**UNIVERSIDADE FEDERAL DO RIO GRANDE – FURG**

**PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS – FISIOLOGIA ANIMAL COMPARADA**

# EFEITOS TÓXICOS DO HERBICIDA ROUNDUP NO PEIXE *Poecilia vivipara* (Bloch e Schneider, 1801)

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**SUMÁRIO**

# RESUMO GERAL

Apesar de se acreditar que herbicidas a base de glifosato são relativamente não tóxicos aos humanos, sua ampla utilização na agricultura e consequente contaminação dos sistemas aquáticos é preocupante. No presente trabalho, parâmetros reprodutivos (qualidade espermática) e bioquímicos (atividade da acetilcolinesterase e da glutationa S-transferase, lipoperoxidação e capacidade antioxidante contra radicais peroxil) foram avaliados em adultos de barrigudinhos (*Poecilia vivipara*) aclimatados em água doce e expostos (96 h) à formulação comercial Roundup em concentrações ambientalmente realísticas de glifosato (130 e 700 $\mu g.L^{-1}$). Barrigudinhos machos expostos ao Roundup apresentaram uma baixa qualidade espermática, mensurada pela redução da integridade da membrana plasmática, funcionalidade mitocondrial, integridade do DNA, motilidade, período de motilidade e concentração das células espermáticas, quando comparados com os machos mantidos na condição controle (sem adição de Roundup na água). A maioria dos parâmetros espermáticos analisados mostrou um forte grau de associação, o que pode auxiliar no entendimento dos mecanismos envolvidos na redução da qualidade espermática. A exposição ao Roundup não alterou os parâmetros bioquímicos analisados, entretanto, diferenças entre os gêneros foram observadas e merecem maiores investigações. Os resultados do presente estudo sugerem que a exposição à concentrações ambientalmente relevantes de Roundup podem negativamente afetar a longo prazo a reprodução de *P. vivipara*, com consequente mudanças nas populações de peixes que habitam ambientes contaminados com o herbicida.

# I. INTRODUÇÃO

Desde o início das atividades relacionadas à agricultura e pecuária, as plantas que infestavam espontaneamente as áreas de ocupação humana, e que não proporcionavam alimentos, fibras ou forragem eram consideradas indesejáveis, tendo sido então rotuladas como plantas daninhas. Estas plantas passaram a ser eliminadas com o uso de controladores químicos, principalmente a partir da segunda metade do século XX com o desenvolvimento da indústria de herbicidas (Galli e Montezuma, 2005). A produção de cultivo intensivo requer o uso de agroquímicos com efeito seletivo em plantas daninhas (Kreutz et al., 2011) e, devido a confiança que os agricultores depositaram nesses produtos, uma grande quantidade de herbicidas, fungicidas e pesticidas passou a ser utilizada nestas áreas (Galli e Montezuma, 2005; Kreutz et al., 2008).

Existem centenas, talvez milhares, de poluentes que afetam o ambiente aquático e, considerando a constante síntese de novos compostos e formulações, este número cresce anualmente (Albinati et al., 2007). A poluição aquática está comumente associada à descarga de efluentes domésticos, industriais e agrícolas, sendo esta última bastante relevante, principalmente devido ao uso inadequado do solo e da intensa utilização de pesticidas e herbicidas (Shioguiri et al., 2012). Em áreas agrícolas, os agroquímicos podem contaminar ecossistemas aquáticos por lixiviação, escoamento superficial ou pela pulverização (direta ou indiretamente), sendo esta última dada pela ação do vento (Ramírez-Duarte et al., 2008; WHO, 1994). Além disto, alguns agroquímicos podem ser adicionados diretamente na água para o controle de macrófitas aquáticas e de insetos predadores (Kreutz et al., 2008; Szarek et al., 2000).

Dentre os contaminantes aquáticos, os agroquímicos são um dos mais perigosos, primeiramente pelo fato de terem sido concebidos para eliminar alguma forma de vida,

o que possibilita a ação de modo letal sobre espécies não-alvo (Albinati et al., 2009); e depois, o uso indiscriminado dos herbicidas, o manuseio descuidado, os derrames acidentais ou a descarga de efluentes não tratados em cursos de água naturais têm efeitos nocivos sobre a vida aquática, o que pode contribuir para efeitos deletérios no ambiente como um todo (Jiraungkoorskul et al., 2002). Apesar de sua natureza declaradamente tóxica, ainda há muito a ser entendido sobre o comportamento desses produtos no ambiente (Galli e Montezuma, 2005), e, além disto, os surfactantes usados nas composições das formulações comerciais dos herbicidas, para reduzir a tensão superficial da água e assim melhorar a sua eficácia (Diamond e Durkin, 1997), podem ser mais tóxicos para os peixes, algas, bactérias, protozoários e crustáceos que o próprio princípio ativo do herbicida (Shiogiri et al., 2010; Tsui e Chu, 2004).

