluth, MN, (1993).

[24] A.H. Lau. Master's thesis. Testes de Genotoxicidade em Planárias - Análise de Aberrações Cromossômicas e Teste Cometa. Programa de Pós Graduação em Genética e Biologia Molecular - Universidade Federal do Rio Grande do Sul. Porto Alegre – RS, 1998.

[25] W. Schmid. The micronucleus test, Mutation Res. 31 (1975) 9-15.

[26] M.L.S. Mello, B.de C. Vidal. A reação de Feulgen, Ciências e Cultura, 30 (1978) 665-676.

[27] J.H. Zar. Biostatistical Analysis, 4th Ed.; Prentice Hall: Upper Saddle River, New Jersey (1999) 932 pp.

[28] T. Nishikawa, M. Haresaku, K. Adachi, M. Masuda, M. Hayashi. Study of a rat skin in vivo micronucleus test: data generated by mitomycin C and methyl metanesulfonate, Mutation Res. 444 (1999) 159-166.

[29] M. Fenech. The *in vitro* micronucleus technique, Mutation Res. 455 (2000) 81–95.

[30] J. da Silva, S.M. Herrmann, V. Heuser, W. Peres, N. Possa Marroni, J. Gonzalez- Gallego, B. Erdtmann. Evaluation of the genotoxic effect of rutin and quercetin by comet assay and micronucleus test, Food Chem. Toxicol. 40 (2002) 941–947.

[31] A. Wojck, M. Kowalska, E. Bouzk, I. Buaczaewska, G. Kobialko, N.Jorocewicz, I. Szumiel. Validation of the micronucleus-centromere assay for biological dosimetry, Genet. Mol. Biol. 23 (2000) 1083-1985.

[32] T. Cavas, N.N. Garanko, V.V. Arkhipchuk. Induction of micronucli and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate, Food Chem. Toxicol. 43 (2004) 569-574.

[33] C. Bolognesi, E. Perrone, P. Roggieri, D.M. Pampanin, A. Sciutto. Assessment of micronuclei induction in peripheral erythrocytes of fish exposed to xenobiotics under controlled conditions, Aquat. Toxicol. 78S (2006) S93–S98.

[34] T. Wolf, N.-P. Luepke. Formation of micronuclei in incubated hen's eggs as a measure of genetoxicity, Mutation Res. 349 (1997) 163-175

[35] M. Solenská, K. Micieta, M. Misik. Plant biossays for an in situ monitoring of air near an industrial area and a municipal solid waste – Zilina (Slovakia), Environ. Monit. Assess. 115 (2006) 499–508.

[36] I. Udroiu. The micronucleus test in piscine erythrocytes, Aquat. Toxicol. 79 (2006) 201-204.

[37] T. Burgeot, E. His, F. Galgani. The micronucleus assay in *Crassostrea gigas* for the detection of seawater genotoxicity, Mutation Res. 342 (1995) 125-140.

[38] E. de la Sienraa, M.A. Armientab, M.E. Gonsebatta. Potassium dichromate increases the micronucleus frequency in the crayfish *Procambarus clarkii*, Environ. Pollut. 126 (2003) 367–370.

[39] I.V.Villela, I.M. de Oliveira, J. da Silva, J.A.P. Henriques. DNA damage and repair in haemolymph cells of golden mussel (*Limnoperna f ortunei*) exposed to environmental contaminants, Mutation Res. 605 (2006) 78–86.

[40] D.Vargas, T. Johannes, S. Jainta, S. Schuster, U Schwarz-Boerger, M. Kiechle, B.P. Garcia, W. Vogel. An automated scoring procedure for the micronucleus test by image analysis. Mutagenesis 19 (2004) 391-397.

[41] P.W. Reddien, A.S. Alvarado. Fundamentals of planarian regeneration, Annu. Rev. Cell Dev. Biol. 20 (2004) 725-757.

[42] P.W. Reddien, N.J. Oviedo, J.R. Jennings, J.C. Jenkin, A.S. Alvarado. SMEDWI-2 is a PIWI-like protein that regulates planarian stem cells, Science 310 (2005) 1327-1330.

[43] J. da Silva, T.R.O. de Freitas, V. Heuser, J.R. Marinho, F. Bittencourt, C.T.S. Cerski, L.M. Kliemann, B. Erdtmann. Effects of chronic exposure to coal in wild rodents (*Ctenomys torquatus*) evaluated by multiple methods and tissues, Mutation Res. 470 (2000) 39–51.

Table 1. Dose-response relationship between MN frequency and γ-rays exposure for intact and regenerating *G. tigrina* planarians. Presented MN frequencies correspond to means of two independent experiments, each of them with at least two replicas.

| Gamma radiation (Gy) | Intact | | | Regenerating | | |
|---|---|---|---|---|---|---|
| | No. of scored neoblasts | MN ‰ Mean (SE) | CV % | No. of scored neoblasts | MN ‰ Mean (SE)** | CV % |
| 0 | 2000 | 2.00 (1.00) | 70.7 | 5042 | 1.39 (0.51)[a] | 81.7 |
| 0.50 | 4000 | 4.67 (1.45) | 53.9 | 5012 | 3.20 (0.66)[b] | 46.3 |
| 1.00 | 4000 | 4.50 (1.50) | 47.1 | 5012 | 4.19 (0.59)[b;c] | 31.3 |
| 1.25 | 2000 | 6.00 (1.00) | 23.6 | 2000 | 6.00 (1.00)[c] | 23.6 |
| Kruskal-Walls Test* | © 12,000 | $\chi^2 = 3.25$ $P = 0.358$ | | © 17,066 | $\chi^2 = 9.49$ $P = 0.023$ | |

(*) P<0.05 in the Kruskal-Wall test indicates means that do not differ significantly. In case they differ (P>0.05), they were individually compared to the control by the Dunn test.

(**) Means indicated by same letter do not differ significantly (Dunn Test, $\alpha = 0.05$).

Table 2. Dose-response relationship between MN frequency and γ-radiation exposure for intact and regenerating *G. schubarti* planarians. Presented MN frequencies correspond to means of two independent experiments, each of them with at least two replicas.

| Gamma Radiation (Gy) | Intact | | | Regenerating | | |
|---|---|---|---|---|---|---|
| | No. of scored neoblasts | MN ‰ Mean (SE) | CV % | No. of scored neoblasts | MN ‰ Mean (SE)** | CV % |
| 0 | 2000 | 0.50 (0.50) | 141.4 | 2440 | 0.83 (0.05)[a] | 51.6 |
| 0.50 | 4000 | 2.00 (0.57) | 57.1 | 2330 | 6.10 (1.30)[a;b] | 30.3 |
| 1.00 | 6000 | 1.50 (0.64) | 86.1 | 3430 | 8.21 (1.14)[b] | 24.7 |
| 1.25 | 5000 | 1.62 (0.55) | 68.2 | 5900 | 11.65 (1.72)[b] | 33.1 |
| Kruskal-Walls Test* | 17,000 | $\chi^2 = 2.50$ $P = 0.475$ | | 14,100 | $\chi^2 = 10.26$ $P = 0.016$ | |

(*) P<0.05 in the Kruskal-Wall test indicates means that do not differ significantly. In case they differ (P>0.05), they were individually compared to the control by the Dunn test.

(**) Means indicated by the same letter do not differ significantly (Dunn Test, $\alpha = 0.05$).

Presented data correspond to means of two independent experiments each them with at least two replicas.

Table 3. Concentration-response relationship between MN frequency and MMS exposure for regenerating *G. tigrina* and *G. schubarti* planarians. Presented MN frequencies correspond to means of two independent experiments, each of them with at least two replicas.

| MMS (∝M) | *G. tigrina* | | | *G. schubarti* | | |
|---|---|---|---|---|---|---|
| | No. of scored neoblasts | MN ‰ Mean (SE) | CV % | No. of scored neoblasts | MN ‰ Mean (SE)** | CV % |
| 0.0 | 9232 | 3.70 (0.40)[a] | 20.9 | 8095 | 1.73 (0.47)[a] | 68.7 |
| 0.8 | 7965 | 6.26 (0.31)[b] | 9.8 | 8136 | 2.47 (0.46)[a;b] | 37.5 |
| 1.2 | 2025 | 9.89 (1.11)[b] | 15.8 | 7019 | 6.70 (1.82)[b] | 47.2 |
| 1.6 | 4170 | 6.93 (1.58)[b;c] | 32.3 | 8120 | 5.12 (1.49)[b] | 50.5 |
| 2.4 | 4025 | 4.22 (0.25)[a;c] | 8.5 | 7219 | 2.70 (0.61)[a;b] | 45.5 |
| 3.2 | 4089 | 3.64 (1.14)[a;c] | 44.4 | 4022 | 1.24 (0.74)[a] | 84.6 |
| Kruskal-Walls Test* | © 29,448 | $\chi^2 = 11.87$ $P = 0.037$ | | © 41,611 | $\chi^2 = 11.09$ $P = 0.050$ | |

(*) P<0.05 in the Kruskal-Wall test indicates means that do not differ significantly. In case they differ (P>0.05), they were individually compared to the control by the Dunn test.

(**) Means indicated by the same letter do not differ significantly (Dunn Test, $\alpha = 0.05$).

Table 4. MN frequency of regenerating *G. tigrina* and *G. schubarti* planarians in different times (from 0 to 144 h) after exposure to a 1 Gy γ-ray dose. Presented MN frequencies correspond to means of two independent experiments, each of them with at least two replicas.

| Hours after exposure | *G. tigrina* | | | *G. schubarti* | | |
|---|---|---|---|---|---|---|
| | No. of scored neoblasts | MN ‰ Mean (SE)** | CV % | No. of scored neoblasts | MN ‰ Mean (SE)** | CV % |
| 0 | 4086 | 2.00 (0.14)[a] | 0.01 | 4029 | 1.25 (0.59)[a] | 0.28 |
| 24 | 4014 | 4.24 (0.61)[a] | 0.09 | 4055 | 3.14 (0.49)[a] | 0.09 |
| 48 | 4041 | 12.11 (1.52)[b] | 0.19 | 4052 | 4.94 (1.19)[a] | 0.29 |
| 72 | 4072 | 11.06 (1.07)[b] | 0.10 | 4008 | 8.73 (1.02)[b] | 0.12 |
| 96 | 4054 | 11.36 (1.25)[b] | 0.14 | 4091 | 11.00 (0.15)[b] | 0.00 |
| 120 | 4009 | 5.99 (0.14)[a] | 0.00 | 4058 | 6.17 (1.96)[a] | 0.63 |
| 144 | 4087 | 5.12 (0.94)[a] | 0.17 | 4044 | 3.70 (1.00)[a] | 0.27 |
| Kruskal-Walls Test* | © 28,363 | $\chi^2 = 22.480$ $P = 0.001$ | | © 28,337 | $\chi^2 = 17.946$ $P = 0.006$ | |

(*) P<0.05 in the Kruskal-Wall test indicates means that do not differ significantly. In case they differ (P>0.05), they were individually compared to the control (0 hour) by the Dunn test.

(**) Means indicated by the same letter do not differ significantly (Dunn Test, $\alpha = 0.05$).

## *3.3 Capílulo III* - Evaluation of copper effects upon *G. tigrina* freshwater planarians *based on a set of biomarkers*

Tanise Knakievicz & Henrique Bunselmeyer Ferreira.

Artigo publicado em Chemosphere

Elsevier Editorial System(tm) for Chemosphere

Manuscript Draft

Manuscript Number: CHEM11215

Title: Evaluation of copper effects upon Girardia tigrina freshwater planarians based on a set of biomarkers.

Article Type: Research Paper

Section/Category: Environmental Toxicology and Risk Assessment

Keywords: heavy metals; biomarkers; teratogenesis; micronucleus assay; regeneration; reproduction.

Corresponding Author: Dr. Henrique Bunselmeyer Ferreira, Ph.D.

Corresponding Author's Institution: Universidade Federal do Rio Grande do Sul

First Author: Tanise Knakievicz, M.Sc.

Order of Authors: Tanise Knakievicz, M.Sc.; Henrique Bunselmeyer Ferreira, Ph.D.

Manuscript Region of Origin:

Abstract: Copper (Cu+2) is a common environmental contaminant, which is particularly toxic to living organisms when in high concentrations. To monitor environmental contaminantion by Cu+2 and other heavy metals, well characterized bioindicator organisms and standardized assays are needed. As a first step toward this end, we have analysed Cu+2 effects upon Girardia tigrina freshwater planarians, based on the assessment of mobility, regeneration performance, MN frequency in regenerating animals, and reproductive performance. These four biomarkers provided complementary information on Cu+2 toxicity, teratogenicity, mutagenicity and chronicle effects, respectively. The LC50 was calculated for newborn, adult and regenerating planarians, and values of 12 ± 0.02 mg*L-1, 42 ± 0.08 mg*L-1, 48 ± 0.13 mg*L-1, respectively, were obtained after 96 h of exposure. Mobility, for intact adults, and time of regeneration and MN frequency, for regenerating animals, were significantly affected by Cu+2 concentrations as low as 0.10 mg*L-1. MN assay for regenerating G. tigrina neoblasts showed higher sensitivities than MN assays performed with other bioindicator freshwater organisms, such as moluscs or fish. Chronic exposure effects were clearly evidenced by assessment of reproductive performance, with significant reduction in fecundity and fertility rates upon exposure to Cu+2 concentrations as low as 0.05 mg*L-1. Therefore, G. tigrina can be regarded as a useful bioindicator species for the detection and evaluation of Cu+2 effects upon freshwater invertebrates, allowing insights on the effects of Cu+2 (an possibly other heavy metals) in a freshwater environment.

assay for regenerating G. tigrina neoblasts showed higher sensitivities than MN assays performed with other bioindicator freshwater organisms, such as moluscs or fish. Chronic exposure effects were clearly evidenced by assessment of reproductive performance, with significant reduction in fecundity and fertility rates upon exposure to Cu+2 concentrations as low as 0.05 mg*L-1. Therefore, G. tigrina can be regarded as a useful bioindicator species for the detection and evaluation of Cu+2 effects upon freshwater invertebrates, allowing insights on the effects of Cu+2 (an possibly other heavy metals) in a freshwater environment.

**Title:** Evaluation of copper effects upon *Girardia tigrina* freshwater planarians based on a set of biomarkers.

**Authors:** Tanise Knakievicz[1] & Henrique Bunselmeyer Ferreira[1,2,*].

**Affiliation:**

[1]Laboratório de Biologia Molecular de Cestódeos, and Programa de Pós-Graduação em Biologia Celular e Molecular, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.

[2]Departamento de Biologia Molecular e Biotecnologia, Instituto de Biociências, UFRGS, Porto Alegre, RS, Brazil.

***Corresponding author:**

Dr. Henrique B. Ferreira

Centro de Biotecnologia. Av. Bento Gonçalves, 9500 - Prédio 43421, salas 204 e 206

Caixa Postal 15005 CEP: 91501-970 Porto Alegre, RS, Brasil.

Tel: +55 51 3308-6070 - Fax: +55 51 3308-7309. E-mail: henrique@cbiot.ufrgs.br

**Abstract**

Copper ($Cu^{+2}$) is a common environmental contaminant, which is particularly toxic to living organisms when in high concentrations. To monitor environmental contaminantion by $Cu^{+2}$ and other heavy metals, well characterized bioindicator organisms and standardized assays are needed. As a first step toward this end, we have analysed $Cu^{+2}$ effects upon *Girardia tigrina* freshwater planarians, based on the assessment of mobility, regeneration performance, MN frequency in regenerating animals, and reproductive performance. These four biomarkers provided complementary information on $Cu^{+2}$ toxicity, teratogenicity, mutagenicity and chronicle effects, respectively. The $LC_{50}$ was calculated for newborn, adult and regenerating planarians, and values of 12 ± 0.02 $mg{\cdot}L^{-1}$, 42 ± 0.08 $mg{\cdot}L^{-1}$, 48 ± 0.13 $mg{\cdot}L^{-1}$, respectively, were obtained after 96 h of exposure. Mobility, for intact adults, and time of regeneration and MN frequency, for regenerating animals, were significantly affected by $Cu^{+2}$ concentrations as low as 0.10 $mg{\cdot}L^{-1}$. MN assay for regenerating *G. tigrina* neoblasts showed higher sensitivities than MN assays performed with other bioindicator freshwater organisms, such as moluscs or fish. Chronic exposure effects were clearly evidenced by assessment of reproductive performance, with significant reduction in fecundity and fertility rates upon exposure to $Cu^{+2}$ concentrations as low as 0.05 $mg{\cdot}L^{-1}$. Therefore, *G. tigrina* can be regarded as a useful bioindicator species for the detection and evaluation of $Cu^{+2}$ effects upon freshwater invertebrates, allowing insights on the effects of $Cu^{+2}$ (an possibly other heavy metals) in a freshwater environment.

**Keywords:** heavy metals; biomarkers; teratogenesis; micronucleus assay; regeneration; reproduction.

## 1. Introduction

Recent studies have suggested that the biodiversity of freshwater ecosystems is being reduced at a fast pace worldwide, and that this reduction is even more accentuated than that observed in terrestrial ecosystems impacted by the anthropic action (Sala et al., 2000). The industrial activity mobilizes and disperses metals in freshwater systems at a rate higher than that expected from natural processes, causing an especially important pollution problem, since both essential and non-essential heavy metals are highly stable and persistent in the environment. Although heavy metals usually become pollutants in the form of chemically simple molecules, their mechanisms of action in biological systems are complex and need to be better studied (Baker-Austin et al., 2005; Costa, 1991). Within this context, studies assessing the genotoxic and teratogenic effects of essential heavy metals or their compounds are of great importance, especially those involving aquatic environments, where they tend to accumulate.

The copper ($Cu^{2+}$) is an essential micronutrient, but is particularly toxic to living organisms when in high concentrations (Vargas et al., 2001). It has been commonly related to water pollution, since copper compounds have been used, for instance, in sewage treatment (Bhadra, 1992) or as antifungics in agriculture (Sonmez et al., 2006).

The use of aquatic organisms as biological sentinels has proven to be useful for environmental monitoring (Bebianno et al., 2004; Hutchinson et al., 1997). Natural occurring endemic species are the organisms of choice, and, for any given monitoring species, it is interesting that its use viabilizes the evaluation of several biological responses that deviate from normality upon exposure to a pollutant. Such biochemical, cellular and/or

physiological parameters describe the concept of biomarkers (Nascimento et al., 2006), which can be utilized as diagnostic screening tools for environmental monitoring.

Among the organisms proposed for monitoring, freshwater planarians present the advantages of being particularly susceptible to pollutants and easy and inexpensive to culture in laboratory, with minimum requirements in terms of infrastructure. Besides, it has been shown that pollutant agents, like heavy metals, cause morphological, locomotive and behavioral alterations in planarians (Kalafatić et al., 1999), allowing the assessment of their effects through the analysis of several different biomarkers (Best and Morita, 1991), such as the activity of enzymes involved in oxidative stress response (Guecheva et al., 2003), the regeneration capacity (Calevro et al., 1998; Franjević et al., 2000), the neoblast abnormal mitotic frequency (Milic-Strkalj et al., 1997), the frequency of chromosomal aberrations (Kalafatić and Taborsak, 1998) and the frequency of cells presenting DNA damage (Guecheva et al., 2001).

The use of any organism or biomarker for environmental monitoring requires previous systematic studies to, first, establish the natural behavior of the organism in nature and in laboratory conditions (Stohler et al., 2004), second, to identify biomarkers altered in response to environmental conditions (Prá et al., 2005), and, third, to establish the degree of susceptibility of the organism to specific agents (Chèvre et al., 2003). Once established the organism's sensitivity to a given agent in concentration ranges just above the level found in natural environments, and identified one or more biomarkers useful for measuring the toxic effects of that agent, the organism can be considered potentially suitable for biomonitoring.

We have assessed the sensitivity of a sexual population of the freshwater planarian *Girardia tigrina* (Platyhelminthes, Tricladida, Paludicola) to the presence of $Cu^{2+}$ in water. The $Cu^{2+}$ toxicity, citotoxicity, and genotoxicity for the planarians were established on

assays that evaluated four biomarkers namely; motility alterations; teratogenic alterations during regeneration; micronucleus (MN) formation in dividing neoblasts, and reproductive performance. Based on these biomarkers, we have been able to demonstrate that subchronically exposure of *G. tigrina* to $Cu^{2+}$ concentrations within a range of 0.04 to 0.20 $mg.L^{-1}$ causes detectable effects in one or more of the standardized assays, depending on the developmental stage (adults, regeneratings or newborns) analysed. This allowed us to propose *G. tigrina* as a potential bioindicator for monitoring pollution, and mobility, regeneration, MN and reproductive performance assays as suitable biomarkers to detect $Cu^{2+}$ contamination in aquatic environments.

## 2. Material and methods

### *2.1 Animals*

The sexual diploid (2n) *Girardia tigrina* population used in this study was originally collected from a lotic freshwater environment of Dois Irmãos, Rio Grande do Sul, Brazil, and has been maintained in our laboratory since 1993 (Knakievicz et al., 2006). Adult (3 to 4-month old, 7-10 mm long) and newborn (3 to 10 days after hatch, 2-4 mm long) specimens were utilized in the experiments.

Planarians were cultivated and the experiments were performed at 20-22$^{o}$C, in tanks with reconstituted water of known composition (Knakievicz et al., 2006; Supplementary data Table S1), which was changed once a week, for maintenance purposes, or daily, during experiments (*2.3*). The planarians were fed once a week with bovine liver, except in the three days prior to and during each experiment, when they were not fed.

For experiments involving regenerating specimens, adult planarians were decapitated essentially as described by Knakievicz et al. (2006). Briefly, each individual was immobilized over a block of ice and a single cut was made, as precisely as possible, immediately behind the auricles. After decapitation individuals were immediately exposed to experimental conditions.

*2.2 Chemicals*

Copper sulphate, sodium citrate (CAS 6132-04-3) and MTTP medium were purchased from Synth (Brazil), ISOFAR (Brazil) and Nutricell (Brazil), respectively. Fast green reagent (CAS 2353-45-9), and gelatin (CAS 9000-70-8) from bovine skin were purchased from Sigma (USA). Fucsin (CAS 632-99-5) and formaldehyde solution (CAS 50-00-0) were purchased from Merck (Germany).

*2.3 $Cu^{2+}$ exposure and toxicity test*

Stock copper solution was freshly prepared in distilled water with $CuSO_4.5H_2O$ dissolved to a concentration of 30 $mg{\cdot}L^{-1}$. The stock solution was dissolved in reconstituted water (*2.1*) to final $Cu^{2+}$ concentrations from 0.05 to 1.60 $mg{\cdot}L^{-1}$, as described in each experiment. During experiments, the $Cu^{+2}$-containing water or the $Cu^{+2}$-less water (controls) was changed daily. In each test, six independent samples of 20 newborn or adult (intact or regenerating) planarians (divided in 4 containers with 20 ml of water and 5 animals each) were cultured for 96 h, with counts and removal of dead animals every 24 h. Values of median lethal concentration ($LC_{50}$) were calculated using the Epa Probit Analysis

Program Version 1.5 (parametric samples) and the Trimmed Spearman-Karber (TSK) Program Version 1.5. (nonparametric samples) (Statistical Analysis for Biological Methods). $LC_{50}$ variation coefficients (VC) were calculated as described in Zar (1999). Interactions between developmental stages and exposure times were verified by variance analysis of repeated measures of $LC_{50}$ data using the General Linear Model (GLM) procedure (SAS® for Windows™ version 9.1, http://www.sas.com). Differences between means were statistically evaluated by the Tukey test (Zar, 1999) using SPSS® for Windows™ version 8.0 (http://www.spss.com). A value of $\alpha < 0.05$ was used in all tests.

*2.4 $Cu^{2+}$ accumulation measurement*

For $Cu^{2+}$ content measurements, samples (n = 150) of intact adult planarians were cultured in water with $Cu^{+2}$ concentration of 0.20 $mg \cdot L^{-1}$ for 24 h, 48 h, 72 h and 96 h, with the $Cu^{+2}$-containg water changed daily. Non-exposed planarians (n = 150) were assumed to have the $Cu^{+2}$ steady-state concentration ($C_{ss}$), used as reference. For assessment of the $Cu^{+2}$ elimination rate, planarian samples were kept for 96 h in water containing 0.2 $mg.L^{-1}$ of $Cu^{+2}$ with daily changes, and, after that, were allowed to recover in reconstituted water for 24 h, 72 h, 120 h or 168 h.

After the exposure or recovering periods, planarian samples were washed 5 times in reconstituted water and dried in a centrifugal concentrator (CentriVap Concentrador Cold Trap, Labconco, U.S.A.). $Cu^{+2}$-content for each sample was determined by acid digesting 0.1 g of a dried planarian sample and submitting it to analysis by flame atomic absorption

spectroscopy in a Perkin Elmer 3300 spectrometer (Perkin Elmer, U.S.A.) calibrated with a titrisol copper-standard solution (Merk). $Cu^{2+}$ concentrations (w/w) are expressed in $\mu g \cdot g^{-1}$.

$Cu^{+2}$ concentrations versus exposure times were plotted in graphs and an ascending curve behavior or a descending one with a tendency to stabilize in levels above the $C_{ss}$ were taken as evidence of $Cu^{+2}$ accumulation. A descending curve, with tendency to stabilize in levels below $C_{ss}$ was taken as evidence of $Cu^{+2}$ elimination. Curve behaviors were mathematically described, as proposed by Luoma and Rainbow (2005), to define accumulation ($K_u$ and K) or efflux ($K_e$) rates (expressed in $\mu g \cdot g^{-1} \cdot d^{-1}$ for any time of exposure or recovery time, respectively). Thus, a $Cu^{+2}$ concentration for any given time, t ($C_t$) of exposure can be calculated from the general exponential function:

$$C_t = K_0 \cdot e^{\lambda t} \quad (1)$$

where $\lambda$ is a rate calculated from measures of $Cu^{+2}$ concentration.

The uptake rate for a given time, t ($K_t$) was derived from the general exponential function above (1) and expressed as:

$$Kt = \frac{dCt}{dt} \quad (2)$$

$Cu^{+2}$ concentrations were expressed differently, depending on the planarian physiological behavior regarding $Cu^{+2}$ uptake and/or efflux in a given period of exposure or recovery. The uptake rate ($K_u$) was derived from the experimental measures of $Cu^{+2}$ concentration after the first 24 h of exposure (0 and 1 day of exposure). This represents a situation in which the $Cu^{+2}$ uptake process is the only one considered, assuming a neglectable efflux effect. Therefore, this situation was represented by the equation:

$$C_{t\,(0\text{-}1)} = K_u \cdot t + Css \quad (3)$$

The accumulation rate (K) was derived from the experimental measures of $Cu^{+2}$ concentration from 24 h to 96 h of exposure (1 to 4 days of exposure). This represents a situation in which both $Cu^{+2}$ uptake and $Cu^{+2}$ efflux are considered, being the observed $Cu^{+2}$ concentrations the results of uptake minus efflux. Such situation was represented by the equation:

$$C_{t\ (1\text{-}4)} = K \cdot e^{-0.156t} \quad (4)$$

The efflux rate ($K_e$) was derived from the experimental measures of $Cu^{+2}$ concentrations from 0 h to 168 h of recovering (0 to 7 days of recovering). This represents a situation in which the $Cu^{+2}$ efflux process is the only one considered, assuming a neglectable uptake effect. Such situation was represented by the equation:

$$C_{t\ (0\text{-}7)} = K_e \cdot e^{-0.463t} \quad (5)$$

*2.5 Mobility assay*

Planarian mobility was measured as previously described (Raffa et al. 2001). Briefly, each individual was placed into a clear plastic Petri dish, which was placed over a 0.6 cm square grid. The mobility was expressed as the number of gridlines crossed by the specimen in 30 s of observation. Each mobility assay was independently repeated 3 times (replicates), each time with 5 different groups of 20 planarians (divided in 4 containers with 20 ml of water and 5 animals each). In a given assay, each planarian group (n = 20) was cultured in $Cu^{+2}$-containing water ($Cu^{2+}$ concentrations from 0.05 to 0.8 $mg \cdot L^{-1}$) or in $Cu^{+2}$-less water (controls). Cultures were maintained for 96 h with daily $Cu^{+2}$ solution/water changes. The mobility data were used to calculate the half-inhibitory concentration ($IC_{50}$) in exposure times of 24 h, 48 h, 72 h or 96 h using the Inhibition Concentration (ICp)

Approach software Version 2.0 (Statistical Analysis for Biological Methods). Interactions between $Cu^{+2}$ concentrations and exposure times were verified by variance analyses of repeated measures using the GLM procedure, as described in *2.3*. The Dunnett's test was used for multiple comparisons to determine the mobility lowest-observed-adverse-effect concentration (mLOAEC) values, calculated using SPSS® for Windows™ version 8.0 (http://www.spss.com) and SAS® for Windown™ version 9.1 (http://www.sas.com). A value of $\alpha < 0.05$ was used in all tests.

*2.6 Regeneration assay*

Each regeneration assay was independently repeated 4 times (replicates), each time with 4 different groups of 20 planarians (divided in 4 containers with 20 ml of water and 5 animals each). In a given assay, each planarian group (n = 20) was cultured in $Cu^{+2}$-containing water ($Cu^{2+}$ concentrations from 0.05 to 0.2 $mg \cdot L^{-1}$) or in $Cu^{+2}$-less water (controls). Regenerating planarians were followed by daily observations for 4 days by directly observation in a Zeiss Axiophot microscope. Four regeneration states were defined according to the sequential appearance of cicatrix, blastema, eyespots and auricles, along the 96 h typically necessary for full regeneration (Calevro et al., 1998; Knakievicz et al., 2006). The number of full regenerated animals in each sample was recorded after 96 h of culture in each given experimental or control condition. The regeneration $IC_{50}$ and LOAEC were calculated as described in *2.4*.

*2.7* In vitro *micronucleus assay*

Micronucleus (MN) assays were performed essentially as described by Knakievicz et al. (manuscript in preparation) using samples of 10 planarians. Intact (adults and newborns) or regenerating (adults) planarians were incubated in $Cu^{+2}$-containing water for 24 h and transferred for further 48 h in $Cu^{+2}$-less water, to allow the occurrence of at least one cell division cycle in a non-inhibitory condition for regeneration. Samples of regenerating planarians were also exposed to $Cu^{+2}$ for 96 h, but, in this case, they were processed immediately after the exposure time, since, in 96 h, most planarians were fully regenerated, and, therefore, had already undergone cell division to allow MN generation.

Planarians were then minced in MTTP medium (Schürmann and Peter, 2001) and cell suspensions were fixed with formaline prior to spread on microscope slides, dehydration, and Feulgen/Fast Green staining. One slide was prepared from each sample of 10 planarians submitted to a given treatment or control condition. Cells were visualized in a Zeiss Axiophot optical microscope and neoblasts were morphologically identified by their characteristic size, shape, and nucleocytoplasmic ratio. Neoblasts containing one or more micronuclei were scored as MN positive cells. To determine the MN frequency for a given condition, at least two slides were analysed. For each slide, two independent counting of at least 1000 neoblasts each were done, and the scored frequency was the mean of these two counts. In all experiments, slides were coded to avoid any counting bias. Differences in MN frequencies among treatments were compared by One-way ANOVA and Dunnett's test for multiple comparisons ($\alpha = 0.05$).

*2.8 Reproductive performance evaluation*

The *G. tigrina* reproduction performance was evaluated by monitoring samples (n = 50) of randomly selected adult planarians. Treated samples were chronically exposed to water with different $Cu^{2+}$ concentrations (0.05 and 0.20 $mg \cdot L^{-1}$) for 5 weeks, while control samples were maintained in the same conditions but without exposure to $Cu^{+2}$. Cocoons and hatchlings produced by sexual reproduction were counted weekly and transferred to separated containers. Fecundity was measured as the number of cocoons and fertility was measured as the number of hatchlings, and the corresponding indexes (Fc and Fr) were calculated as described (Knakievicz et al., 2006).

Comparisons of reproductive performances were performed using the Friedman´s test, followed by the Nemenyi test (Zar, 1999). A $P$ value < 0.05 was considered for statistical significance.

## 3. Results

### *3.1* G. tigrina *$Cu^{+2}$ accumulation rate*

In order to obtain information on the dynamics of $Cu^{+2}$ uptake and efflux in *G. tigrina*, $Cu^{+2}$ concentration was analysed both for the exposure (from 0 to 96 h) and the recovery (from 0 to 168 h) periods and the obtained results are shown in Figure 1. The planarian body $Cu^{+2}$ concentration during exposure periods to a $Cu^{+2}$ concentration of 0.20 $mg.L^{-1}$ (i.e. lower than $LC_{50}$, see item 3.2 below) (Figure 1A) and the corresponding rates of uptake and accumulation ($K_u$ and K) (Supplementary data Table S2) demonstrated that,

initially (in the first 24 h of exposure), *G. tigrina* accumulates $Cu^{+2}$ in a high rate (91.70 $\mu g \cdot g^{-1} \cdot d^{-1}$), without a detectable effect of $Cu^{+2}$ loss. Later (from 24 to 96 h of exposure), the curve descending behavior (K values from -27.93 to -6.96 $\mu g \cdot g^{-1} \cdot d^{-1}$) indicates the operation of $Cu^{+2}$ efflux mechanism(s). However, the balance between uptake and efflux continued to favor accumulation, demonstrated by the calculated tendency of the curve to stabilize in $Cu^{+2}$ concentrations above the $C_{ss}$ (26.3 $\mu g \cdot g^{-1}$) (Supplementary data Table S2).

The planarian body $Cu^{+2}$ concentration during recovering periods (Figure 1B), which varied from 73.9 $\mu g \cdot g^{-1}$ (0 h) to 16.6 $\mu g \cdot g^{-1}$ (168 h), and the corresponding efflux rates ($K_e$) (Supplementary data Table S2) indicate that *G. tigrina* undergone progressive $Cu^{+2}$ elimination by mechanisms that lead $Cu^{+2}$ concentration to stabilize in a level below the $C_{ss}$. This is understandable, since the reconstituted water does not contain $Cu^{+2}$, and the return to the $C_{ss}$ would be expected only upon feeding on a $Cu^{+2}$-containing source.

*3.2 $Cu^{2+}$acute toxicity and teratogenicity*

Once established that, under exposure to $Cu^{+2}$-containing water, *G. tigrina* stays with a body $Cu^{+2}$ concentration above the $C_{ss}$, we assessed the $Cu^{+2}$ lethal effects for this planarian species. Therefore, we determined the $LC_{50}$ for newborn and intact or regenerating adult *G. tigrina* planarians for exposure times of 24 h, 48 h, 72 h and 96 h (Table 1). Newborn, intact and regenerating planarians differed in their sensitivities to $Cu^{+2}$, with the mean $LC_{50}$ values showing statistically significant interactions between developmental stages and exposure times ($P < 0.001$, Supplementary data Table S3). However, it was not possible to detect any clear correlations between developmental stages,

exposure times and $LC_{50}$. For instance, there was no significant difference in $Cu^{+2}$ mean $LC_{50}$ between newborn, intact or regenerating planarians in a 48 h exposure time, while, in the other exposure times, newborns are significantly more sensitive than intact adults (24 h) or than regenerating planarians (72 h and 96 h). $Cu^{+2}$ mean $LC_{50}$ from intact and regenerating planarians did not differ significantly in any of the exposure times. Overall, our $LC_{50}$ data suggest that newborns are more sensitive to $Cu^{+2}$, but their degree of sensitivity in relation to intact and regenerating adults may vary with the time of exposure.

To evaluate $Cu^{+2}$ sub-lethal effects after exposure times from 24 h to 96 h, two physiological biomarkers, mobility and regeneration performance, were evaluated in *G. tigrina* planarians. The results obtained for both mobility and regeneration performance (Tables 2, 3 and 4) were indicative of significant alterations in response to $Cu^{+2}$.

The mobility $IC_{50}$ ($mIC_{50}$) analysis (Table 2) showed that newborns are apparently more sensitive to $Cu^{+2}$, since in many experimental conditions their locomotion was seriously impaired. Although statistical validation was not possible (see Table 2 footnotes), this developmental stage can, therefore, be regarded as inadequate to assess the mobility biomarker in the tested concentrations. For regenerating animals, mobility was impaired in 24 – 72 h of exposure to $Cu^{+2}$. However, we can not exclude that the observed reduction in mobility was due, at least in part, to the lack of a full regenerated head, since full regenerated animals (96 h after decapitation) presented $mIC_{50}$ values comparable to those of intact adults. Intact adult mobility is apparently less affected by $Cu^{+2}$ exposure, but the analysis of intact adults would be more informative about the actual effect of $Cu^{+2}$ on this biomarker, than those of newborn or regenerating adult *G. tigrina* planarians.