Atualmente, dentre os vários agroquímicos existentes no mercado, àqueles a base de glifosato são os mais extensivamente usados, representam 60% do mercado mundial de herbicidas não seletivos, contabilizando um total de US$ 1.2 bilhão por ano com vendas dos produtos (Amarante Jr et al., 2002; Cavalcante et al., 2008; Ramírez-Duarte et al., 2008; Romano et al., 2008). A propriedade herbicida dessa molécula foi descoberta pela empresa Monsanto em 1970 e a primeira formulação comercial foi lançada nos Estados Unidos em 1974, com o nome comercial de Roundup (Galli e Montezuma, 2005). O glifosato é um herbicida pertencente ao grupo químico das glicinas substituídas, pós-emergente, sistêmico, de amplo espectro, não seletivo, usado no combate de plantas indesejáveis na agricultura, na indústria, em áreas urbanas e aquáticas (Albinati et al., 2007; Amarante Jr et al., 2002; Cavalcante et al., 2008; Galli e Montezuma, 2005; Silva et al., 2003; WHO, 1994).

Desde 1978, o glifosato tem sido utilizado no Brasil (Galli e Montezuma, 2005) e, segundo a Agência Nacional de Vigilância Sanitária (ANVISA) (2010), em 2008 o

Brasil assumiu o posto de maior consumidor de agrotóxicos do mundo, posição antes ocupada pelos Estados Unidos. Apesar da resolução CONAMA 357/05 permitir as concentrações de 65 $\mu g.L^{-1}$ (água doce - classe 1) e 280 $\mu g.L^{-1}$ (água doce - classe 3) de glifosato na água doce (Brasil, 2005), Silva et al. (2003) detectaram altas concentrações (>100 $\mu g.L^{-1}$) de glifosato em águas próximas à áreas de cultivo intenso de milho em semeadura direta no sul do Brasil. Na revisão realizada por Guilherme et al. (2010), as concentrações de Roundup, medido como ácido equivalente de glifosato, em corpos de águas naturais variou entre 0,01 e 0,7 $mg.L^{-1}$, atingindo 1,7 $mg.L^{-1}$ em situações extremas após a aplicação direta na água.

Nas culturas tradicionais, a aplicação do glifosato é realizada antes ou após o plantio com uso de equipamentos adequados para evitar o contato com a planta cultivada (Romano et al., 2009). Em contato com ervas daninhas, este composto é rapidamente absorvido pelas folhas, atuando como potente inibidor da atividade da 5-enolpiruvilshiquimato-3-fosfato sintase (EPSPS), uma enzima catalisadora responsável pela biossíntese de corismato, um intermediário na síntese dos aminoácidos aromáticos fenilalanina, tirosina e triptofano (Galli e Montezuma, 2005; Romano et al., 2009, 2008; Silva et al., 2003). Assim, este herbicida compromete a produção de clorofila e carotenóides, causando danos celulares irreversíveis. Entre os danos mais comuns observados, a ruptura parcial do cloroplasto e a perda de água do retículo endoplasmático rugoso são os mais importantes (Silva et al., 2003). Esta via para a biossíntese de aminoácidos aromáticos não é expressa por nenhum membro do reino animal, tornando esse mecanismo de ação exclusivo às plantas (Cerdeira et al., 2007; Romano et al., 2008; 2009; Williams et al., 2000).

Roundup é o nome comercial do herbicida em que o glifosato é formulado como sal de isopropilamina (IPA) juntamente com o surfactante catiônico denominado

polietoxileno amina (POEA), que é adicionado para aumentar a eficácia deste herbicida (Cavalcante et al., 2008; Langiano e Martinez, 2008; Releya, 2005; Tsui e Chu, 2004) e confere as propriedades toxicológicas diferentes do glifosato (Ramírez-Duarte et al., 2008). A principal rota de degradação do glifosato são os microrganismos de solo e água, que o decompõem por processos aeróbicos e anaeróbicos em compostos naturais, sendo o primeiro e principal produto da degradação o ácido aminometilfosfônico (AMPA) (Galli e Montezuma, 2005; Silva et al., 2003). No solo, o glifosato é fortemente retido na forma de resíduo ligado, já na água, ele é altamente solúvel, sendo a volatilidade e evaporação insignificantes (Galli e Montezuma, 2005). Devido a sua alta solubilidade em água e seu uso extensivo, a exposição de organismos aquáticos não-alvo a este herbicida é um problema, especialmente em sistemas de águas rasas (Cavalcante et al., 2008).

As substâncias contidas nos produtos químicos existentes nos pesticidas, herbicidas, inseticidas e fertilizantes, bem como seus adjuvantes, podem ser responsáveis por inúmeras alterações nos organismos. Estes efeitos podem ser agudos ou crônicos, na dependência do tempo de exposição, concentração no ambiente, modo de contato com o produto e tipo de degradação (Romano et al., 2008).