The mobility LOAEC (mLOAEC) of $Cu^{+2}$ for newborn and intact or regenerating adult *G. tigrina* planarians was also calculated (Table 3; Supplementary data Tables S4-

S11). Values of mLOAEC as low as 0.10 $mg.L^{-1}$ were obtained for newborn (24 h and 72 h) and regenerating adult (24 h, 72 h and 96 h) planarians. Another mLOAEC behavior was observed for intact adults, with higher values (0.40 $mg.L^{-1}$) at 24 h and 72 h and lower ones at 48 and 96 h (0.20 $mg.L^{-1}$). These apparent discrepancies, that do not allow clear correlations between developmental stage, exposure time and mLOAEC, are possibly a consequence of the $Cu^{+2}$ biodynamics in *G. tigrina*, which may differ for each of the analysed developmental stages.

Regarding regeneration performance (Table 4), the appearance of eyespot and auricles in regenerating animals was significantly affected by $Cu^{+2}$ exposure in comparison to controls, with a mean $rIC_{50}$ value of 0.11 $mg{\cdot}L^{-1}$ for both sensorial structures. An apparent inverse correlation between eyespots or auricles appearance and $Cu^{+2}$ concentration was observed. Planarians not fully regenerated after 96 h normally accomplished their regeneration upon transfer to $Cu^{+2}$-less water and culture for additional 3 days. Therefore, although $Cu^{+2}$ caused a delay in the time of appearance of eyespots and auricles in a significant number of individuals, no teratogenic effect, in the form of malformations, was observed.

### *3.3 $Cu^{+2}$ mutagenic effects*

To evaluate $Cu^{+2}$ acute mutagenicity to *G. tigrina*, MN frequency, a cellular biomarker, was assessed for planarians exposed to water with different $Cu^{+2}$ concentrations for 24 h or 96 h. Initially, newborn and intact and regenerating adults had MN frequencies analysed after 24 h of exposure (Table 5). Apparent increases in MN frequency were observed both for newborns and regenerating planarians, although it was significantly

different from control just in one experimental condition (0.10 mg·$L^{-1}$ of $Cu^{+2}$ concentration for regenerating planarians). For planarians in these two developmental stages, exposure to $Cu^{+2}$ concentrations above 0.20 mg·$L^{-1}$ apparently caused a cytotoxic effect, reverting the effect of MN increase observed for lower concentrations. Intact adults, on the other hand, were apparently non-responsive, in terms of MN generation, to the used $Cu^{+2}$ concentration, showing in all of them MN frequencies practically identical to that of the control ($P$ = 0.805).

The MN frequencies of the more responsive regenerating planarians were then analysed after exposure for a longer period (96h). Considering the means of 5 independent experiments (Table 6), it was possible to observe a tendency of increase in MN frequency (from 3.4 to 6.2 MN/1000 cells) in response to the increase in $Cu^{+2}$ concentration. However, a statistically significant difference from the control (mean MN frequency of 2.96 MN/1000 cells) was observed only for the exposure to a $Cu^{+2}$ concentration of 0.8 mg·$L^{-1}$.

*3.4 Effect of $Cu^{2+}$ chronic exposure*

To evaluate the effect of $Cu^{2+}$ chronic exposure, the reproductive performance of adult *G. tigrina* planarians was evaluated during a period of 5 weeks of exposure. $Cu^{+2}$ concentrations of 0.05 and 0.20 mg·$L^{-1}$ were used, since concentrations 0.4 mg·$L^{-1}$ or higher were lethal within 2 weeks or less (data not shown). Significant reductions in mean fecundity and fertility rates were observed in response to both $Cu^{+2}$ concentrations tested (Table 7). A clear detectable effect in reproductive performance upon exposure to $Cu^{+2}$ as

low as 0.05 $mg \cdot L^{-1}$ makes fecundity and fertility rates good biomarkers for assessment of $Cu^{+2}$ chronic exposure effects.

## 4. Discussion

Organisms with well defined biodynamics and adequate biomarkers regarding pollutants, such heavy metals, are needed for environmental monitoring. Here, we evaluated alterations in some biomarkers in the freshwater planarian *G. tigrina* in response to exposure to $Cu^{+2}$ sub-lethal concentrations. $Cu_2SO_4$ is a reference substance for toxicity assays and has been used to measure the sensitivity of test organisms prior to their use in environmental monitoring assays (Zagatto, 2006). The sub-lethal $Cu^{+2}$ concentrations used in our study are within the limits usually found in many natural environments subjected to pollution, such as those near $Cu^{+2}$ smelters or mines, or cropping areas treated with $Cu^{+2}$-containing fertilizers or herbicides (Bhadra, 1992; Lukina and Nikonov, 1995; Sonmez et al., 2006). They are, however, below the $Cu^{+2}$ water concentration (2.00 $mg \cdot L^{-1}$) regarded as acceptable by the corresponding environmental Brazilian legislation (Ministério da Saúde, BR), based on less sensitive biomarkers and bioindicator organisms than those used in this work.

*G. tigrina* planarians were evaluated in conditions ($Cu^{+2}$ concentrations and exposure times) in which the $Cu^{+2}$ biodynamics (uptake versus loss) determined $Cu^{+2}$ accumulation above its steady state concentration in the control condition. The bioassays for $Cu^{+2}$ accumulation, performed for intact adult planarians, were assumed to be valid, at least to some extant, for newborns and regenerating adults.

The values of $Cu^{+2}$ $LC^{50}$, $mIC_{50}$ and mLOAEC for newborns and intact or regenerating adults did not show any clear correlations with developmental stages or exposure times. This apparent lack of clear correlations, despite the clearly detectable effects upon viability or mobility, may be consequence of differences in the type or activity of mechanisms of $Cu^{+2}$ detoxification that operate in different *G. tigrina* developmental stages (newborn or adult) or physiological states (intact or regenerating). For instance, it has been demonstrated that newborns, as a function of their higher body surface/volume rate in comparison to that of adult animals, would have a different osmotic balance, possibly more prone to soluble heavy metal uptake (Preza and Smith, 2001).

Despite of the above discussed variations, *G. tigrina* can be regarded as a useful organism for assessment of $Cu^{+2}$ (and other similar environmental pollutants). *G. tigrina* mobility and regeneration performance were both informative of the heavy metal presence and effect on living organisms. Intact adult mobility (significantly reduced upon $Cu^{+2}$ exposure) and the time of regeneration (delayed upon $Cu^{+2}$ exposure) would be, therefore, two good biomarkers for $Cu^{+2}$ exposure.

*G. tigrina* regeneration was significantly delayed by $Cu^{+2}$-exposure in comparison to non-exposed control animals. Despite the observed delay, overall regeneration was normal in $Cu^{+2}$-exposed planarians, since no teratogenic effects (i. e. malformations) were observed. The observed regeneration delay could be explained by a possible $Cu^{+2}$ citotoxic effect, which would not cause malformations. $Cu^{+2}$ may be a poor teratogenic agent for *G. tigrina* in comparison to other heavy metals, since $Cr^{+3}$ presented clear teratogenic effects in regeneration tests, in the form of malformed eyes and auricles, but without causing any detectable delay in the overall regeneration process (T. Knakievicz & H.B. Ferreira, unpublished results).

$Cu^{+2}$ mutagenicity was assessed by MN assays, demonstrating that, in the tested conditions, $Cu^{+2}$ was poorly mutagenic. This observation is in agreement with the observation that, in *Girardia schubarti*, $Cu^{+2}$ would not be directly harmful to DNA, with its genotoxic effect being essentially upon DNA repair (Guecheva et al., 2001). Despite of the $Cu^{+2}$ low mutagenicity, our MN assay was able to detect $Cu^{+2}$ effects in concentrations as low as 0.10 $mg.L^{-1}$ for regenerating planarians. The higher sensitivity of regenerating planarians in MN assays was probably due to the fact that they undergo a higher proliferation rate than intact newborn or adult individuals (Baguñà, 1976). Due to its practicity and sensitivity, the regenerating *G. tigrina* MN assay present advantages in comparison to MN assays performed with other bioindicator aquatic organisms, such as moluscs (Villela et al., 2006) or fish (Cavas et al., 2005).

$Cu^{+2}$ also caused detectable effects on reproductive performance upon chronicle exposure to concentrations as low as 0.05 $mg{\cdot}L^{-1}$. Both fecundity and fertility rates are, therefore, good biomarkers for assessment of $Cu^{+2}$ chronic exposure effects, in agreement with results in similar tests performed with the planarian *Polycelis temuis* (Indeherberg et al., 1999). These tests with planarians present similar sensitivity and advantages in simplicity in comparison, for instance, to the sea urchin sperm cell bioassay, used as an indirect parameter to evaluate reproductive performance upon chronicle exposure to pollutants in marine environments (Bielmyer et al., 2005).

## 5. Conclusion

Overall, we can conclude that *G. tigrina* is a good bioindicator organism for the detection and evaluation of $Cu^{+2}$ effects in freshwater environments. Assays of adult

mobility, regeneration performance, MN frequency in regenerating animals, and reproductive performance allow to obtain complementary information on toxicity, teratogenicity, mutagenicity and chronicle effects upon populations, respectively. These assays provide evidence of $Cu^{+2}$ effects upon different levels of biological organization, i.e. from the cellular to the population level.

**Acknowledgements**

We thank Dr. T. O. de Freitas for allowing the use of his microscopes, and Dr. Irene S. Schrank for the critical reading of this manuscript. This work was supported by Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). T. K. is a recipient of a predoctoral fellowship from CNPq.

**References**

Baguñà, J., 1976. Mitosis in the Intact and regeneranting planarian *Dugesia mediterranea* n.sp. II mitosis studies during regeneration, and a possible mechanism of blastema formation. J. Exp. Zool. 195, 65-80.

Baker-Austin, C., Dopson, M., Wexler, M., Sawers, R.G., Bond, P.L., 2005. Molecular insight into extreme copper resistance in the extremophilic archaeon '*Ferroplasma acidarmanus*' Fer1. Microbiology 151, 2637–2646.

1 Bebianno, M.J., Geret, F., Hoarau, P., Serafin, M.A., Coelho, M.R., Gnassia-Barelli, M.,
2 Romeo, M., 2004. Biomarkers in *Ruditapes dexussatus*: a potential bioindicator species.
3 Biomarkers 9, 305-330.
4 Best, J.B., Morita, M., 1991. Toxicology of planarians. Hydrobiology 227, 375-383.
5 Bhadra, A.K., 1992. Control of odor and septicity of sewage, United States Patent 5141647.
6 http://www.freepatentsonline.com/5141647.html, accessed in 04/09/2007.
7 Bielmyer, G.K., Brix, K.V., Capo, T.R., Grosell. M., 2005. The effects of metals on
8 embryo-larval and adult life stages of the sea urchin, *Diadema antillarum*. Aquat. Toxicol.
9 74, 254–263.
10 Calevro, F., Filippi, C., Deri, P., Albertosi, C., Batistoni, R., 1998. Toxic effects of
11 Aluminum, Chromium and Cadmium in intact and regenerating freshwater planarians.
12 Chemosphere 37, 651-659.
13 Cavas, T., Garanko, N.N., Arkhipchuk, V.V., 2005. Induction of micronuclei and binuclei
14 in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and
15 copper sulphate. Food Chem. Toxicol. 43, 569-574.
16 Chèvre, N., Gagné, F., Gagnon, P., Blaise, C., 2003. Application of rough sets analysis to
17 identify polluted aquatic sites based on a battery of biomarkers: a comparison with classical
18 methods. Chemosphere 51, 13–23.
19 Costa, M., 1991. Molecular mechanisms of nickel carcinogenesis. Annu. Rev. Pharmacol.
20 Toxicol. 31, 321–337.
21 Franjević, D., Krajna, A.,Kalafatić, M., Ljubesić, N., 2000. The effects of zinc the survival
22 and regeneration of planarian *Polycelis feline*. Biologia (Bratisl) 55, 689-694.

1 Guecheva, T., Henriques, J.A.P., Erdtmann, B., 2001. Genotoxic effects of copper sulphate
2 in freshwater planarian in vivo, studied with the single-cell gel test (comet assay). Mutation
3 Res. 497, 19-27.
4 Guecheva, T.N., Erdtmann, B., Benfato, M.S., Henriques, J.A.P., 2003. Stress protein
5 response and catalase activity in freshwater planarian *Dugesia (Girardia) schubarti*
6 exposed to copper. Ecotoxicol. Environ. Saf. 56, 351–357.
7 Hutchinson, T.H., Hutchinson, M.J., Moore, K.W., 1997. A review of the effects of
8 bromate on aquatic organisms and toxicity of bromate to oyster (*Crassastrea gigas*)
9 embryos. Ecotoxicol. Environ. Saf. 38, 238-243.
10 Indeherberg, M.B.M., Straalen, N.M. van, Schockaert, E.R., 1999. Combining life-history
11 and toxicokinetic parameters to interpret differences in sensitivity to cadmium between
12 population of *Polycelis tenuis* (Platyhelminthes). Ecotoxicol. Environ. Saf. 44, 1-11.
13 Kalafatić, M., Taborsak, S., 1998. Effects of chromium upon neoblast division in the
14 regenerantes of *Polycelis feline*. Biologia (Bratisl) 53, 321-325.
15 Kalafatić, M., Tomaskovic, I., 1999. Toxic effects of aluminium in neutral acidic media on
16 the planarian *Polycelis feline*. Biologia 54, 713-718.
17 Knakievicz, T., Erdtmann, B., Vieira, S.M., Ferreira, H.B., 2006. Reproduction modes and
18 life cycle of freshwater planarians (Plathyhelminthes, Tricladida, Paludicula) from
19 Southern Brazil. Invertebrate Biology 125, 212–221.
20 Louma, S.N., Rainbow, P.S., 2005. Why is metal bioaccumulation so variable?
21 Biodynamics as a unifying concept. Environ. Sci. Technol. 39, 1921-1930.
22 Lukina, N., Nikonov, V., 1995. Acidity of poszolic soils subjected to sulphur pollution near
23 a Cu-Ni smelter at the Kola Peninsula. Water Air Soil Pollut 85, 1057-1062.

Milic-Strkalj, I., Kalafatić, M., 1997. Effects of the herbicide Dicuran 500 FL upon the mitosis of the planarian *Polycelis feline* (Daly.). Period. Biol. 99, 549-553.

Ministério da Saúde. *Portaria Nº.518/2004 - BR*. http://portal.saude.gov.br/portal/arquivos/pdf/portaria_518_2004.pdf. Accessed in 04/03/2007.

Nascimento, I.A., Pereira, S.A., Leite, M.B.N.L., 2006. Biomarcadores como instrumentos preventivos de poluição. In: Zagatto, P.A., Bertoletti. E.B., (Eds.), Ecotoxicologia Aquática - Princípio e Aplicações. Ed. RiMa, São Carlos, pp. 413-429.

Prá, D., Lau, A.H., Knakievicz, T., Carneiro, F.R., Erdtmann. B., 2005. Environmental genotoxicity assessment of an urban stream using freshwater planarians. Mutation Res. 585, 79–85.

Preza, D.L.C., Smith, D.H., 2001. Use of newborn *Girardia tigrina* (Girard, 1950) in acute toxicity tests. Ecotoxicol. Environ. Saf. 50, 1-3.

Raffa, R.B., Holland, L.J., Schulingkamp, R.J., 2001. Quantitative assessment of dopamine D2 antagonist activity using invertebrate (Planaria) locomotion as a functional endpoint. J. Pharmacol. Toxicol. Methods 45, 223-226.

Sala, O.E., Chapin III, F.S., 2000. Armesto, J.J. Global biodiversity scenarios for the year 2100. Science 287, 1770-1774.

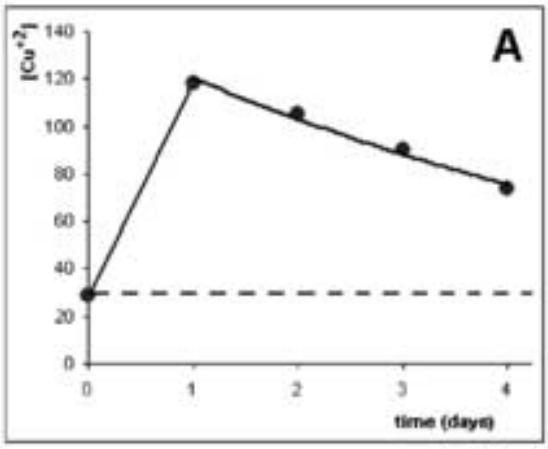
Schürmann, W., Peter, R., 2001. Planarian cell culture: a comparative review of methods and an improved protocol for primary cultures of neoblasts. Belg. J. Zool. 131, 123-130.

Sonmez, S., Kaplan, M.,Sonmez, N.K., Kaya, H., Uz, I., 2006. High level of copper application to soil and leaves reduce the growth and yield of tomato plants. Sci. Agric. 63, 213-218.

1 Statistical Analysis for Biological Methods. http://www.epa.gov/nerleerd/stat2.htm,
2 accessed in 03/10/2007.
3 Stohler, R.A., Curtis, J., Minchella, D.J., 2004. A comparison of microsatellite
4 polymorphism and heterozygosity among field and laboratory populations of *Schistosoma*
5 *mansoni*. Int. J. Parasitol. 34, 595–601.
6 Vargas, V.M.F., Migliavacca, S.B., Melo, A.C., Horn, R.C., Guidobono, R.R., Ferreira,
7 I.C.F.S., Pestana, M.H.D., 2001. Genotoxicity assessment in aquatic environments under
8 the influence of heavy metals and organic contaminants. Mutation Res. 490, 141–158.
9 Villela, I.V., Oliveira, I.M. de, Silva, J. da, Henriques, J.A.P., 2006. DNA damage and
10 repair in haemolymph cells of golden mussel (*Limnoperna fortunei*) exposed to
11 environmental contaminants. Mutation Res. 605, 78–86.
12 Zagatto, P.A., 2006. O uso de substâncias de referência no controle do Qualidade de
13 Ensaios Ecotoxicologicos. In: Zagatto, P.A., Bertoletti. E.B., (Eds.), Ecotoxicologia
14 Aquática - Princípio e Aplicações. Ed. RiMa, São Carlos, pp. 184-197.
15 Zar, J.H., 1999. Biostatistical Analysis. Prentice Hall, New Jersey, pp 267-301

**Figure caption**

Figure 1. The *G. tigrina* biodynamics of $Cu^{+2}$ concentration in periods of exposure (A) and recovery (B), as described in Materials & Methods. Dots represent experimental mesurements of Cu+2 concentration after each given time (days) of exposure or recovery. The curves (continuous lines) were calculated using formulas (3), (4) and (5) (see Material & Methods), in order to describe periods of $Cu^{+2}$ uptake (from day 0 to 1, in A), $Cu^{+2}$ accumulation (from day 1 to 4, in A), and $Cu^{+2}$ efflux (from day 0 to 7, in B), respectively. The traced lines correspond to the steady-state $Cu^{+2}$ concentration ($C_{ss}$), measured for non-exposed planarians.



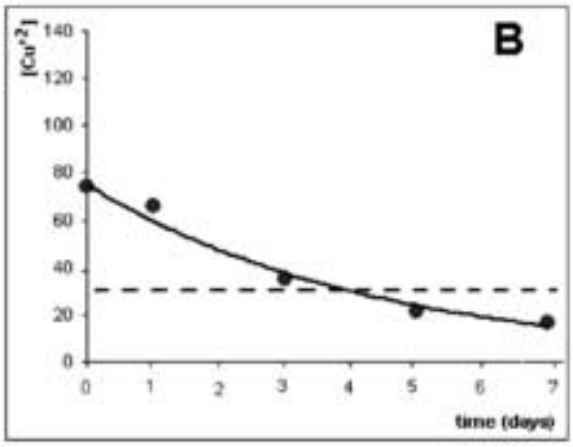



Table 1. $Cu^{2+}$ lethal concentrations ($LC_{50}$) for newborn and adults (regenerating and intact) planarians exposed to water with different $Cu^{2+}$ concentrations. Each replicate (I, II or III) consisted of six samples of 20 planarians, with each sample cultured in $Cu^{2+}$-less water (control) or in water with a given $Cu^{2+}$ concentration (0.10, 0.20, 0.40, 0.8, or 1.6 $mg.L^{-1}$). Cultured planarians were followed for 96 h, with counts every 24 h for death recording.

| Replicate | $LC_{50}$ ($mg \cdot L^{-1}$) [(1)] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Newborn (intact) | | | | Adult (intact) | | | | Adult (regeneranting) | | | |
| | 24 h | 48 h | 72 h | 96 h | 24 h | 48 h | 72 h | 96 h | 24 h | 48 h | 72 h | 96 h |
| I | 0.32 | 0.24 | 0.15 | 0.14 | 0.76 | 0.5 | 0.47 | 0.48 | 0.60 | 0.60 | 0.59 | 0.59 |
| II | 0.24 | 0.19 | 0.15 | 0.11 | 0.62 | 0.5 | 0.50 | 0.44 | 0.56 | 0.54 | 0.54 | 0.53 |
| III | 0.27 | 0.24 | 0.11 | 0.11 | 0.46 | 0.33 | 0.33 | 0.33 | 0.32 | 0.36 | 0.32 | 0.32 |
| Mean $LC_{50}$ ± SD[(2)] | 0.27 ± 0.04[(a)] | 0.22 ± 0.03[(c)] | 0.14 ± 0.02[(d)] | 0.12 ± 0.02[(f)] | 0.61± 0.15[(b)] | 0.44 ± 0.10[(c)] | 0.43 ± 0.09[(d,e)] | 0.42 ± 0.08[(f,g)] | 0.49 ± 0.12[(a,b)] | 0.50 ± 0.12[(c)] | 0.48 ± 0.13[(e)] | 0.48 ± 0.13[(g)] |
| $VC_{LC50}$ | 0.15 | 0.13 | 0.17 | 0.14 | 0.24 | 0.22 | 0.21 | 0.19 | 0.25 | 0.26 | 0.29 | 0.28 |

SD, standard deviation; $VC_{LC50}$, $LC_{50}$ variation coefficients.

[(1)] $Cu^{2+}$ $LC_{50}$ values were independently calculated in each test for exposure times of 24 h, 48 h, 72 h and 96 h.

[(2)] Each mean $LC_{50}$ value ($mg \cdot L^{-1}$) corresponds to the mean of the $Cu^{+2}$ $LC_{50}$ values of the 3 replicates for a given exposure time.

Statistically significant differences between mean $LC_{50}$ values from newborn, intact and regenerating planarians in corresponding exposure times are significantly different when indicated by different letters (a, b, for 24 h; c, for 48 h; d, e, for 72 h; f, g, for 96 h).

Table 2. $Cu^{2+}$ mobility inhibitory concentration ($mIC_{50}$) for newborn and adults (regenerating and intact) planarians exposed to water with different $Cu^{2+}$ concentrations. Each replicate (I, II or III) consisted of five samples of 20 planarians, with each sample cultured in $Cu^{2+}$-less water (control) or in water with a given $Cu^{2+}$ concentration (0.05, 0.10, 0.20, 0.40, or 0.8 $mg.L^{-1}$). Planarians were cultured for 96 h, with the mobility of each individual in a sample assessed at 24 h intervals.

| **Replicates** | **$mIC_{50}$ ($mg \cdot L^{-1}$) (1)** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Newborn (intact)(2)** | | | | **Adult (intact)** | | | | **Adult (regeneranting)(3)** | | |
| | 24 h | 48 h | 72 h | 96 h | 24 h | 48 h | 72 h | 96 h | 48 h | 72 h | 96 h |
| **I** | 0.25 | 0.15 | NC | NC | 0.36 | 0.36 | 0.36 | 0.21 | 0.25 | 0.16 | 0.50 |
| **II** | NC | NC | NC | NC | 0.47 | 0.51 | 0.42 | 0.36 | 0.27 | 0.35 | 0.30 |
| **III** | 0.22 | 0.19 | 0.09 | NC | 0.38 | 0.37 | 0.31 | 0.25 | 0.20 | 0.10 | 0.10 |
| **Mean $mIC_{50}$ ± SD(4)** | 0.23 ± 0.02 | 0.17± 0.03 | 0.09 | NC | 0.40 ± 0.06 | 0.41 ± 0.08 | 0.36 ± 0.06 | 0.27 ± 0.08 | 0.24 ± 0.04 | 0.20 ± 0.13 | 0.30 ± 0.20 |
| **$mVC_{IC50}$** | 0.09 | 0.17 | NC | NC | 0.15 | 0.20 | 0.15 | 0.28 | 0.15 | 0.64 | 0.67 |

SD, standard deviation; $mVC_{IC50}$, $mIC_{50}$ variation coefficients. .

(1) $Cu^{2+}$ $mIC_{50}$ values were independently calculated in each test for exposure times of 24 h, 48 h, 72 h and 96 h.

(2) In certain experimental conditions ($Cu^{+2}$ concentrations and exposure times), *G. tigrina* newborns did not locomote, inviabilizing

the mIC50 calculation. These situations are denoted by NC (not calculated).

(3) Within 24 h of decapitation, *G. tigrina* planarians little or do not locomote; therefore this exposure time was excluded from mobility analysis for regenerating planarians.

(4) Each mean $mIC_{50}$ value ($mg \cdot L^{-1}$) corresponds to the mean of the $Cu^{+2}$ $mIC_{50}$ values of the 3 replicates for a given exposure time.

Table 3. $Cu^{2+}$ Lowest-observed-adverse-effect concentration (mLOAEC) values concerning mobility for newborns and adults (intact or regenerating).

| **Developmental stage** | **mLOAEC[a] of $Cu^{2+}$ $mg.L^{-1}$** | | | |
|---|---|---|---|---|
| | **24 h** | **48 h** | **72 h** | **96 h** |
| **Newborn** | 0.10 | 0.20 | 0.10 | NC |
| **Adult (intact)** | 0.40 | 0.20 | 0.40 | 0.20 |
| **Adult (regenerating)** | 0.10 | 0.20 | 0.10 | 0.10 |

NC, not calculated.

[a] LOAEC values were calculated by Variance analyses through General Linear Model (GLM) procedure for repeated measured of mobility data, following by Dunnett's test for multiple comparison (supplementary material).

Table 4. $Cu^{2+}$ regeneration inhibitory concentration ($rIC_{50}$) for newborn and adults (regenerating and intact) planarians exposed to water with different $Cu^{2+}$ concentrations. Each replicate (I, II, III or IV) consisted of four samples of 20 planarians, with each sample cultured in $Cu^{2+}$-less water (control) or in water with a given $Cu^{2+}$ concentration (0.05, 0.10 and 0.20, $mg \cdot L^{-1}$). Planarians were cultured for 96h, with the regeneration stage of each individual in a sample scored by the end of that time.

| **$Cu^{2+}$ mg·L$^{-1}$** | **Eyespots** | | | | | **Auricles** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Test[1]** | | | | **Mean ± SD[2]** | **Test[1]** | | | | **Mean ± SD[2]** |
| | **I** | **II** | **III** | **IV** | | **I** | **II** | **III** | **IV** | |
| **Control** | 20 | 18 | 17 | 15 | 17.6 ± 2.0 | 20 | 18 | 17 | 14 | 17.3 ± 2.5 |
| **0.05** | 17 | 15 | 18 | 9 | 14.8 ± 4.0 | 17 | 15 | 18 | 9 | 14.8 ± 4.0 |
| **0.10** | 18 | 7 | 7 | 10 | 10.8 ± 5.0* | 20 | 7 | 8 | 8 | 10.8 ± 6.2 |
| **0.20** | 5 | 5 | 5 | 9 | 5.3 ± 2.9* | 4 | 5 | 2 | 9 | 5.0 ± 2.9* |
| **$rIC_{50}$[3]** | 0.16 | 0.08 | 0.09 | NC | 0.11 ± 0.04 | 0.16 | 0.09 | 0.09 | NC | 0.11 ± 0.04 |

NC, not calculated.

[1] Number of animals in each sample with eyespots or auricles recorded after 96 h of culture in each given experimental condition.

[2] An asterisk (*) denotes means differ significantly of control compared by Dunnett's test for multiple comparisons ($\alpha = 0.05$) to ANOVA, $P = 0.004$ for eyespots and $P = 0.007$ for auricles.

[(3)] $Cu^{2+}$ $rIC_{50}$ values, expressed in $mg \cdot L^{-1}$, were independently calculated in each test and means ± SD values for both eyespots and auricles for exposure time of 96 h.

Table 5. Neoblast MN frequency of the newborn, regenerating and intact adult *G. tigrina* exposed to water without $Cu^{+2}$ (control) or with $Cu^{+2}$ concentrations of 0.05 or 0.80 $mg.L^{-1}$ for 24 h and they were following in cultive water for over 48 h.

| **$Cu^{+2}$ ($mg \cdot L^{-1}$)** | **Newborn** | | **Adult (intact)** | | **Adult (regenerating)** | |
|---|---|---|---|---|---|---|
| | **MN (cell)[1]** | **MN/1000 Mean ± SD** | **MN (cell) [1]** | **MN/1000 Mean ± SD** | **MN (cell) [1]** | **MN/1000 Mean ± SD[2]** |
| **0.00** | 13 ± 4064 | 3.19 ± 0.98 | 8 (4086) | 1.97 ± 0.03 | 14 (4091) | 3.42 ± 0.05 |
| **0.05** | 13 ± 4037 | 3.22 ± 0.34 | 9 (4050) | 2.23 ± 1.05 | 15 (4059) | 3.68 ± 0.34 |
| **0.10** | 22 ± 4060 | 5.45 ± 2.15 | 11 (4007) | 2.74 ± 0.35 | 30 (4043) | 7.43 ± 2.87* |
| **0.20** | 25 ± 4049 | 6.19 ± 1.83 | 11 (4048) | 2.71 ± 1.01 | 20 (4018) | 4.98 ± 0.03 |
| **0.40** | 25 ± 4048 | 6.19 ± 0.39 | 11 (4052) | 2.72 ± 1.07 | 21 (4134) | 5.06 ± 0.90 |
| **0.80** | 23 ± 4135 | 5.51 ± 0.26 | 13 (4085) | 3.16 ± 0.94 | 8 (4050) | 1.98 ± 0.71 |
| **Statistic analysis[3]** | NA | $F_{5,24} = 2.48$ $P = 0.070$ | NA | $F_{5,24} = 0.45$ $P = 0.805$ | NA | $F_{5,24} = 5.34$ $P = 0.003$ |

NA, not applicable.

[1] Total number of micronucleated neoblasts and within parenthesis total number of analysed neoblasts from two tests.

[2] An asterisk (*) denotes mean differ significantly of control compared by Dunnett's test for multiple comparisons ($\alpha = 0.05$).

[3] Variance analysis performed as described in the Materials and Methods section.

Table 6. Neoblast MN frequency of the regenerating *G. tigrina* exposed to water without $Cu^{+2}$ (control) or with $Cu^{+2}$ concentrations of 0.10 or 0.80 $mg.L^{-1}$ for 96 h.

| Test | Control | | 0.10 $mg.L^{-1}$ | | 0.20 $mg.L^{-1}$ | | 0.40 $mg.L^{-1}$ | | 0.80 $mg.L^{-1}$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MN (cell)[1] | MN/1000 neoblast | MN (cell)[1] | MN/1000 neoblast | MN (cell)[1] | MN/1000 neoblast | MN (cell)[1] | MN/1000 neoblast | MN (cell)[1] | MN/1000 neoblast |
| I | 7 (2029) | 3.45 | 7 (2002) | 3.50 | 7 (2022) | 3.46 | 12 (2001) | 6.00 | 9 (2001) | 4.50 |
| II | 8 (2054) | 3.89 | 8 (2024) | 3.95 | 10 (2052) | 4.87 | 17 (2029) | 8.38 | 9 (2082) | 4.32 |
| III | 5 (2029) | 2.46 | 5 (2015) | 2.48 | 11 (2078) | 5.29 | 16 (2014) | 7.94 | 17 (2007) | 8.47 |
| IV | 6 (3029) | 1.98 | 7 (2016) | 3.47 | 6 (2000) | 3.00 | 6 (2369) | 2.53 | 11 (2232) | 4.93 |
| V | 6 (2005) | 2.99 | 13 (3016) | 4.31 | 9 (2007) | 4.48 | 14 (3142) | 4.46 | 18 (2045) | 8.80 |
| Mean ± SD[2] | NA | 2.96 ± 0.76 | NA | 3.54 ± 0.69 | NA | 4.22 ± 0.96 | NA | 5.86 ± 2.44 | NA | 6.20 ± 2.23* |

NA, not applicable.

[1] Total number of micronucleated neoblasts and within parenthesis total number of analysed neoblasts in each test.

[2] The asterisk (*) denotes mean differ significantly of control (One-way ANOVA, $F_{4,25} = 3.930$, $P = 0.016$) compared by Dunnett's test for multiple comparisons ($\alpha = 0.05$).

Table 7. Reproductive performance of *G. tigrina* adult samples exposed to water without $Cu^{+2}$ (control) or with $Cu^{+2}$ concentrations of 0.05 or 0.20 mg.L-1. Samples (n = 50) were followed for five weeks with weekly counts of cocoons and newborns. Fc and Fr indexes were calculated and statistical analyses were performed as described by [21].

| Exposure condition | Mean Fc (SD)[(1)] | Fr (SD)[(2)] |
|---|---|---|
| Control | 0.92 (0.76) | 0.64 (0.33) |
| 0.05 $mg.L^{-1}$ | 0.44 (0.56)* | 0.31 (0.21)* |
| 0.20 $mg.L^{-1}$ | 0.49 (0.56)* | 0.24 (0.31)* |
| Friedman test | $\chi^2 = 6.52$<br>$P = 0.04$<br>N = 5 [(3)] | $\chi^2 = 7.60$<br>$P = 0.02$<br>N = 5 [(3)] |

[(1)] Mean ± standard deviation (SD) of Fc indexes calculated weekly for each experimental sample. An asterisk (*) denotes Fc or Fr means which differ significantly from the corresponding index calculated for the control sample (Nemenyi test, $\alpha = 0.05$).

[(2)] Mean ± SD of Fr indexes calculated weekly for each experimental sample. An asterisk (*) denotes Fr means which differ significantly from the corresponding index calculated for the control sample (Nemenyi test, $\alpha = 0.05$).

[(3)] For the Friedman test, N corresponds to the number of different Fc or Fr indexes (five, one for each week for each sample) used to calculate the corresponding means.

**Electronic Supplementary Material (online publication only)**
**Click here to download Electronic Supplementary Material (online publication only): Knakievicz_SupplementaryMaterial.doc**

## *3.4 Capítulo IV* - Avaliação da capacidade de regeneração e da longevidade de *G. tigrina* e *G. schubarti*

### 3.4.1 Introdução

Planárias são organismos únicos no reino animal quanto à presença de uma população especial de células totipotentes, os neoblastos (REDDIEN & SÁNCHEZ ALVARADO, 2004). A importância dos neoblastos na biologia dos turbelários é amplamente reconhecida. Eles são responsáveis pelo restabelecimento de todos os tipos celulares durante o desenvolvimento, crescimento, regeneração e reposição de células mortas. Para o estudo dos mecanismos moleculares da manutenção dos neoblastos das planárias, é pré-requisito conhecer a longevidade das planárias e as alterações na capacidade regenerativa e reprodutiva decorrentes do aumento da idade. A capacidade regenerativa e a capacidade reprodutiva são dois parâmetros indiretos que permitem avaliar a homeostase dos neoblastos ao longo da vida das planárias.

Para atingir os objetivos, este trabalho foi subdividido em duas etapas:

1ª Verificar a capacidade de regeneração de indivíduos juvenis e adultos das espécies *G. tigrina* (GtPop1, linhagem sexuada) e *G. schubarti* (GsPop1, linhagem sexuada, e GsPop2 e GsPop3, linhagens assexuadas) através da quantificação do tempo de regeneração da cabeça em indivíduos previamente decapitados.

2ª Acompanhar o ciclo de vida da população *G. tigrina* sexuada (GtPop1) e das populações *G. schubarti* assexuadas (GsPop2 e GsPop3) para verificar o efeito da longevidade sobre a reprodução sexuada através dos índices de fertilidade e fecundidade, e sobre a reprodução assexuada através dos índices de fissiparidade, respectivamente.

### 3.4.2 Materiais e Métodos

#### 3.4.2.1 Planárias

As espécies e populações de planárias estudadas foram *G. tigrina,* diplóides adultas (GtPop1, com idade de 3 a 4 meses e com 10 - 13 mm de comprimento); *G. schubarti*, em diplóides jovens (GsPop1, com idade de 3 a 4

meses e com ~ 8 - 10 mm de comprimento), em diplóides adultos (com idade superior a 18 meses e com 20 - 30 mm de comprimento) e em mixoplóides (população fissiparitária) (GsPop2 e GsPop3, com idade indeterminada e com ~ 15 mm de comprimento).