Estudos do efeito do glifosato e das formulações comerciais em peixes já foram realizados, evidenciando tanto alterações na reprodução quanto efeitos bioquímicos nestes organismos. Soso et al. (2007) constataram o efeito deletério na reprodução de *Rhamdia quelen*, através da diminuição da concentração de 17β-estradiol nas fêmeas da espécie e na diminuição da viabilidade de seus ovos, enquanto Hued et al. (2012) observaram a redução da atividade sexual dos machos de *Jenynsia multidentata* expostos ao Roundup.

As respostas bioquímicas variaram de acordo com o composto, a concentração utilizada, o tempo de exposição, a espécie e o tecido escolhido. A acetilcolinesterase (AChE) é uma enzima que catalisa a hidrólise da acetilcolina em colina e acetato nas fendas sinápticas, sendo a sua atividade um parâmetro frequentemente utilizado para o monitoramento ambiental, principalmente em áreas contaminadas por poluentes (Cattaneo et al., 2011). Glusczak et al. (2006) não verificaram alteração na atividade da AChE no músculo de *Leporinus obtusidens* expostos ao Roundup por 96 h, entretanto, uma exposição mais prolongada (90 dias) permitiu que Salbego et al. (2010) verificassem um aumento da atividade enzimática neste tecido. A inibição da AChE foi a resposta mais frequente nos estudos realizados nas diferentes espécies de peixes (Glusczak et al., 2007, 2006; Modesto e Martinez, 2010; Salbego et al., 2010; Sandrini et al., 2013), mas o aumento e a não alteração da atividade enzimática também foram encontrados (Glusczak et al., 2007; Rendón-von Osten et al., 2005; Salbego et al. 2010).

A lipoperoxidação (LPO) é comumente utilizada como marcador de estresse oxidativo (Lushchak et al., 2009). O aumento da lipoperoxidação foi verificado no músculo, fígado e cérebro de *R. quelen* (Glusczak et al., 2007; Menezes et al., 2011), no sangue de *Anguilla anguilla* (Guilherme et al., 2010) e no cérebro e músculo de *Cyprinus carpio* (Cattaneo et al., 2011).

A glutationa S-transferase (GST) é uma enzima de detoxificação de fase II que catalisa a conjugação da glutationa reduzida (GSH) com uma variedade de compostos eletrofílicos (Ferreira et al., 2010). Esta enzima apresentou atividade inalterada em *Gambusia yucatana* (Rendón-von Osten et al., 2005), *Prochilodus lineatus* (Langiano e Martinez, 2008), *Carassius auratus* (Lushchak et al., 2009), *A. anguilla* (Guilherme et al., 2010), aumentada em *P. lineatus* (Modesto e Martinez, 2010) e reduzida em *R. quelen* (Menezes et al., 2011) e no fígado de *C. auratus* (Lushchak et al., 2009).

A contaminação da água com grande quantidade de pesticida lidera a causa da mortalidade de peixes, mas os efeitos em pequenas quantidades são praticamente desconhecidos (Kreutz et al., 2008) e poucas são as investigações toxicológicas de agentes químicos que utilizam espécies nativas do Brasil (Albinati et al., 2009).

*Poecilia vivipara* é um peixe pertencente à família Poeciliidae, caracterizado como bentopelágico, não migratório, encontrado em ambientes dulcícolas e estuarinos (Froese e Pauly, 2011). É uma das espécies de peixes mais comuns em pequenas lagoas, rios e ecossistemas lagunares costeiros do Brasil (Santos et al., 2011), sendo encontrado ao longo da costa da América do Sul, desde a Venezuela até o Rio da Prata (Argentina) (Froese e Pauly, 2011). Apesar de sua relevância ecológica e abundância, existem poucos estudos toxicológicos neste potencial biomonitor.

Considerando os aspectos descritos acima, no presente estudo foram avaliados parâmetros reprodutivos (avaliação da qualidade espermática) e bioquímicos (determinação das atividades da acetilcolinesterase e da glutationa S-transferase, capacidade antioxidante total e lipoperoxidação) em *P. vivipara* expostos ao Roundup em concentrações de glifosato ambientalmente relevantes.