3.4.2.2 Avaliação da capacidade regenerativa das planárias *G. tigrina* e *G. schubarti*.

A capacidade de regeneração de *G. tigrina* (diplóides) e *G. schubarti* (diplóides e mixoplóides) foi avaliada através da determinação do tempo necessário para o surgimento das aurículas e ocelos após decapitação, como descrito no Capítulo I, e através da mobilidade das planárias durante o processo de regeneração, como descrito no Capítulo III. Durante os experimentos, as planárias regenerantes eram mantidas em condições padrões de temperatura (21 ± 2ºC), fotoperíodo (C:E, 12h:12h) e luminosidade (35 FC), ou em condições modificadas de temperatura (28 ± 2$^{o}$C), de luminosidade (0; 0,1; 8; 40,5 e 46,2 FC) de fotoperíodo, (claro constante, C:C, e escuro:escuro, E:E) ou na presença de campo eletromagnético (CEM) fraco (834 MHz, 32-54 V/m) por 8 h/dia durante o período de regeneração.

3.4.2.3 Avaliação da ciclo de vida das planárias *G. tigrina*.

A sobrevivência, a fertilidade e a fecundidade de populações de *G. tigrina* diplóides foram analisadas como descrito no Capítulo I, para avaliação do efeito da densidade populacional, da quantidade de sais na água de cultivo e da longevidade. Para verificar o efeito da densidade populacional sobre a reprodução, populações com 20 a 60 indivíduos$\cdot L^{-1}$ foram monitoradas por 14 semanas. Para verificar o efeito da quantidade de sais na água sobre a reprodução e a longevidade, populações com inicialmente 60 indivíduos$\cdot L^{-1}$ foram monitoradas por ~189 semanas em água com concentração padrão, com redução de 50% e com acréscimo de 3x na quantidade de sais.

3.4.2.4 Análises estatísticas.

Os procedimentos estatísticos utilizados estão descritos nos Capítulos I e

III, secção *Material and Methodos – 2.5 Mobility assay* e *2.6 Regeneration assay*.

### 3.4.3 Resultados

3.4.3.1 Avaliação da capacidade regenerativa de planárias *G. tigrina* e *G. schubarti*.

Em condições padrões, 21 ± 2ºC, fotoperíodo C:E (12h:12h) e luminosidade de 35 FC, para planárias diplóides e adultas da espécie *G. tigrina* foi observado que o surgimento de ocelos e aurículas ocorre no 3º ou no 4º dia após a decapitação (Capítulo III, Table 4), assim como para planárias jovens diplóides e as mixoplóides da espécie *G. schubarti* (Figura 2E,D e 3E,D). No entanto, para planárias diplóides e adultas da espécie *G. schubarti* foi requerido o dobro do tempo para o surgimento dessas estruturas (ver Capítulo I). Portanto, para a espécie *G. schubarti*, os jovens diplóides e os mixoplóides têm similar capacidade de regeneração e regeneram mais rápido do que os adultos diplóides.

No entanto, alterações no tempo de regeneração podem ser introduzidas através de alterações das condições ambientais. O efeito da luminosidade de 0 a 46,2FC sobre o tempo de regeneração da cabeça de planárias *G. tigrina* diplóides adultas decapitadas foi avaliado. Não houve diferenças significativas no tempo de regeneração nas luminosidades testadas (0,1 a 46,2). No entanto, luminosidade constante (C:C) de 35 FC acelerou o tempo de regeneração (Figura 4A), enquanto que a ausência total de luz (0 FC) retardou a regeneração em mais de 4 dias. As alterações na regeneração causadas pela presença de CEM foram similares em *G. tigrina* e *G. schubarti* (Figura 4B). O aumento na temperatura causou redução no tempo de regeneração em *G. tigrina* (2n) (Figuras 4C e 4D) e em *G. schubarti* (2n/3n) (dados não-mostrados).

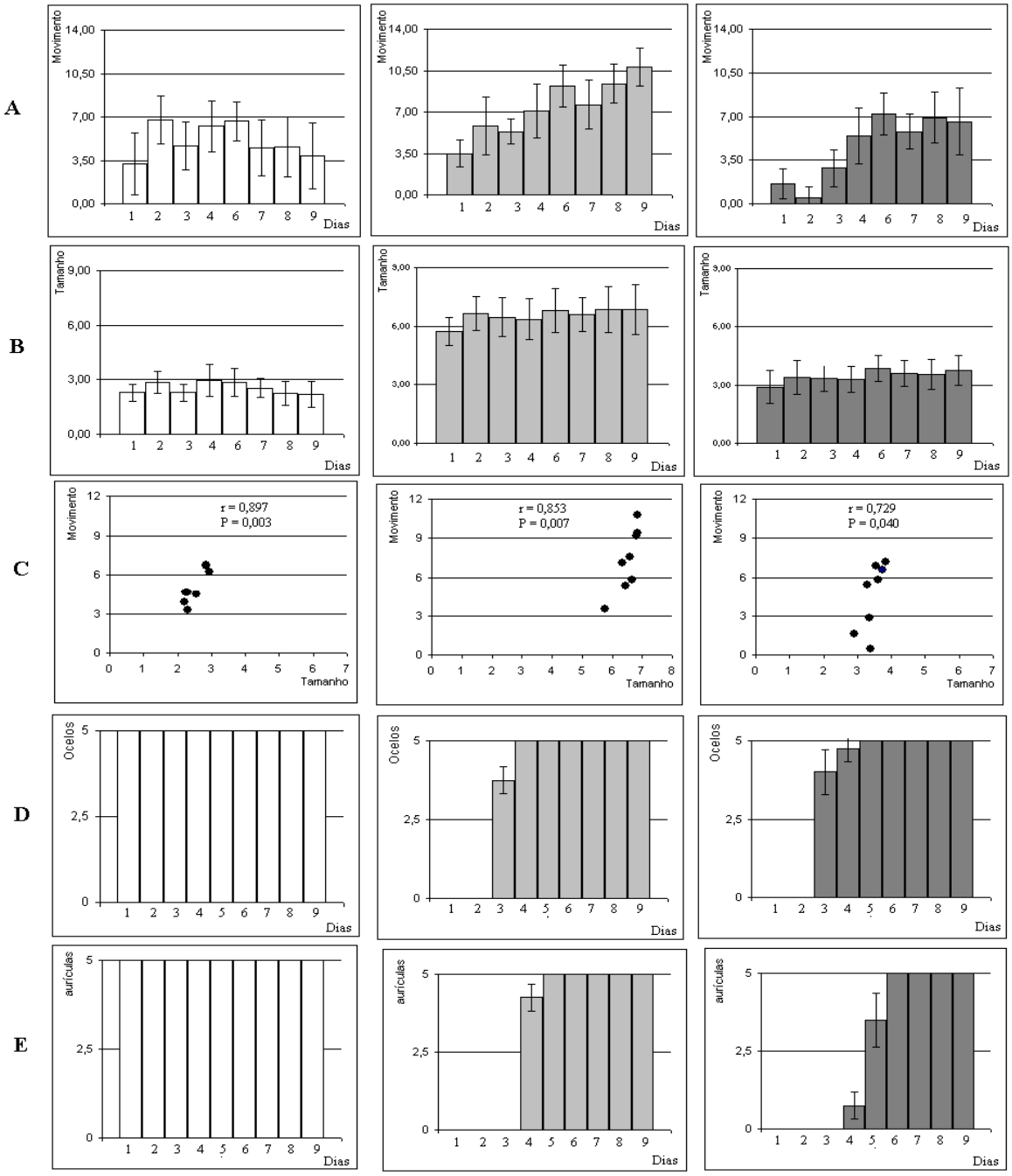



**Figura 2**. Análise de diferentes parâmetros para acompanhamento do processo de regeneração de *G. schubarti* 2n jovem (2 - 3 meses de idade). Os gráficos da coluna da esquerda correspondem aos fragmentos da cabeça, os da coluna central correspondem aos fragmentos do meio do corpo e os da direita correspondem aos fragmentos da cauda. Parâmetros analisados: **A**) movimento dos regenerantes (número de linhas cruzadas por unidade de tempo); **B**) tamanho dos regenerantes (cm); **C**) Correlação de Pearson entre mobilidade e o tamanho dos regenerantes; **D**) tempo necessário (dias) para surgimento (detectado visualmente, em lupa) dos primeiros rudimentos dos ocelos; **E**) tempo necessário (dias) para surgimento (detectado visualmente, em lupa) dos primeiros rudimentos das aurículas.

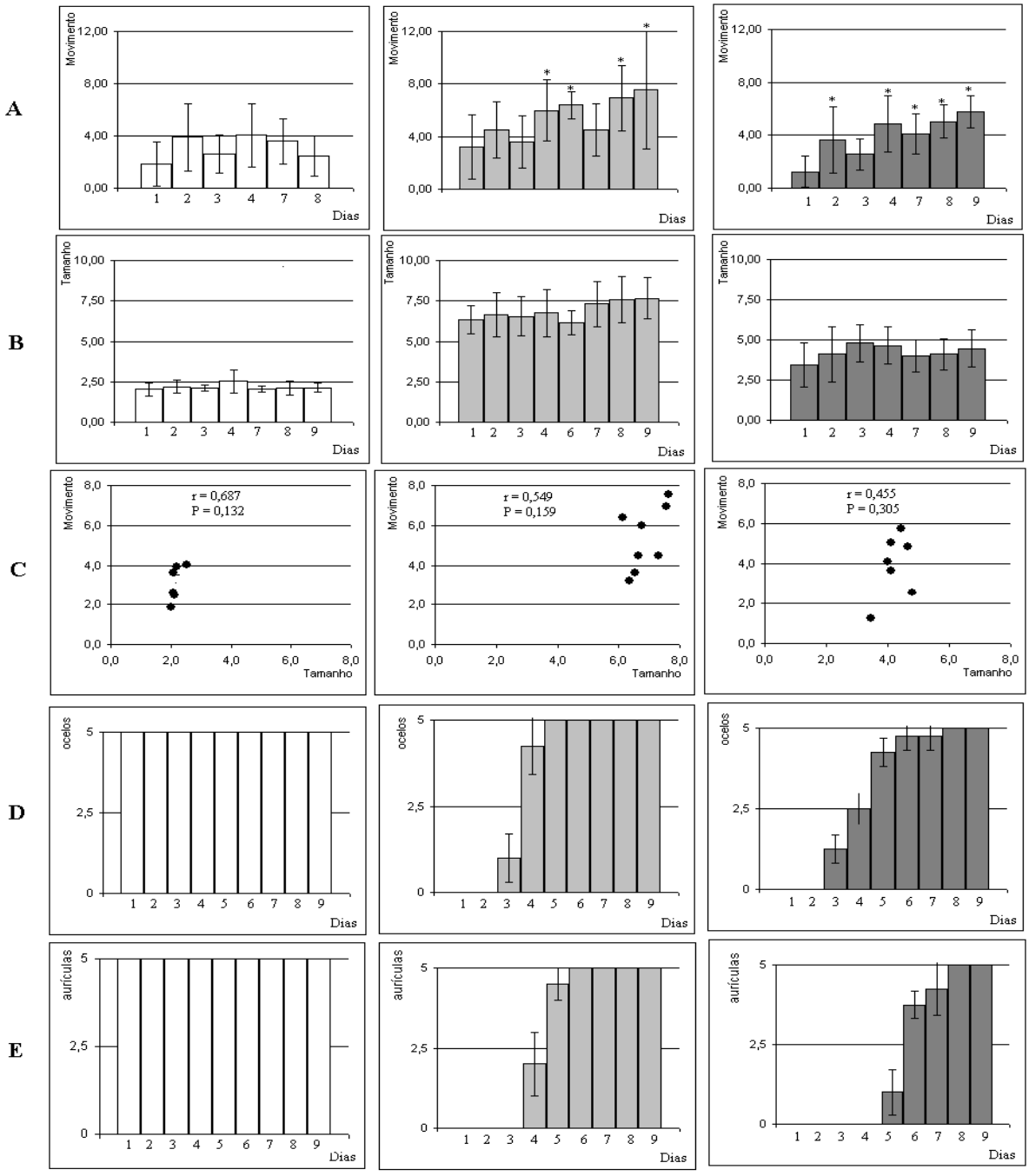



**Figura 3**. Análise de diferentes parâmetros para acompanhamento do processo de regeneração de *G. schubarti* mixoplóide (população clonal). Os gráficos da coluna da esquerda correspondem aos fragmentos da cabeça, os da coluna central correspondem aos fragmentos do meio do corpo e os da direita correspondem aos fragmentos da cauda. Parâmetros analisados: **A**) movimento (número de linhas cruzadas por unidade de tempo); **B**) tamanho (cm); **C**) Correlação de Pearson entre mobilidade e o tamanho dos regenerantes; **D**) tempo necessário (dias) para surgimento (detectado visualmente, em lupa) dos primeiros rudimentos dos ocelos; **E**) tempo necessário (dias) para surgimento (detectado visualmente, em lupa) dos primeiros rudimentos das aurículas.

Quanto ao tamanho do fragmento em regeneração, para *G. tigrina* não foram observadas alterações significativas no comprimento dos fragmentos regenerantes (dados não mostrados), assim como para ambas as raças cromossômicas diplóide e mixoplóide de *G. schubarti* (Figuras 4B e 5B, respectivamente), independentemente da região do corpo (regiões cefálica, média e caudal) regenerada. Portanto, em ambas as espécies, durante o processo de regeneração, foi(ram) formada(s) a(s) parte(s) perdida(s) e foi restabelecido o padrão corporal sem alterações do comprimento dos regenerantes.

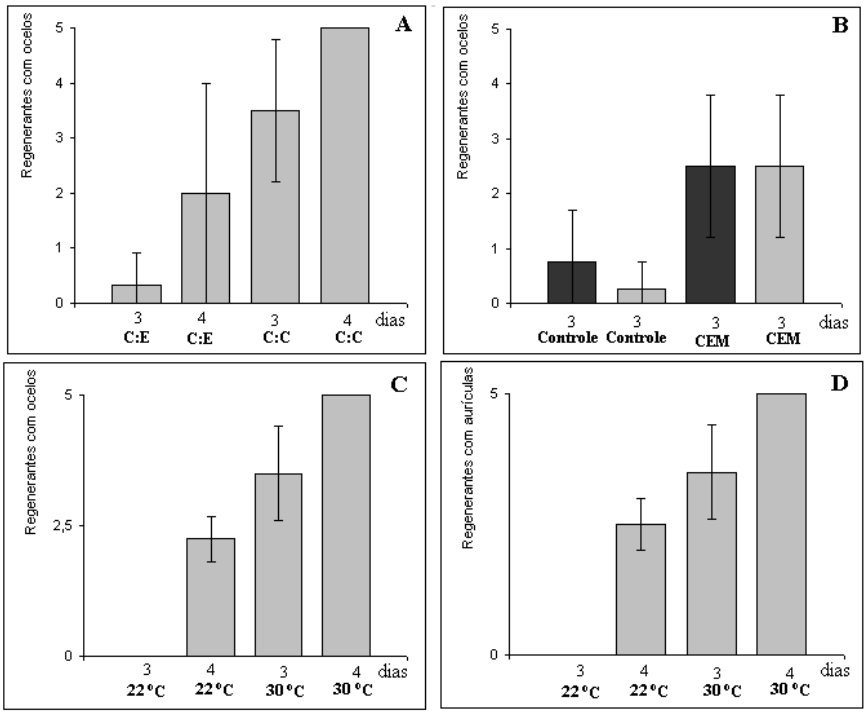



**Figura 4**. Efeito da temperatura, do CEM e da temperatura sobre a regeneração de planárias. As barras claras representam regenerantes *G. tigrina* diplóides e as barras pretas representam regenerantes *G. schubarti* (mixoplóide). **A**) Efeito do fotoperíodo; **B**) efeito do CEM; **C**) efeito da temperatura sobre a regeneração dos ocelos e **D**) efeito da temperatura sobre a regeneração das aurículas.

Quanto à mobilidade dos regenerantes, foi observado para *G. tigrina* (Capítulo III, Seção Supplementary Material – Table 5S) e para ambas as raças cromossômicas de *G. schubarti* (Figuras 2A e 3A, respectivamente) que a velocidade de deslocamento é retomada simultaneamente com o surgimento dos ocelos e das aurículas. Para a raça cromossômica diplóide *G. schubarti*, há

correlação significativa entre a quantidade de movimento e o tamanho dos fragmentos regenerantes da cabeça, do tronco ou da cauda (Figura 2C). Entretanto, para os mixoplóides não há correlação entre mobilidade e o tamanho dos fragmentos regenerantes para quaisquer partes do corpo (Figura 3C). Essa diferença de mobilidade entre as raças sexuadas e assexuadas de *G. schubarti* pode refletir diferenças de pressões adaptativas dos indivíduos diplóides e mixoplóides.

### 3.4.3.2 Densidade e estabilidade populacional em *G. tigrina*.

Para verificar o efeito da densidade sobre a estabilidade populacional e a reprodução, populações de *G. tigrina* com 20 a 60 indivíduos/litros, de diferentes idades, foram monitoradas por ~14 semanas. Foi observado que populações jovens (3 a 6 meses) são menos férteis que populações com um ano ou mais de vida (Figura 5A). E populações com mais de 60 indivíduos·$L^{-1}$ apresentam maior mortalidade, estabilizando o número de indivíduos em ~ 40 indivíduos·$L^{-1}$ (Figura 5B). Também foi observado que os maiores e menores índices de Fc e Fr ocorreram em populações menos e mais densas, respectivamente (Figuras 5C e 5D).

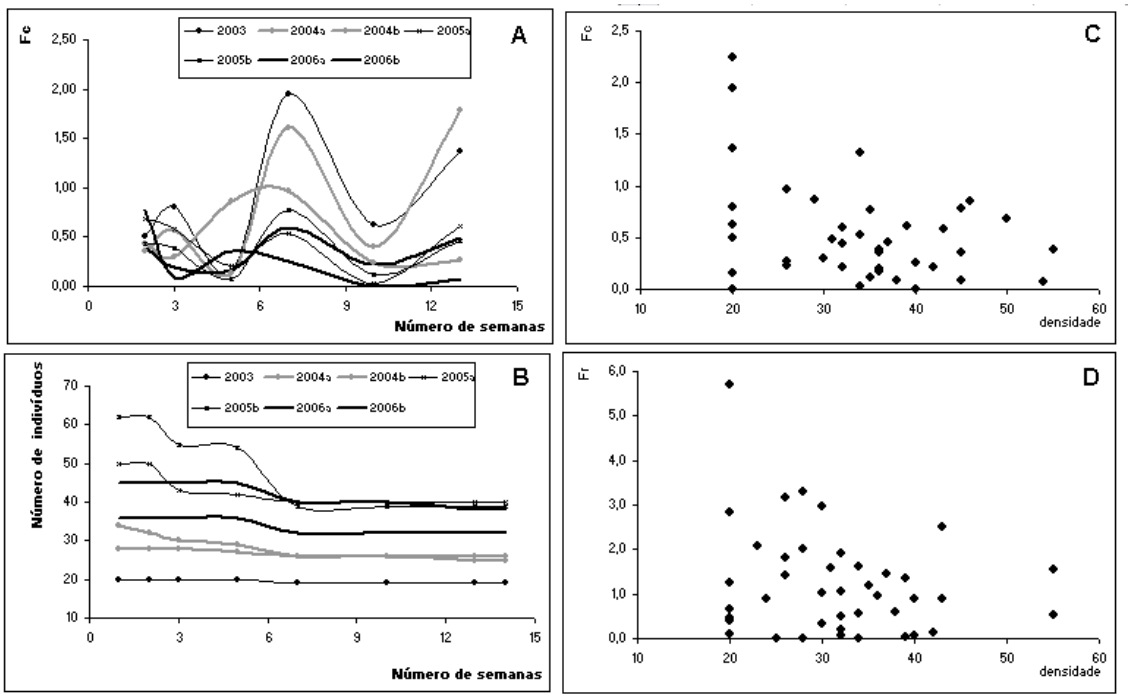


**Figura 5**. Efeito da densidade populacional sobre a reprodução sexuada e a sobrevivência de *G. tigrina*. **A**) Efeito da idade sobre a Fc (linhas representam o número de casulos/indivíduo/semana); **B**) efeito da densidade populacional sobre a sobrevivência (linhas representam o número de indivíduos); **C)** correlação entre a densidade populacional e o Fc (número de casulos/indivíduo/semana); **D**) correlação da densidade populacional e o Fr (número de filhotes/indivíduo/semana).

O cultivo de populações de *G. tigrina* pelo período de ~200 semanas de 2002 a 2007 foi monitorado. Nas duas populações (Figuras 6A e 6B) mantidas em quantidade de sais padrão, o aumento da idade dos indivíduos aparentemente diminui a taxa de reprodução (Figuras 6E e 6F); no entanto, não ocorre diminuição da produção de gametas (dados não mostrados).

A redução de 50% da quantidade de sais na água de cultivo causa uma redução similar no número de indivíduos (Figura 6C), e um aumento de 3x na concentração de sais reduz a longevidade das planárias a ~ 60 semanas (Figura 6D). No entanto, a concentração de sais parece não afetar a Fc (Figura 6E,F,G,H). Dentro da janela de tempo monitorada, observou-se que o aumento da idade dos indivíduos aparentemente diminui a taxa de reprodução (Fc), independentemente da concentração de sais da água de cultivo.

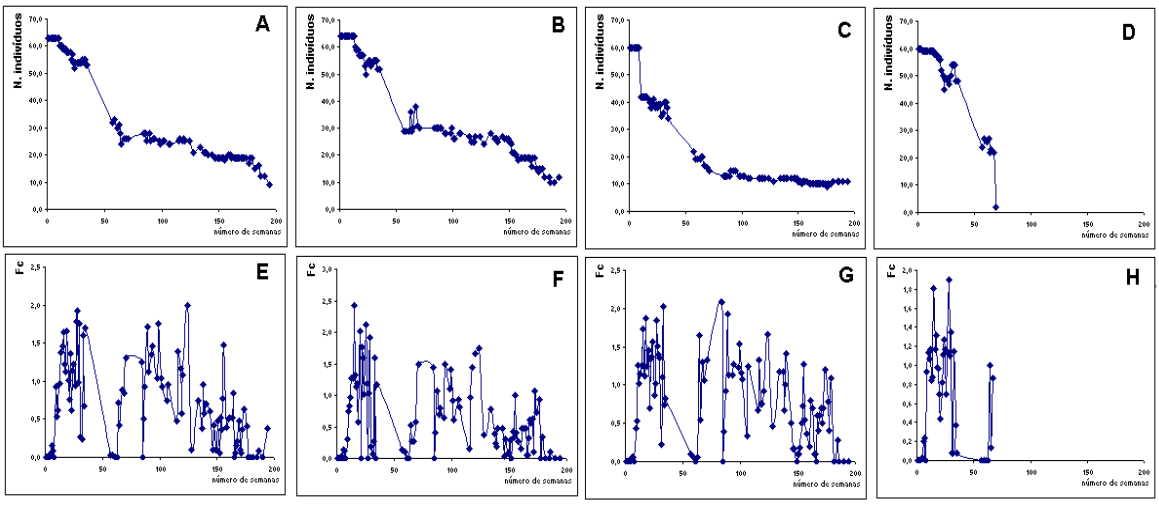



**Figura 6**. .A sobrevivência de *G. tigrina* e sua capacidade reprodutiva (Fc) foram monitoradas por ~200 semanas através do número de indivíduos sobreviventes e de sua fertilidade (Fc). **A** e **B**) sobrevivência de indivíduos cultivados em água de cultivo com quantidade de sais padrão; **C**) sobrevivência de indivíduos cultivados em água de cultivo com 50% de redução na quantidade de

sais; **D**) sobrevivência de indivíduos cultivados em água de cultivo com 150% de acréscimo na quantidade de sais; **E, F, G** e **F**) fertilidade de indivíduos cultivados nas condições descritas em A, B, C e D, respectivamente.

Esses resultados sugerem que uma porcentagem dos indivíduos de *G. tigrina* é mais longeva do que outros. A produção de gameta nessas populações (Figura B, com ~5 anos de idade) não difere da que ocorre em outras mais jovens (com ~4 e ~3 anos de idade). Provavelmente, a diminuição da fertilidade observada pode ser devido à redução da viabilidade dos gametas produzidos, e não devido à sobrevivência diferencial dos indivíduos menos férteis.

# 4. Discussão final

A caracterização biológica (ciclo de vida, modo de reprodução e ploidia) das populações nativas de *G. tigrina* e *G. schubarti* e o desenvolvimento de novos métodos de diagnóstico de fenótipos diferenciados (testes de toxicidade, teratogenicidade e mutagenicidade) são relevantes para o estudo das células-tronco, uma vez que estas são responsáveis não só pela capacidade de regeneração (BAGUÑÀ *et al*., 1989), mas também pela homeostase de todos os tecidos desses organismos (REDDIEN et al 2005a; 2005b) e pela produção de suas células germinativas (SATO *et al*., 2006).

A espécie *G. tigrina* apresenta duas raças cromossômicas - a diplóide e a triplóide, e a espécie *G. schubarti* apresenta três raças cromossômicas - a diplóide, a triplóide e a mixoplóide (KNAKIEVICZ *et al*., 2007). Em ambas as espécies, as raças triplóides e a raça mixoplóide da espécie *G. schubarti* distinguem-se das raças diplóides por serem exclusivamente fissiparitárias e terem tamanho corporal menor do que as raças cromossômicas diplóides (ver Capítulo 1).

É amplamente conhecido que mudanças no tamanho do genoma por multiplicações do genoma causam diversidade morfológica em animais e em plantas (GREGORY, 2005). Em anfíbios, o aumento da ploidia causa uma simplificação do sistema neuronal e diminuição do número de células nervosas (GREGORY, 2003) e, em planárias, causa diminuição considerável do tamanho corporal, assexualidade e diferenças de adaptabilidade à temperatura ambiental (ver Capítulo 1). Sabe-se que o número de células nervosas de planárias está intimamente correlacionado com o tamanho corporal (OVIEDO *et al*., 2003) e com a manutenção do número de células-tronco (GUO *et a*l., 2006). Por exemplo, o gene *cintillo* de *S. Mediterranea* é expressado em neurônios sensoriais que variam em número proporcionalmente ao tamanho do animal (OVIEDO *et al*.,

2003). Além disso, HORI & KISHIDA (1998) observaram que a decapitação de planárias acelera a freqüência de fissiparidade, provavelmente devido ao decréscimo nas concentrações de neurotransmissores (YOSHIZAWA *et al*., 1991). É provável que a proporção de tecido específico, número de células, tamanho do genoma, tamanho do animal e condições ambientais sejam intrinsecamente regulados. A interligação desses fatores pode ser parte do mecanismo de determinação do modo de reprodução (sexuado ou assexuado) em planárias. Como a interligação das funções-chave das células diferenciadas e indiferenciadas, tais como herança e adaptação, respondem à seleção natural permanece a ser esclarecido (HSIN & KENYAN, 1999).

Além disso, sabe-se que em indivíduos mixoplóides da espécie *G. schubarti* o aumento na disponibilidade de nutrientes aumenta a proporção de células 3n; entretanto, um aumento na proporção de células 3n parece não afetar a freqüência de fissiparidade (Figura 7). Assim, os organismos mixoplóides, nos quais as células 2n e 3n compartilham o mesmo nicho, também podem ser úteis na compreensão das inter-relações número de células/tamanho do genoma, e dos processos de diferenciação celular.

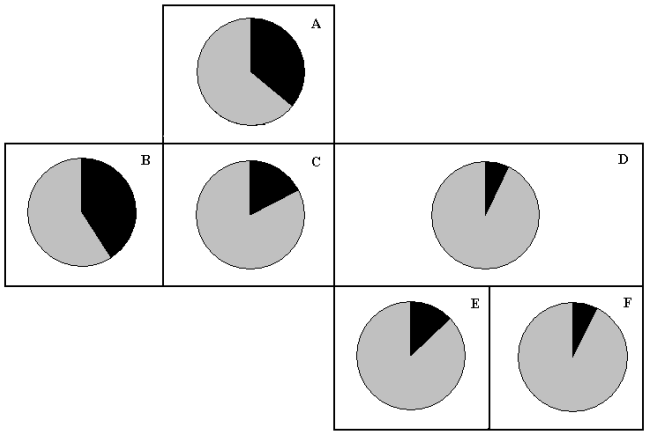



**Figura 7**. Flutuações na proporção de células triplóides (cinza) e diplóides (preto) em indivíduos *G. schubarti* mixoplóide (GsPop2). DN = dieta normal, alimento fornecido semanalmente; RC = restrição calórica, alimento fornecido a cada 20 dias; DC = dieta calórica, alimento fornecido a cada 4 dias. **A**) RC constante, de 1993 a 1996 (UFRGS); **B**) RC constante, de 1983 a 2000 (UNISINOS); **C**) DN, de 1996 a 1999; **D**) DC, durante seis meses em 1999; **E**) população

amostrada em **D**, submetida a RC por 9 meses, 2001; **F**) população amostrada em **D**, sob DC por mais 9 meses, 2001. (modificado de KNAKIEVICZ, 2001).

Outra questão interessante é a que envolve a elucidação dos mecanismos determinantes da longevidade dos organismos. Há uma correlação positiva entre a proporção tamanho do cérebro/tamanho do corpo e a longevidade dos organismos, especialmente em mamíferos (MATTSON *et al*., 2002). Isso sugere um papel para o cérebro na determinação da longevidade, provavelmente associado à capacidade do cérebro de estimular rotas de sinalização celular que aumentam a resistência a estresses oxidativos (KARBOWNIK *et al*., 2001; REITER, 2003) e/ou por sua interação com as células-tronco (GUO *et al*., 2006; REUTER & KRESHCHENKO, 2004). Em *C. elegans,* um aumento na longevidade do organismo ocorre quando as células-tronco da linhagem germinativa são mortas (HSIN & KENYAN, 1999). Em *G. schubarti*, os indivíduos assexuados (triplóides e/ou mixoplóides) são mais longevos e menores do que os sexuados (diplóides) (ver Capítulo I).

O aumento da idade, tanto para a *G. tigrina* sexuada (Figura 6) quanto para a *G. schubarti* assexuada, parecem ter pouco efeito sobre a reprodução (ver Capítulo I). Provavelmente, planárias contam com mecanismos eficientes de auto-seleção de neoblastos normais e eliminação dos anormais (KALAFATIC *et al*., 2004; ver Capítulo II), os quais podem compensar as desvantagens da reprodução assexuada a longo prazo (RICE, 2002). Se a proporção tamanho do cérebro/tamanho do corpo e/ou tamanho/quantidade de células fazem parte de mecanismos que regulam a manutenção das células-tronco e sua progênie (células germinativas e células somáticas) permanece para ser esclarecido. Também devem ser investigadas as possíveis correlações de tais mecanismos com o modo de reprodução (sexualidade e/ou assexualidade) e a longevidade em planárias.

Devido à excepcional capacidade de regeneração das planárias e à presença de uma grande população de células-tronco proliferativas, inclusive nos organismos adultos, o uso desses organismos para avaliação dos riscos biológicos dos poluentes tem se mostrado promissor (GUECHEVA *et al*., 2001,

2003; PRÁ *et al*., 2005). As planárias fornecem informações complementares do efeito tóxico, teratogênico, mutagênico e crônico das amostras avaliadas através de bioensaios simultâneos (Ver Capítulo III).

Esse conjunto de bioensaios (Figura 8), além ser útil à ecotoxicologia, pode auxiliar na obtenção de fenótipos diferenciados para o uso em estudos de desenvolvimento. A obtenção de fenótipos diferenciados bem caracterizados, aliada aos métodos avançados já disponíveis para outras espécies de planárias, tais como RNAi (SANCHEZ ALVARADO & NEWMARK, 1999), marcação com BrdU, (NEWMARK & SANCHEZ ALVARADO, 2000), bancos de dados de EST (ZAYAS *et al*., 2005), microRNA (PALAKODETI *et al*., 2006) e outras; podem ser recursos complementares úteis. Por exemplo, o conhecimento dos mecanismos moleculares envolvidos no processo de regeneração da SNC de planárias disponíveis (MINETA *et al*., 2003; INOUE *et al*., 2004), aliado à análise comparativa do efeito simultâneo tóxico, teratogênico e/ou mutagênico de agentes que interferem em rotas de sinalização conhecidas, pode auxiliar na obtenção de linhagens sensíveis a poluentes ambientais específicos de interesse para a saúde humana ou dos ecossistemas.

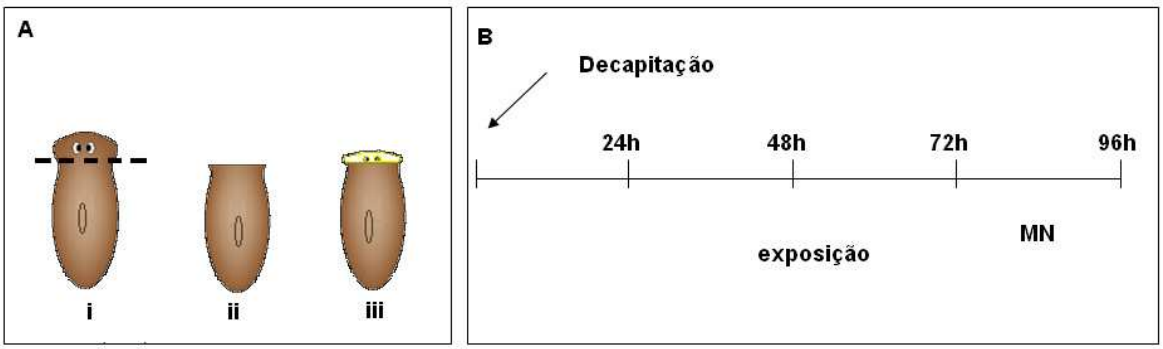



**Figura 8**. Esquema de exposição de planárias a substâncias de interesse. **A**) Planárias regenerantes (ii) são obtidas pela decapitação de planárias intactas (i) que completam a regeneração em 72 h a 96 h (iii). B) Procedimento de exposição de planárias intactas (A-i) ou regenerantes (A-ii) e troca de solução-teste a cada 24 h, seguida de análise de sobrevivência, mobilidade, regeneração e/ou freqüência de MN.

Conhece-se pouco a respeito de como o ciclo celular de células-tronco

somáticas de planárias adultas pode ser regulado. No entanto, existe uma série de evidências de que o ciclo celular dos neoblastos pode ser modulado por outros fatores além daqueles ativados pela regeneração (BAGUÑÀ, 1976). A proliferação dos neoblastos também responde a fatores ambientais, tais como disponibilidade de nutrientes (BAGUÑÁ, 1974; NIMETH *et al*., 2004), CEM (Figura 4A), luminosidade (Figura 4B), temperatura (Figuras 4C e 4D) e ritmo circadiano. Na regulação pelo ritmo circadiano, a fase S do ciclo celular de neoblastos é sincronizada com o período claro do dia, ao menos para *G. schubarti* (dados não mostrados), ao contrário do que é observado em vertebrados (MATSUO *et al*., 2003; MERROW & ROENNEBERG, 2004). Além disso, o ciclo celular de neoblastos 2n e 3n de planárias mixoplóides parece ser diferentemente modulado pela disponibilidade de nutrientes (Figura 7).

As células-tronco de planárias, conhecidas como neoblastos, assim como as de outros organismos apresentam várias características únicas quando comparadas com células diferenciadas, tais como a presença de corpos cromatóides (material citoplasmático eletrodenso) (SHIBATA *et al*., 1999), capacidade de proliferação (NEWMARK & SANCHEZ ALVARADO, 2000), citosol altamente basófilo (SÁNCHEZ ALVARADO & KANG, 2005), além de sensibilidade a raios X (AGATA, 2003; REDDIEN *et al*., 2005a). No entanto, a relativa uniformidade morfológica dos neoblastos totipotentes e da sua progênie (Figura 10) dificulta a determinação da heterogeneidade existente nessa população celular (REDDIEN & SÁNCHEZ ALVARADO, 2004).