# II. ARTIGO

(a ser submetido ao periódico Aquatic Toxicology)

## Toxic effects of the herbicide Roundup in the guppy *Poecilia vivipara* acclimated to fresh water

**Toxic effects of the herbicide Roundup in the guppy *Poecilia vivipara* acclimated to fresh water**

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Abstract

Although it is believed that glyphosate-based herbicides are relatively nontoxic to humans, its broad use in agriculture and consequent contamination of aquatic systems is a concern. In the present study, reproductive (sperm quality) and biochemical parameters (acetylcholinesterase and glutathione S-transferase activity, lipoperoxidation, and antioxidant capacity against peroxyl radicals) were evaluated in adult guppies (*Poecilia vivipara*) acclimated to fresh water and exposed (96 h) to environmentally realistic concentrations of glyphosate (130 and 700 µg $L^{-1}$) as the commercial formulation Roundup. Male guppies exposed to Roundup showed a poorer sperm quality, measured as reduced plasmatic membrane integrity, mitochondrial functionality, DNA integrity, motility, motility period and concentration of spermatic cells, than those kept under control condition (no Roundup addition to the water). Most of the spermatic parameters analyzed showed strong association, which may help to understand the mechanisms underlying the observed reduction in sperm quality. Exposure to Roundup did not alter the biochemical parameters analyzed, though differences between genders were observed and deserve further investigations. Findings from the present study suggest that exposure to environmentally relevant concentrations of Roundup may negatively affect at long-term the reproduction of *P. vivipara*, with consequent changes in fish populations inhabiting environments contaminated with the herbicide.

Keywords**:** biomarkers, glyphosate, herbicide, guppy, Roundup

## 1. Introduction

During the last decades, the inadequate use of soil and the intense utilization of pesticides and herbicides to increase agricultural productivity have contributed to water contamination (Shioguiri et al., 2012). Indeed, the indiscriminate use of herbicides, careless handling, accidental spillage, or discharge of untreated effluents into natural waterways have caused harmful effects on aquatic life and may contributed to long-term biological effects (Jiraungkoorskul et al., 2002).

Glyphosate is one of the problematic organic contaminants reaching the aquatic systems. It is a weak organic acid consisting of a glycine and a phosphonomethyl moiety. Glyphosate is a broad-spectrum post-emergent, systemic and non-selective herbicide that is used in both agricultural and non-agricultural areas all around the world (WHO, 2005, 1994). The use of glyphosate as an herbicide was first proposed by Monsanto Company in 1970 (Lushchak et al., 2009) as the commercial formulation named Roundup. In this formulation, glyphosate is presented as the isopropylamine salt with addiction of surfactants, usually polyoxyethylene amine (POEA), and inert compounds (WHO, 1994).

Although it is believed that glyphosate-based herbicides are relatively nontoxic to humans (WHO, 1994), its broad application to aquatic systems and pollution of terrestrial ecosystems is of ecotoxicological concern (Lushchak et al., 2009). For aquatic systems, fish species are good indicators of the effects of noxious compounds because of their ecological and economical relevance (Jiraungkoorskul et al., 2002). In this context, changes at cellular and biochemical levels are among the monitored biological responses induced by pollutants in these aquatic organisms (Glusczak et al., 2007; Sandrini et al., 2013).

Due to its effectiveness and low cost, Roundup has been employed all over the world and since 1978 glyphosate has been used in Brazil (Galli and Montezuma, 2005). According to the Brazilian National Health Surveillance Agency (ANVISA) (2010), Brazil is the largest consumer of agrichemicals in the world since 2008. In fact, Silva et al. (2003) detected high concentrations of glyphosate in water near to intense cultivation areas in southern Brazil. In turn, Guilherme et al. (2010) reviewed the concentrations of Roundup, measured as glyphosate acid equivalents, in natural water bodies and found concentrations ranging between 0.01 and 0.7 mg $L^{-1}$, reaching the maximum value of 1.7 mg $L^{-1}$ in extreme situations after direct application of the herbicide into the water.

Although the intense use of glyphosate in Brazil, there is a lack of investigations using Brazilian native species in sublethal toxicity tests (Albinati et al., 2009). In this context, the guppy *Poecilia vivipara* (Bloch and Schneider, 1801) has been employed in ecotoxicological studies with both inorganic and organic contaminants (Ferreira et al., 2012; Machado et al., 2013). This guppy belongs to the Poeciliidae family, being characterized as benthopelagic and non-migratory fish. It is euryhaline, being found in fresh water and estuarine environments along the coast of South America, from Venezuela to Argentina (Froese and Pauly, 2011). Indeed, it is one of the most common species of fish found in small ponds, rivers and coastal lagoon ecosystems of Brazil (Santos et al., 2011). Despite its ecological relevance and abundance, there are still only few toxicological studies performed with this potential biomonitor. In light of the above, reproductive (sperm quality) and biochemical parameters [acetylcholinesterase (AChE) and glutathione S-transferase (GST) activity, lipoperoxidation (LPO), and total antioxidant capacity (ACAP)] were evaluated in *P. vivipara* acclimated to fresh water and exposed to environmentally realistic concentrations of glyphosate (130 and 700 µg $L^{-1}$) as the commercial formulation Roundup.

## 2. Material and methods

### 2.1. Fish collection and acclimation

Adults of *P. vivipara* were collected at the Gelo Creek (Cassino Beach, Rio Grande, RS, Southern Brazil) with nets and minnow traps. They were transferred to the animal care room of the Institute of Biological Sciences at the Federal University of Rio Grande (FURG) and acclimated for at least 7 days in continuously aerated and dechlorinated tap water. Room photoperiod (12L:12D) and temperature (28ºC) were fixed. Fish were daily fed with commercial food until apparent satiation. Feeding was stopped 24 h prior to the beginning of the experiments. Fish were fasten during the experimental period.

### 2.2. Fish exposure to Roundup

Due to the sexual dimorphism, 24 males [body length (mean ± standard deviation): 3.8 ± 1.2 cm; body weight: 0.54 ± 0.06 g; n = 8 fish per treatment] and 21 females (body length: 3.5 ± 0.9 cm; body weight: 0.41 ± 0.03 g; n = 7 fish per treatment) were individually kept under control condition (no Roundup addition into the water) or exposed (96 h) to Roundup (130 and 700 µg $L^{-1}$ of glyphosate). Other experimental conditions were kept as described above for the acclimation period (section 2.1.). Every 24 h, exposure media were completely renewed. Every 24 h and after fish transfer to the experimental tank, water samples (n = 24) from control and treatments were collected, filtered (0.2 µm-mesh filter; Millipore, Merck; São Paulo, SP, Brazil), and stored at 4$^{o}$C in glass bottles until analysis.

After exposure, fish were euthanized by decapitation (AVMA, 2001) and tissue (brain, muscle, gills and liver) were dissected and stored at -80 ºC for biochemical assays (section 2.4.). In males, testes were also dissected and immersed in Hanks balanced-salt solution (HBSS; 0.137 M NaCl, 5.4 mM KCl, 0.25 mM $Na_2HPO_4$, 0.44 mM $KH_2PO_4$, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 4.2 mM $NaHCO_3$) for sperm analysis (section 2.3.).

The present work was approved by the Ethics Committee on Animal Use of Federal University of Rio Grande (CEUA - FURG; reference # P054/2011).

## 2.3. Sperm analysis

Testis samples were placed in 1.5 ml bullet tubes containing HBSS and shaken for the release of spermatozeugmatas (sperm bundles). Sperm was released by gently and repeatedly disrupting spermatozeugmatas with a 10-µL pipette tip (Sun et al., 2010). The sperm suspension was used for analyses described below.

For estimation of sperm motility and motility period, 10 µL of sperm suspension were placed on a glass microscope slide with a cover slip. Sperm motility was estimated visually at 200X magnification using a phase contrast microscope (Olympus BX 51; América, São Paulo, SP, Brazil). Results of sperm motility were expressed as percentage of cells actively moving forward. Sperm vibration without effectively moving forward was not considered as being motile (Sun et al., 2010). The evaluation of the motility period, which is the period to achieve the complete lack of sperm motility, was performed in parallel with the sperm motility estimation. It was determined using a digital chronometer and expressed in seconds (Varela Junior et al., 2012). The sperm concentration was determined using a Neubauer chamber (Varela Junior et al., 2012).

Garner et al. (1986) have described the use of carboxyfluorescein diacetate (CFDA) and propidium iodide (PI), which are two fluorescent probes, to assess the plasma membrane integrity. A stock solution was prepared with 950 µL sodium citrate 3%, 20 µL PI (≥ 95%, Sigma-Aldrich, São Paulo, SP, Brazil), 20 µL CFDA (~95%, Sigma-Aldrich, São Paulo, SP, Brazil) and 10 µL formaldehyde made up not more than 1 h before use. An aliquot (10 µL) of sperm suspension and 40 µL of stock solution were incubated for 10 min at 20ºC. After incubation, 10 µL of the mixture were placed on a glass microscope slide with a cover slip and the membrane integrity was verified under 400X magnification using an epifluorescence microscope (Olympus BX 51, América, São Paulo, SP, Brazil). For quantitative analysis of membrane integrity, 200 cells were counted and classified according to their color. Green cells indicated cells with intact plasma membrane while red or green/red cells were classified as injured cells (Harrison and Vickers, 1990).

Rhodamine 123 (Rh123) (≥ 95%, Sigma-Aldrich, São Paulo, SP, Brazil) was used to evaluate mitochondrial functionality. An aliquot (10 µL) of sperm suspension and 40 µL of Rh123 solution (13 µM) were incubated for 10 min at 20ºC. Evaluation was performed using an aliquot (1 µL) of the mixture placed on glass microscope slide with a cover slip. For quantitative assessment of mitochondrial functionality, 200 cells were counted under 400X magnification with an epifluorescence microscope (Olympus BX 51, América, São Paulo, SP, Brazil). Data were expressed as percentage. Cells exhibiting green fluorescence were classified as presenting functional mitochondria while sperm showing no fluorescence was classified as containing cells with dysfunctional mitochondria (He and Woods, 2004). The rate of mitochondrial functionality was determined by considering the proportion of sperm emitting green fluorescence compared with the total sperm analyzed (Varela Junior et al., 2012).