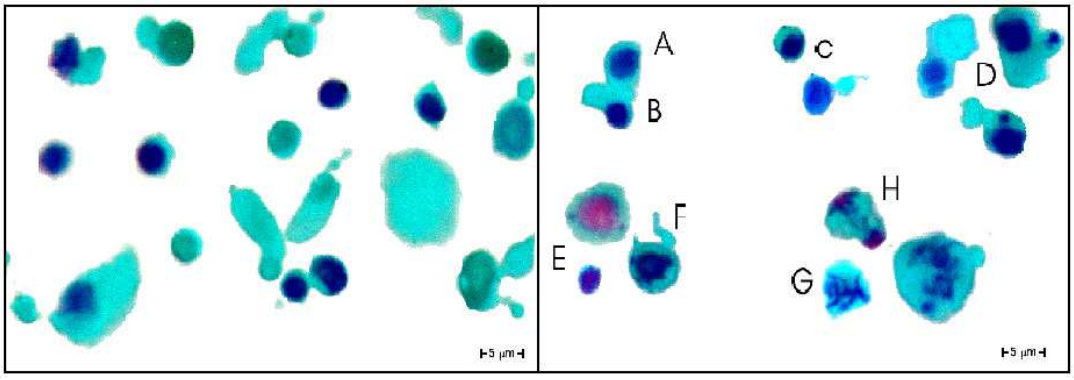



**Figura 10**. Neoblastos de planárias *Girardia tigrina*. Neoblasto eucromático (A) e heterocromático (B). Os micronúcleos (MN) pequenos (C), grandes (D) ou múltiplos (E,F). Anomalias cromossômicas, pontes (H) ou mitoses C (G).

Os corpos cromatóides (ricos em RNAs que codificam enzimas modificadoras de cromatina) dos neoblastos parecem estar comprometidos com o processo de diferenciação celular tecido-específico e posição-dependente, promovendo a transição da cromatina inativa para a ativa em domínios cromossômicos específicos (ROSSI *et al*., 2001; AGATA, 2003), os quais são induzidos a se formar por neuropeptídeos (HORI & KISHIDA, 2003). Recentemente, foi observado em células-tronco germinativas de *D. melanogaster* que a herança diferencial do centrômero assegura divisões mitóticas assimétricas (SPRADLING & ZHENG, 2007). A retenção permanente do centrômero-mãe promove considerável estabilidade e longevidade a essas células, sugerindo que esse mecanismo pode ser essencial à biologia das células-tronco. É provável que os fatores controladores da arquitetura da cromatina desempenhem um papel fundamental na definição do destino de desenvolvimento da progênie dos neoblastos (SÁNCHEZ ALVARADO & KANG, 2005).

Apesar da grande capacidade de regeneração, foram observados poucos indivíduos com crescimento desordenado de tecidos (Figura 11). As planárias, provavelmente, contam com mecanismos eficientes de eliminação dos neoblastos anormais (KALAFATIC *et al*., 2004). Observamos em ambas as espécies que a freqüência de MN em neoblastos aumenta de 48 h a 96 h após a exposição a raios $\gamma$. Após 120 h da exposição ao agente clastogênico, a freqüência de neoblastos micronúcleados diminui consideravelmente (ver Capítulo II). Essa diminuição na freqüência de neoblastos micronúcleados pode ser via diferenciação celular (REDDIEN *et al*., 2005b) e/ou por morte celular (UNGER *et al*., 1994; SABLINA *et al*., 1998; BAATOUT & DERRADII, 2004). O requerimento de um compartimento específico (mesênquima) para a manutenção das células-tronco (SALVETTI *et al*., 2000; OGAWA *et al*., 2002; ORII *et al*., 2005) e a diferenciação da progênie dos neoblastos dependente de migração tecido-específico (AGATA, 2003; REDDIEN & SÁNCHEZ ALVARADO, 2004) provavelmente sejam mecanismos importantes para a manutenção da homeostase de todos os tecidos desses organismos.

**Figura 11**. Planária *G. tigrina* com crescimento desordenado de tecidos. O tecido anormal surgiu em um animal com ~ 3 anos de idade e cultivado em condições-padrão (foto). Depois de ~ 6 meses, a planária sofreu fissões desprendendo o tecido anormal. O tecido anormal desprendido do corpo se degradou, e os fragmentos anterior e posterior regeneraram as partes perdidas, dando origem a dois novos organismos normais (foto: Prof. L.C. Amato)

Portanto, planárias são organismos promissores para a realização de uma ampla gama de estudos para investigar o papel das células-tronco, pois permitem múltiplas abordagens experimentais. Planárias podem ser organismos interessantes para o estudo das inter-relações entre proliferação celular, tamanho genômico e tamanho corporal, prováveis fatores responsáveis pela modulação/regulação da fissiparidade/assexualidade, e modo de reprodução (sexualidade e/ou assexualidade) e longevidade.

As planárias podem ser úteis para investigar a separação fisiológica das funções das células indiferenciadas e diferenciadas, as quais sofrem pressões operacionalmente opostas para produzir ganhos apropriados em adaptabilidade respectiva. Elas também servem para o estudo das inter-relações entre compartimento específico dos neoblastos e sua progênie, prováveis fatores responsáveis pela manutenção da homeostase de todos os tecidos. A compreensão dos mecanismos de manutenção da homeostase celular nesses seres pode contribuir para o desenvolvimento de terapias de interesse para a saúde humana.

# 5. Conclusões

**Quanto à ploidia e ao modo de reprodução**

- Em *G. tigrina* e *G. schubarti*, o modo de reprodução correlaciona-se com a ploidia; indivíduos diplóides são geralmente sexuados e os indivíduos triplóides e mixoplóides são exclusivamente fissiparitários.

- Em ambas as espécies, a progênie sexuada é maior (até ~ 14 vezes em *G. tigrina* e ~ 2 vezes em *G. schubarti*) do que a progênie assexuada; no entanto, a Fs (prole assexuada) nas planárias fissiparitárias é similar. Indivíduos mixoplóides da espécie *G. schubarti* morrem em temperaturas menores do que 6ºC.

- *G. tigrina* e *G. schubarti* diferem entre si em relação a alguns aspectos reprodutivos. Quanto às raças cromossômicas diplóides ou linhagens sexuadas, *G. tigrina* é em torno de 10 vezes mais fértil e/ou fecunda do que *G. schubarti*. Os casulos de *G. tigrina* são ~100% viáveis, enquanto que em *G. schubarti* a viabilidade dos casulos é de 77%. A freqüência de malformações em filhotes de *G. schubarti* é ~ 4 vezes maior do que aquela observada em filhotes de *G. tigrina*; e ~20% dos filhotes eram mixoplóides.

- A freqüência de reprodução sexuada e a longevidade em ambas as espécies sofrem influência das condições ambientais, tais como concentração de sais e aeração da água de cultivo, densidade populacional, tipo de alimento; no entanto, não foi observada troca entre os modos de reprodução sexuada e assexuada nos indivíduos.

**Quanto à longevidade**

- Em ambas as espécies, a densidade populacional e o tipo de alimento

afetam a longevidade. Populações diplóides de *G. tigrina* com ~ 40 indivíduos·$L^{-1}$ são naturalmente estabelecidas por morte de indivíduos supernumerários dentro de algumas semanas de cultivo. Populações diplóides de *G. schubarti* são estáveis com até ~ 50 indivíduos·$L^{-1}$; no entanto, o aumento da densidade populacional acima de ~12 indivíduos·$L^{-1}$ leva à diminuição na fertilidade.

- Em populações diplóides de *G. tigrina,* o aumento da densidade populacional não altera a Fc e a Fr; no entanto, o aumento da idade diminui a Fc e Fr, mas parece não afetar a produção de gametas.

- Ao menos em *G. schubarti*, as populações poliplóides (com indivíduos triplóides e mixoplóides) são mais longevas do que as diplóides em temperaturas entre 13 e 21ºC; e são sensíveis a baixas temperaturas (> 6ºC);

- Em populações mixoplóides de *G. schubarti*, o aumento populacional não reduz a freqüência de fissiparidade (Fs), a densidade tende a aumentar até ocorrer a morte simultânea de praticamente todos os indivíduos da população. O aumento na idade das populações parece não afetar a Fs.

**Quanto à regeneração**

- *G. tigrina* e *G. schubarti,* quando decapitadas, regeneram suas cabeças em ~ 4 dias, exceto para planárias diplóides *G. schubarti* adultas (com mais de 12 meses de idade e com 20 mm a 30 mm de comprimento), que requerem o dobro do tempo.

- Em ambas as espécies, durante a regeneração os fragmentos de qualquer parte do corpo (da cabeça, do tronco ou da cauda) mantêm o comprimento do fragmento inicial;

- Em ambas as espécies, condições ambientais, tais como temperatura, intensidade luminosa e CEM afetam a regeneração.

**Quanto à suscetibilidade a agentes tóxicos e à identificação de biomarcadores.**

- Em *G. tigrina* e *G. schubarti*, os agentes clastogênicos raios γ, MMS e CP induzem aumento significativo na freqüência de formação de MN somente em neoblastos de planárias regenerantes.

- Em ambas as espécies, a exposição a agentes clastogênicos concomitantemente com o início da regeneração leva a um aumento significativo na freqüência de MN entre 72 h e 96 h, seguido de diminuição após 120 h;

- *G. tigrina,* quando exposta a solução de sulfato de cobre, apresentou fluxo dinâmico de entrada e saída desse metal e permitiu avaliações múltiplas de toxicidade através dos bioensaios simultâneos de mortalidade/mobilidade, de regeneração e de MN; e da avaliação do desempenho reprodutivo. Esse conjunto de bioensaios demonstrou ser um método sensível e fácil para monitorar o efeito da poluição aguda e/ou crônica.

# 6. Perspectivas

Este trabalho tem como perspectivas:

- realizar a normatização dos testes ecotoxicológicos já padronizados para *G. schubarti* e *G. tigrina* de acordo com as normas técnicas da ABNT;

- caracterizar o ciclo celular normal de neoblastos de *G. schubarti* e de *G. tigrina* e suas possíveis variações em função do estágio de desenvolvimento, idade ou ploidia, durante a regeneração e em diferentes condições nutricionais e de fotoperíodo;

- padronizar o cultivo *in vitro* de neoblastos de *G. schubarti* e de *G. tigrina*;

- estudar comparativamente o comportamento de neoblastos das duas espécies em cultura;

- desenvolver ensaios de citotoxicidade, teratogenicidade e mutagenicidade baseados em neoblastos em cultura;

- realizar estudos de proteômica comparativa para identificar proteínas expressadas diferencialmente em planárias intactas e regenerantes e/ou em diferentes estágios de desenvolvimento.

# 7. Referências Bibliográficas


AGATA, K. Molecular e Cellular approaches to regeneration of planarians. *Zoological Sci.ence*, 19: 1391-1392, 2002.

AGATA, K. Regeneration and gene regulation in planarians. *Current opinion in genetics & development*, 13: 492–496, 2003.

AGATA, K., SOEJIMA, Y., KATO, K., KOBAYASHI, C., UMESONO, Y. & WATANABE, K:, Structure of the planarian central nervous system (CNS) revealed by neuronal cell markers. *Zoological Science*, 15: 433-440, 1998.

ARAGÃO, M. A. & ARAÚJO, R. P. A. Métodos de ensaios de Toxicologia com organismos aquáticos In: Zagatto, P. A. & Bertoletti, E. (ed), 2006, Ecotoxicologia aquática – Princípios e aplicações. Ed. RiMa, São Carlos, p 117-152. 2006.

BAATOUT, S. & DERRADJI, H. Cytometric methods to analyses radiation effects. *Journal of Biological regulators and homeostatic agents*, 18: 101-105, 2004.

BAGUÑÁ, J. Dramatic mitotic response in planarians after feeding, and hypothesis for the control mechanism. *Journal experimental zoology*, 190: 117-122, 1974.

BAGUÑÀ, J. SALÓ, E. & AULADELL, C. Regeneration and pattern formation in planarians. III Evidence that neoblasts are totipotent stem cells and the source of blastema cells. *Development*, 107: 77-86, 1989.

BAGUÑÀ, J. Mitosis in the Intact and regeneranting planarian *Dugesia mediterranea* n.sp. II mitosis studies during regeneration, and a possible mechanism of blastema formation. *Journal experimental zoology*, 195: 65-80, 1976.

BEST, J. B. & MORITA, M. Toxicology of planarians. *Hydrobiology*, 227: 375-383, 1991.

CALEVRO, F., FILIPPI, C., DERI, P., ALBERTOSI, C. & BATISTONI, R. Toxic effects of

Aluminum, Chromium and Cadmium in intact and regenerating freshwater planarians. *Chemosphere*, 37: 651-659, 1998.

CALEVRO, F., CAMPINI, S., FILIPPI, C., BATISTONI, R., DERI, P., BUCCI, S., RAGGHIANTI, M. & MANCINO G. Bioassays for testing effects of Al, Cr and Cd using development in the amphibian *Pleurodeles waltl* and regeneration in the planarian *Dugesia etrusca*. *Aquatic Ecosystem Health and Management*, 2: 281-288, 1999.

CEBRIÀ, F., KOBAYASHI, C., UMESONO, Y., NAKAZAWA, M., MINETA, K., IKEO, K., GOJOBORI, T., ITOHK, M., TAIRAK, M., SÁNCHEZ ALVARADO A. & AGATA, K. FGFR-related gene nou-darake restricts brain tissues to the head region of planarians, *Nature*, 419: 620-624, 2002.

D´SOUZA, T.G., STORHAS, M., SCHUENBURG, H., BEUKEBOOM, L.W. & MICHIELS, N.K. Ocasional sex in an ´asexual´ polyploid hermaphrodite. *Proceedings: Biological science*, 271: 1001-1007, 2004.

ERICHSEN JONES, J. R. A further study of the relations between toxicity and solution pressure, with *Polycelis nigra* as test animal. *Journal of Experimental Biology*, 17: 408-415, 1940.

FAIRWEATHER I. & SKUCE, P.J. Flatworm neuropeptides – present status, future directions. Hydrobiologia 305: 309-316, 1995.

GAMO, J. & NOREÑA-JANSSEM, C. Old and new records of turbellarians from the central areas of Spain. *Hydrobiologia*, 383: 299-305, 1998.

GONZÁLEZ-ESTÉVEZ C., MOMOSE, T., GEHRING, W. J., & SALO, E. Transgenic planarian lines obtained by electroporation using transposon-derived vectors and an eye-specific GFP marker. *Proceedings of the National Academy of Sciences of the United States of America*, 100: 14046–14051, 2003.

GOULART, M. & CALLISTO, M. Bioindicadores de qualidade de água como ferramenta em estudos de impacto ambiental. *Revista da FEPAM*, 2 (1) 2003.

GREGORY, T.R. Variation across amphibian species in the size of the nuclear genome supports a pluralistic, hierarchical approach to the C-value enigma. *Biological*

*journal of the Linnean society*, 79: 329–339, 2003.

GREGORY, T. R. The C-value Enigma in Plants and Animals: A Review of Parallels and an Appeal for Partnership. *Annals of botany*, 95: 133–146, 2005.

GSCHEWENTNER, R., LADURNER, P., NIMETH, K., • RIEGER, R. Stem cells in a basal bilaterian S-phase and mitotic cells in Convolutriloba longifissura (Acoela, Platyhelminthes). *Cell Tissue Research*, 304:401–408, 2001.

GUECHEVA, T., HENRIQUES, J. A. P. & ERDTMANN, B. Genotoxic effects of copper sulphate in freshwater planarian in vivo, studied with the single-cell gel test (comet assay). *Mutation research*, 497: 19-27, 2001.

GUO, T., PETERS, A. H. F. M. & NEWMARK, P. A. A bruno-like gene is required for stem cell maintenance in planarians. *Developmental Cell*, 11, 159–169, 2006.

HOLSTEIN, T.W., HOBMAYER, E. & DAVID, C.N. Pattern of epithelial cell cycling in hydra. *Developmental biology*, 148: 602-611, 1991.

HORI I, & KISHIDA Y. Quantitative changes in nuclear pores and chromatoid bodies induced by neuropeptides during cell differentiation in the planarian *Dugesia japonica*. *Journal submicroscopic cytology pathology*, 35(4): 439-44, 2003.

HORI, I. & KISHIDA, Y. A fine structural study of regeneration after fission in the planarian *Dugesia japonica*. *Hydrobiologia*, 383: 131-136, 1998.

HSIN, H. & KENYAN, C. Sinals from the reproductive system regulate the lifespan of *C. elegans*. *Nature*, 399: 326-362, 1999.

INDEHERBERG, M. B. M., VAN STRAALEN, N. M. & SCHOCKAERT, E. R. Combining life-history and toxicokinetic parameters to interpret differences in sensitivity to cadmium between population of *Polycelus tenuis* (Platyhelminthes). *Ecotoxicology and environmental safety*, 44: 1-11, 1999.

INOUE, T., KUMAMOTO, H., OKAMOTO, K., UMESONO, Y. SAKAI, M. SANCHEZ ALVARADO A. & AGATA K. Morfological and funcional recovery of planarian photosensig system during head regeneration. *Zoological science*, 21: 275 – 283, 2004.

ITOH, M. T., SHINOZAWA, T. & SUMI, Y. Circadian rhythms of melatonin-synthesizing enzyme activities and melatonin levels in planarians. *Brain research*, 830: 18-25, 1999.

KALAFATIĆ, M., KOPJAR, N., BESENDORFER, V. The impairments of neoblast division in regenerating planarian *Polycelis felina* (Daly.) caused by in vitro treatment with cadmium sulfate. *Toxicology in vitro*. 18: 99-107, 2004.

KARBOWNIK, M., LEWINSKI, A. & REITER, R. J. Anti-carcinogenic actions of melatonin which involve antioxidative processes: comparison with other antioxidants. *International journal of biochemistry and molecular biology*, 33: 735–753, 2001.

KATO, K., ORII, H., WATANABE, K. & AGATA, K. The role of dorsoventral interaction in the onset of planarian regeneration. *Development,* 126: 1031-1040, 1999.

KNAKIEVICZ, T. Avaliação citogenética, reprodutiva e biogeográfica das espécies de planárias (Platelhimenthes, Tricladida, Paludicola) no Rio Grande do Sul. Dissertação de Mestrado. Curso de Pós Graduação em Genética e Biologia Molecular - Universidade Federal do Rio Grande do Sul. Porto Alegre – RS, 2001.

KNAKIEVICZ, T., LAU, A. H., PRÁ D. & ERDTMANN, B. Biogeography and Karyotypes of Freshwater Planarians (Platyhelminthes, Tricladida, Paludicola) in Southern Brazil. *Zoological Science*, 24: 123-129. 2007.