The acridine orange (AO) fluorescence method described by Tejada et al. (1984) was used to assess the DNA integrity of spermatic cells. Sperm smears were dried in air and fixed in Carnoy solution (3 parts of methanol and 1 part of glacial acetic acid). Slides were rinsed several times with distilled water after being dipped in citric acid solution (0.1 M; pH 2.5) and stained with AO solution (0.2 mg $mL^{-1}$ in distilled water) during 5 min. Smears were washed again with distilled water and covered with a cover slip (Gandini et al., 2006). For quantitative analysis, 200 cells were counted under 400X magnification using an epifluorescence microscope (Olympus BX 51, América, São Paulo, SP, Brazil) without exceeding 1 min of slide exposure. Data were expressed as percentage. Sperm with green fluorescence were considered with normal DNA (bicatenary DNA) and those showing red, orange or yellow fluorescence were considered as having damaged DNA (monocatenary DNA, denatured).

## 2.4. Biochemical analyses

Tissue (brain, muscle, gill and liver) samples were homogenized in phosphate buffer (0.1 M; pH 7.75) and divided into aliquots for AChE activity in muscle and brain samples, and for LPO, ACAP and GST activity in muscle, gill and liver samples. Protein content in tissue homogenates was determined using a commercial reagent kit (Sigma-Aldrich, São Paulo, SP, Brazil) based on the Bradford's method (Bradford, 1976).

AChE activity was measured in brain and muscle samples using a spectrophotometric method following the yellow color yielded by thiocholine after reaction with the dithiobisnitrobenzoate ion (Ellman et al., 1961). Data were normalized considering the protein content in the tissue homogenate.

LPO was determined based on the reaction between the malondialdehyde (MDA) resulting from damage caused to lipids by free radicals and the 2-thiobarbituric acid (TBA) under conditions of high temperature and acidity. The chromogen generated was measured by spectrofluorometry (Oakes and Van Der Kraak, 2003). Data were normalized considering the wet mass (mg) of the tissue sample employed for analysis.

ACAP determination and data expression were performed as described by Amado et al. (2009). The method employed is based on the fluorometric detection of reactive oxygen species using 2',7'-dichlorofluorescein diacetate ($H_2$DCF-DA) as substrate.

GST activity was determined by the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) following procedures described by Keen et al. (1976). Data were normalized considered the protein content in the tissue homogenate.

2.5. Glyphosate concentration in water samples

Glyphosate concentration in filtered water samples was determined by ion chromatograph (IC Compact 881, Metrohm, Herisau, Switzerland) with conductometric detector, using an ion exchange column (Metrosep A Supp 5 150/4.0) and a chemical suppressor. The mobile phase was made with 9.6 mmol $L^{-1}$ of $Na_2CO_3$ and 3 mmol $L^{-1}$ of $NaHCO_3$ degassed for 30 min in an ultrasound bath. The solution for suppressor regeneration was prepared with ultrapure water and 0.1 mol $L^{-1}$ of sulfuric acid. A calibration curve (0.05 to 2.0 mg $L^{-1}$ glyphosate) was built and used for glyphosate concentration determination in water samples from the experimental media. All injections were performed with a loop injection of 20 µL. The quantification and detection limit were 0.05 and 0.01 mg $L^{-1}$, respectively. The method showed good linearity for the calibration curve in both ultrapure water ($r^2$ = 0.999) and the matrix

(dechlorinated tap water) ($r^2$ = 0.998) (Amarante Jr et al., 2002; Queiroz et al., 2011). The selectivity was determined by injecting major anions (fluoride, chloride, bromide, sulfate and phosphate) in water, none of them showing overlaping with the retention time of glyphosate. Data collection and treatment was performed using the Software MagicNet 2.3 (Metrohm, Herisau, Switzerland).

### 2.6.Statistical analysis

Data were expressed as mean ± standard error (SEM). All statistical analyses were done using the software BioEstat 5.0. Data normality was verified using the Shapiro-Wilk test. Parametric data were analyzed using Analysis of Variance (ANOVA) followed by the Tukey test. In turn, non-parametric data were analyzed using the Kruskal-Wallis ANOVA followed by the Dunn test. For all analyses, the significance level adopted was 95% ($p < 0.05$). Also, the Spearman correlation coefficient was used for semen data analysis in order to determine the level of association among the parameters analyzed (Ayres et al., 2007).

## 3. Results

Water analysis data showed that control water used to prepare the exposure media did not contain detectable levels of glyphosate (< 0.05 mg $L^{-1}$). The concentration measured for the nominal 130 and 700 µg $L^{-1}$ treatments were 144.8 ± 14.1 and 723.9 ± 21.8 µg $L^{-1}$, respectively. No fish mortality was observed over the experimental period.