KOSTELECKY, J., ELLIOTT, B. & SCHAEFFER, D. J. Planarians in toxicology. I. Physiology of sexual-only *Dugesia dorotocephala*: Effects of diet and population density on adult weight and cocoon production. *Ecotoxicology and environmental safety* 18 (3): 286-295, 1989.

MARTELLY, I. & FRANQUINET, R. Planarian regeneration as a model for cellular activation studies. *Trends in biological science*, 9: 468-471, 1984.

MATSUO, T., YAMAGUCHI, S., MITSUI, S., EMI, A., SHIMODA, F. & OKAMURA, H. Control mechanism of the circadian clock for timing of cell division in vivo. *Science*, 302: 255-259, 2003.

MATTSON, M. P., DUAN, W. & MASWOOD, N. How does the brain control lifespan? Ageing Research Reviews, 1: 155-165, 2002.

MERROW, M. & ROENNEBERG, T. Cellular Clocks: Coupled Circadian and Cell Division Cycles. *Current biology*, 14: R25–R26, 2004.

MINETA, K., NAKAZAWA, M., CEBRIA, F., IKEO, K., AGATA, K. & GOJOBORI, T. Origin and evolutionary process of the CNS elucidated by comparative genomics analysis of planarian ESTs. Proceedings of the National Academy of Sciences of the United States of America, 100: 7666–7671, 2003.

MORGAN T. H. Experimental studies of the regeneration of *Planaria maculata*. *Arch Entw Mech Org* 7: 364-397, 1898. in: NEWMARK, P. A. & ALVARADO, A. S. Not your father's planarian: a classic model enters the era of functional genomics. *Nature reviews*, 3: 210-220, 2002.

MORITA, M. & BEST, J. B. Effects of photoperiods and melatonin on planarian asexual reproduction. *Journal experimental zoology*, 231: 273-282, 1984

NASCIMENTO, I. A., PEREIRA, S. A. & LEITE M. B. N. L. Biomarcadores como instrumentos preventivos de poluição. In: ZAGATTO P. A. & BERTOLETTI E. (ed), 2006, Ecotoxicologia aquática – Princípios e aplicações. Ed. RiMa. São Carlos, pp. 413-432, 2006.

NEWMARK, P. A. & ALVARADO, A. S. Not your father's planarian: a classic model enters the era of functional genomics. *Nature Reviews*, 3: 210-220, 2002.

NEWMARK P. A. & SÁNCHEZ ALVARADO A. Bromodeoxyuridine specifically labels the regenerative stem cells of planarians. *Developmental Biology,* 220: 142-153, 2000.

NIMETH, K. T., MAHLKNECHT, M., MEZZANATO, A., PETER, R., RIEGER, R. & LADURNER, P. Stem cell dynamics during growth, feeding, and starvation in the basal flatworm *Macrostomum sp*. (Platyhelminthes). *Developmental dynamics*, 230: 91-99, 2004.

OGAWA, K., ISHIHARA, S., SAITO, Y., MINETA, K., NAKAZAWA, M., IKEO, K., GOJOBORI, T., WATANABE, K. & AGATA, K. Induction of a noggin-like gene by ectopic DV interaction during planarian regeneration. *Developmental biology*, 250: 59–70, 2002.

ORII, H., SAKURAI, T. & WATANABE, K. Distribution of the stem cells (neoblasts) in the planarian *Dugesia japonica*. *Developmental genes evolution*, 215: 143–157, 2005.

ORII, H., KATO, K., UMESONO, Y., SAKURAI, T., AGATA, K. & WATANABE, K. The planarian HOM/HOX homeobox genes (Plox) expressed along the anteroposterior Axis. *Developmental biology*, 210: 456–468, 1999.

OVIEDO, N. J., NEWMARK, P. A. & SÁNCHEZ ALVARADO, A. Allometric scaling and proportion regulation in the freshwater planarian *Schmidtea mediterranea*. *Developmental dynamics*, 226: 326–333, 2003.

PALAKODETI, D., SMIELEWSKA, M. & GRAVELEY, B. R. MicroRNAs from the planarian *Schmidtea mediterranea*: a model system for stem cell biology. *RNA*, 12(9): 1640-1649, 2006.

PENNISI, E. RNAi takes evo-devo world by storm. *Sciences*, 304: 384, 2004.

PRÁ, D., LAU, A. H., KNAKIEVICZ, T., CARNEIRO, F. R. & ERDTMANN, B. Environmental genotoxicity assessment of an urban stream using freshwater planarians. *Mutation research*, 585: 79–85, 2005.

PREZA, D. L. C. *Girardia tigrina* (Girard, 1850) (Turbellaria: Tricladida: Paludicola): aspectos biológicos e seu emprego em testes de toxicidade. Dissertação de Mestrado. UFBA Salvador-BA, 1995.

REDDIEN, P. W. & SÁNCHEZ ALVARADO, A. Fundamentals of planarian regeneration. *Annual review of cell and developmental biology*, 20: 725–757, 2004.

REDDIEN, P. W., BERMANGE, A. L., MURFITT, K. J., JENNINGS, J.R. & SÁNCHEZ ALVARADO, A. Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planarian. *Developmental cell*, 8: 635–649, 2005a.

REDDIEN, P. W., OVIEDO, N. J., JENNINGS, J. R., JENKIN, J.C. & SÁNCHEZ ALVARADO, A. SMEDWI-2 is a PIWI-like protein that regulates planarian stem cells. *Science*, 310: 1327–1330, 2005b.

REITER, R. J. Melatonin: clinical relevance. *Best practice & research clinical endocrinology and metabolism*, 17: 273–285, 2003.

REUTER, M. & KRESHCHENKO, N. flatworm asexual multiplication implicates stem cells and regeneration. *Canadian journal zoological*, 82: 334-356. 2004.

RICE, W. R. Experimental tests of the adaptive significance of sexual recombination. *Nature reviews*, 3: 241-241, 2002.

ROSSI, L., BATISTONI, R., SALVETTI, A., DERI, P., BERNINI, F. ANDREOLI, I. FALLENI, A. & GREMIGNI, V. Molecular aspects of cell proliferationand neurogenesis in planarians. *Belgian journal zoology*, 131: 83-87, 2001.

RUPPERT, E. E., FOX, R. S. & BARNES, R. D. Zoologia dos invertebrados, 7th ed. Editora Roca. São Paulo. pp. 259-308, 2005.

SABLINA, A. A., ILYINSKAYA, G. V., RUBTSOVA, S. N., AGAPOVA, L. S., CHUMAKOV, P. M. & KOPNIN, B.P. Activation of p53-mediated cell cycle checkpoint in response to micronuclei formation, Journal cell science, 111: 977–984, 1998.

SALA, O. E., CHAPIN, III, F. S. & ARMESTO, J.J. Global biodiversities scenarios for the year 2100. *Science*, 287: 1770-1774, 2000.

SALVETTI, A., ROSSI, L., DERI, P. & BATISTONI, R. An MCM2-related gene is expressed in proliferating cells of intact and regenerating planarians. *Developmental dynamics*, 218: 603-614, 2000.

SÁNCHEZ ALVARADO, A. & KANG, H. Multicellularity, stem cells, and the neoblasts of the planarian *Schmidtea mediterranea. Experimental cell research*, 306: 299 – 308, 2005.

SANCHEZ ALVARADO A. & NEWMARK, P. A. Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proceedings of the National Academy of Sciences of the United States of America*, 96: 5049–5054, 1999.

SÁNCHEZ ALVARADO, A., NEWMARK, P. A., ROBB, S. M. C. & JUSTE, R. The *Schmidtea mediterranea* database as a molecular resource for studying platyhelminthes, stem cells and regeneration. *Development*, 129: 5659-5665, 2002.

SÁNCHEZ ALVARADO, A. Regeneration in the metazoans: why does it happen? *Bioessays*, 22: 578–590, 2000.

SATO, K., SHIBATA, N., ORII, H., AMIKURA, R., SAKURAI, T., AGATA, K., KOBAYASHI,

S. & WATANABE, K. Identification and origin of the germline stem cells as revealed by the expression of nanos-related gene in planarians. *Development, growth & differentiation*, 48: 615–628, 2006.

SCHÜRMANN, W. & PETER, R. Planarian cell culture: a comparative review of methods and an improved protocol for primary cultures of neoblasts. *Belgian journal of zoology*, 131 (Supplement 1): 123-130, 2001.

SCHÜRMANN, W. & PETER, R. Inhibition of regeneration in the planarian *Dugesia polychroa* (*Schmidt*) by treatment with magnesium chloride: a morphological study of wound closure. *Hydrobiologia*, 383: 111- 116, 1998.

SHIBATA, N., UMESONO, Y., ORII, H., SAKURAI, T., WATANABE, K. & AGATA, K. Expression of *vasa* (vas)-related genes in germline cells and totipotent somatic stem cells of planarians. *Developmental biology*, 206:73-87, 1999.

SLUYS, R., HAUSER, J. & WIRTH, Q. J. Deviation on the grounplan: a unik new species of freshwater planarians from South Brazil Platyhelminthes: Tricladida: Paludicola. *Journal of zoology*, 241: 593-601, 1997.

SLUYS, R. Phylogenetic relationships of the triclads (Platyhelminthes, Seriata, Tricladida). *Bijdragen dierk*, 59: 3-25, 1989.

SALO, E. The power of regeneration and the stem-cell kingdom: freshwater planarians (Platyhelminthes). *Bioessays*, 28(5): 546-559, 2006.

SPRADLING, A. C. & ZHENG, Y. The Mother of All Stem Cells? *Science*, 315: 469-470, 2007.

STOHLER, R. A., CURTIS, J. & MINCHELLA, D. J. A comparison of microsatellite polymorphism and heterozygosity among field and laboratory populations of *Schistosoma mansoni*. *International journal for parasitology,* 34: 595–601, 2004.

STORHAS, M., WEINZIERL, R. P. & MICHIELS, N. K. Paternal sex in parthenogenetic planarians: a tool to investigate the accumulation of deleterious mutations. *Journal evolutionay biology*, 13: 1-8, 2000.

UNGER, C., KRESS, S., BUCHMANN, A. & SCHWARZ, M. Gamma irradiation-induced micronuclei from mouse hepatoma cells accumulate high levels of the tumour

suppressor protein p53, Cancer research, 54 (14): 3651–3655, 1994.

VARGAS, V. M. F., MIGLIAVACCA, S. B., MELO, A. C., HORN, R. C., GUIDOBONO, R. R., FERREIRA, I. C. F. S. & PESTANA, M. H. D. Genotoxicity assessment in aquatic environments under the influence of heavy metals and organic contaminants, *Mutation research*, 490: 141–158, 2001.

VREYS, C. & MICHIELIS, N. K. Sperm trading by volume in a Platyhelminthe platworm with mutal penis intromission. *Animal behaviour*, 56: 777-785, 1998.

VRIES, E. L. de & SLUYS, R. Phylogenetic relationships of the genus Dugesia (Platyhelminthes, Tricladida, Paludicola). *Journal of zoology (London, England: 1987)*, 223: 103-116, 1991.

YOSHIZAWA, Y., WAKABAYASHI, K. & SHINAZAWA, T. Inibition of planarian regeneration by melatonin. *Hydrobiologia*, 227: 31-40, 1991.

ZAGATTO P. A. & BERTOLETTI E. (ed) Ecotoxicologia aquática – Princípios e aplicações. Ed. RiMa. São Carlos, pp 464, 2006.

ZAYAS, R. M., HERNANDEZ, A., HABERMANN, B., WANG, Y., STARY, J. M. & NEWMARK, P. A. The planarian *Schmidtea mediterranea* as a model for epigenetic germ cell specification: Analysis of ESTs from the hermaphroditic strain. *Proceedings of the National Academy of Sciences of the United States of America*, 102 (51): 18491–18496, 2005.

# 8. Anexos

## *8.1 Condições de cultivo, manutenção e teste*

A seguir são apresentadas as condições otimizadas para a manutenção das populações de planárias em laboratório e para a realização de testes ecotoxicológicos de curta e longa duração.

**Tabela 1A. Requerimentos para aceitabilidade de testes de toxicidade de 5 dias com planárias *G. tigrina* ou *G. schubarti***. (Lista de exigências compilada a partir dos resultados obtidos durante os procedimentos para padronização de testes desenvolvidos na elaboração dos resultados apresentados nos Capítulos I, II e III da seção de Resultados).

1. A idade dos organismos-teste no início dos ensaios deve estar dentro dos limites requeridos de 0 a 7 dias para filhotes recém-nascidos e de 3 meses para adultos.

2. Os níveis de dureza, alcalinidade, pH da água de cultivo não devem variar mais do que 50% durante os testes.

3. Mensalmente, ou no início de testes com poluentes, o lote de organismos utilizado deve ser avaliado usando-se uma substância tóxica de referência (por exemplo, o sulfato de cobre). A duração do teste poderá ser de 48 h. É aceitavel para utilização em testes o lote de organismos onde houver sobrevivência de 90% dos organismos do controle (água de cultivo).

4. A temperatura da água de cultivo deve ser medida diariamente, e semanalmente o pH da água de cultivo, dureza, alcalinidade e condutividade da água de cultivo devem ser medidos mensalmente.

## Tabela 1A. Continuação

5. A qualidade dos alimentos (fígado bovino congelado) e a contaminação da água devem ser caracterizadas se forem observados problemas na cultura dos organismos ou durante os testes.

6. Medidas fisiológicas, tais como idade da maturidade sexual e taxas de fertilidade e fecundidade, podem fornecer informações úteis sobre a saúde das populações cultivadas.

7. Todos os organismos em um teste devem ter a mesma origem.

8. É recomendável o início dos testes o mais breve possível após a coleta das amostras de água no campo.

9. Todos os recipientes devem ter o mesmo tamanho e compartilhar o mesmo número de organismos e de volume de amostra.

10. Um controle negativo e controles positivos apropriados devem ser incluídos no teste. A média de sobrevivência de planárias no controle deve ser maior ou igual a 80% no final dos testes.

11. Diariamente, organismos mortos devem ser removidos dos recipientes-teste e as soluções devem ser renovadas.

12. A temperatura durante os testes deve ser a mesma da cultura das populações. A aclimatação dos organismos-teste na água-teste é requerida.

13. A variação média diária da temperatura-teste dever estar entre ± 1C° da temperatura desejada. Alterações instântaneas podem osciliar em até ± 3C° em relação à temperatura desejada.

14. As características físico-químicas naturais das amostras de água coletadas para serem testadas devem estar dentro dos limites de tolerância dos organismos-teste. Relacionado ao item 8.

**Tabela 2A. Resumo das condições de cultivo de planárias em laboratório.**

| Condições de cultivo | Recomendado |
|---|---|
| Sistema | Semi-estático |
| Temperatura | 20 ± 2 ºC |
| Qualidade da luz | Luz fria, tipo fluorescente |
| Intensidade luminosa | 35 FC |
| Fotoperíodo | 12 h luz;12 h escuro |
| Tipo/capacidade do recipiente | Recipente plástico de 2 L, com tampa fosca |
| Água de manutenção | Reconstituda com dureza de 0 a 2 mg $CaCO_3 \cdot L^{-1}$, pH 7,2 a 7,6 e condutividade de 62 a 63 $mS \cdot cm^{-1}$ |
| Volume de água de manutenção por recipiente | Aproximadamente 1000 ml |
| Aeração | Não |
| Troca de água | 1 vez por semana |
| Nº de organismos/recipiente | 40 |
| Tipo de organismos/recipiente | Organismos matrizes ou organismos-teste |
| Nº de recipientes com organismos matrizes | Mínimo de 6, sendo que cada um dever conter organismos de determinada idade, ou seja, de 3 a 48 meses |
| Nº de recipientes com organismos-teste | Mínimo de 3, sendo que cada um deve conter organismos de determinada idade |
| Idade dos organismos | conhecida |
| Alimentação | Semanal, num mesmo dia da semana |
| Tipo e qualidade de alimento/recipiente/ semana | Fígado bovino congelado por, no máximo, um mês |
| Duração das culturas | indeterminado |
| Controles diários | Temperatura da água e máxima e míminia do ar do ambiente |
| Controles no dias da troca de água | sobrevivência dos organismos nas culturas, pH, temperatura da água de manutenção.<br>Contagem e remoção do nº de casulos para um recipiente datado. Os filhotes eclodidos dos casulos também são contados e removidos para outro recipiente para início de uma nova população (matriz ou testes). |
| Controle da sensibilidade das culturas | Teste mensal com uma substância de referência. A $CL_{50}$ obtida deve estar em um intervalo de ± 2 desvios-padrão em relação aos valores médios anteriormente obtidos |
| Controles adicionais | Observação periódica da presença ou não de contaminantes por fungos ou outros organismos. Anotar essas datas |

**Tabela 3A. Resumo das condições de testes de toxicidade aguda com *Girardia tigrina* e *G. schubarti***

| Condições-teste | *G. tigrina* (diplóide sexuada) | *G. schubarti* (mixoplóide assexuada) |
|---|---|---|
| Sistema de teste | Semi-estático | |
| Duração | 24 a 96 | |
| Temperatura | 20 ± 2 ºC | |
| Qualidade de luz | Luz fria, tipo fluorescente | |
| Intensidade luminosa | 35 FC | |
| Fotoperíodo | 12 h luz;12 h escuro | |
| Tamanho do frasco-teste | Recipente plástico de 100 mL, com tampa | |
| Volume da solução-teste | 20 mL | |
| Renovação da solução-teste | diária | |
| Idade dos organismos | Conhecida - filhotes (de 0 a 7 dias de vida) ou adultos (3 meses de idade) | Não estabelecido (tamanho de 7 a 10 mm) |
| Nº organismo/réplica | 5 | |
| Nº réplicas/concentração | 4 | |
| Nº de soluções-teste | 4 a 5 | |
| Fator de diluição | 0,3 ou 0,5 | |
| Alimentação durante os testes | Não | |
| Troca dos frascos-teste | Não | |
| Aeração das soluções-teste | Não | |
| Água de diluíção | Reconstituída | |
| Critérios de avaliação de efeito | Mortalidade, mobilidade, regeneração de cabeça, freqüência de MN | |
| Biomarcadores (*endpoint*) de exposição aguda | Testes de mobilidade, regeneração e MN | |
| Biomarcadores (*endpoint*) de exposição crônica | Avaliação de desempenho reprodutivo | |
| Expressão dos resultados | $DL_{50}$, $mCI_{50}$, $rCI_{50}$ Teste de MN, Fc e Fr (24 a 96 h) | |
| Critérios de aceitação do teste | > 80% de sobrevivência dos organismos-controle | |