Regarding spermatic responses, a decrease in the integrity of sperm plasma membrane was observed in fish exposed to any of the concentrations of Roundup tested when compared with fish from the control group. The effect observed was similar in

both herbicide concentrations (Fig. 1A). Sperm mitochondrial functionality decreased with increasing concentrations of Roundup, being significantly lower in fish exposed to 700 µg $L^{-1}$ of glyphosate than in control ones (Fig. 1B). Sperm DNA integrity was also reduced in male fish exposed to the highest concentration of Roundup tested (Fig. 1C). As observed for mitochondrial functionality, sperm motility was also reduced in fish exposed to 700 µg $L^{-1}$ of glyphosate (Fig. 1D). Motility period showed a trend of reduction with increasing concentrations of Roundup, but no significant change was observed (Fig. 1E). Also, the spermatic cells density was not significantly altered by exposure to the concentrations of the herbicide and the experimental period tested (Fig. 1F).

Spearman correlation analysis showed an association between most of the sperm quality parameters analyzed, which may help to understand the mechanisms underlying the observed reduction in sperm quality. Roundup exposure was correlated with membrane integrity ($r^2$ = 0.44), mitochondrial functionality ($r^2$ = 0.92), DNA integrity ($r^2$ = 0.38), sperm motility ($r^2$ = 0.66) and motility period ($r^2$ = 0.32). In turn, membrane integrity was correlated with mitochondrial functionality ($r^2$ = 0.37) and sperm motility ($r^2$ = 0.44), while mitochondrial functionality showed association with DNA integrity ($r^2$ = 0.39), sperm motility ($r^2$ = 0.56) and motility period ($r^2$ = 0.32). Finally, sperm motility was correlated with the motility period ($r^2$ = 0.42) (Fig. 2).

With respect to biochemical parameters, no significant differences in AChE activity was observed in muscle (Fig.3A) and brain (Fig. 3B) of guppies kept under control conditions or exposed to Roundup. A similar result was observed for both male and female fish. However, the enzyme activity was lower in muscle of female fish exposed to 700 µg $L^{-1}$ of glyphosate than in those exposed to 130 µg $L^{-1}$ of glyphosate (Fig. 3A). Males showed higher levels of muscle AChE activity than females (Fig. 3A)

while no significant difference in brain AChE activity was observed between genders (Fig. 3B).

No significant difference was observed in ACAP between control and Roundup-exposed fish. This lack of response was observed for muscle (Fig. 4A), gills (Fig. 4B), and liver (Fig. 4C) of both male and female fish. Control males showed higher gill ACAP than females (Fig. 4B). Females exposed to 130 µg $L^{-1}$ of glyphosate showed higher muscle ACAP than males (Fig. 4A), while males exposed to 700 µg $L^{-1}$ of glyphosate showed higher gill ACAP than females (Fig. 4B).

No significant effect of Roundup exposure was observed in muscle (Fig. 5A) and gill (Fig. 5B) LPO of male guppies. However, liver LPO was lower in male fish exposed to 700 µg $L^{-1}$ of glyphosate than in control male fish (Fig. 5C). In females, no significant difference was observed in tissue LPO between control and Roundup-exposed fish (Fig. 5). In all cases (control or Roundup-exposed fish), males showed higher liver LPO levels than females (Fig. 5C).

No significant effect of Roundup exposure was observed in GST activity in muscle (Fig. 6A), gills (Fig. 6B) and liver (Fig. 6C) of male and female guppies. In all groups (control and Roundup-exposed fish), females showed higher muscle GST activity than males (Fig. 6A). Also, control females showed higher gill GST activity than control males (Fig. 6B). No significant gender difference was observed in liver GST activity (Fig. 6C).

## 4. Discussion

As reviewed by Guilherme et al. (2010), the herbicide Roundup (measured as glyphosate acid equivalents) has been detected in natural water bodies at concentrations

ranging from 0.01 to 0.7 mg $L^{-1}$, reaching up to 1.7 mg $L^{-1}$ in extreme situations after direct application of the herbicide into the water. It is important to note that no fish mortality was observed in the present study after exposure of male and female guppies (*P. vivipara*) to Roundup at 130 and 700 µg $L^{-1}$ for 96 h. Therefore, these concentrations can be considered as being sublethal to *P. vivipara* and of ecotoxicological relevance.

Findings from the present study showed that sperm of *P. vivipara* acutely (96 h) exposed to Roundup showed a lower quality, measured as a reduction in the following parameters: integrity of sperm plasma membrane, mitochondrial functionality, DNA integrity, sperm motility and motility period. An effect of the herbicide on all of these parameters was homogeneously observed in male fish exposed to the highest concentration of Roundup tested (700 µg $L^{-1}$ of glyphosate). This herbicide concentration also induced a slight but not significant increase in spermatic cells density, which could be a response to the poorer sperm quality observed in fish exposed to this condition.

Concerning the association among the different sperm parameters analyzed, there was a correlation between the reduced membrane integrity and the reduced mitochondrial functionality. This is consistent with a possible damage induced by exposure to Roundup and/or its metabolites on sperm membrane, which would likely imply the impairment of the mitochondria functionality. In turn, the reduced mitochondrial functionality paralleled with the reduced membrane integrity could explain the observed decrease in sperm motility. Additionally, the observed damage to the mitochondria induced by Roundup exposure can be also responsible for the increased DNA damage and reduced motility period of fish sperm. In fact it is reported that mitochondrial dysfunction can lead simultaneously to production of reactive

oxygen species and lower energy availability in mammalian cancer cells (Pelicano et al., 2009). It is important to note that changes in the structural and functional integrity of plasma membrane and mitochondria, as well as reduced sperm motility, are critical endpoints of the fertilization process in teleost fish eggs (He and Woods, 2004). Finally, the strong correlation observed between the exposure concentration of Roundup and fish sperm quality reinforces the idea that realistic environmental levels of Roundup could cause relevant damages to fish reproduction.

It is worth to mention that the poorer quality of the semen found in *P. vivipara* exposed to Roundup can be due to the direct effect of this herbicide and/or its metabolites on sperm. The direct effect of the herbicide would represent a direct damage in sperm plasma membrane. In other hand, the indirect effect would be related to a disruption of steroid hormones regulation. In fact, it has been reported that Roundup decreases steroidogenesis in Leydig cells by reducing the StARS levels, thus contributing for the development of a reproductive dysfunction (Walsh et al., 2000). Both direct and indirect effects clearly indicate that Roundup exposure is disrupting the fish sperm quality.

The findings described above point out to the potential effects of Roundup on male fish reproduction. In this context, it is important to note that Soso et al. (2007) reported that females of the catfish *R. quelen* exposed to Roundup showed a disruption in steroidogenesis characterized by a decreased level of 17β-estradiol. This hormone is produced by the ovarian follicular layer and stimulates vitellogenin production and secretion. Besides the impairment in ovarian follicle function, these authors also reported a reduction in egg viability, since a low number of viable swim-up fries were obtained. Moreover, Hued et al. (2012) reported a lower sexual activity, measured by a decreased number of copulations and matching success in the guppy *Jenynsia*

*multidentata*, another viviparous fish, after exposure to Roundup. It is worth to note that this guppy species also show internal fertilization, as observed in *P. vivipara*.

In summary, findings from the present study along with those already described in the literature support the idea that fish reproduction could be impaired in aquatic environments contaminated with Roundup. Furthermore, parameters analyzed in the present study to assess the semen quality can be considered as potential biomarkers of fish exposure to this herbicide.

Male and female *P. vivipara* showed differential levels of some of the biochemical parameters analyzed. In viviparous fish, males tend to be more mobile than females (Magurran and Maciás Garcia, 2000). The higher swimming activity increases the metabolic rate, which could explain the higher muscle AChE activity found in males of *P. vivipara* in the present study. In turn, an elevated metabolic rate stimulates the production of reactive oxygen species (ROS), demanding a higher tissue ACAP to protect cells against the oxidative damage induced to macromolecules. The higher levels of ACAP in gills and LPO in liver of male *P. vivipara* reported in the present study are consistent with this idea. Similarly, Vega-López et al. (2007) reported higher levels of LPO in liver of male *Girardinichthys viviparous*, another viviparous fish. They explained the observed gender difference considering a higher cytochrome P450 content and CYP1A catalytic activity in males than in females. This would lead to an increased generation of ROS and consequent higher LPO levels. In fact, a positive response of the enzymatic antioxidant defenses (superoxide dismutase and catalase activities) in response to the increased LPO level was observed in liver of males *G. viviparous*, indicating that they were more subjected to oxidative stress than females. In turn, GSTs are detoxifying enzymes of phase II that catalyze the conjugation of GSH with a variety of electrophilic compounds (Ferreira et al., 2010). The higher GST activity found in

female *P. vivipara* can be associated with its viviparous reproduction, once during gestation the maternal system must provide oxygen to and remove metabolic wastes from the embryo (Timmerman and Chapman, 2003).

In the present study, none of the biochemical parameters analyzed was significantly affected by Roundup exposure, except for a reduced LPO level observed in liver of male *P. vivipara* exposed to the highest concentration of Roundup. Similar result was reported by Rendón-von Osten et al. (2005) for the mosquitofish *Gambusia yucatana* after exposure to Rival, a commercial formulation of a glyphosate-based herbicide like Roundup. These authors reported no significant effects of the herbicide on GST and AChE activity, and suggested that GSTs are not involved in the detoxification of the agrichemical.

Biochemical responses in fish exposed to Roundup were already described and varied depending on the species and the tissue analyzed (Glusczak et al., 2007, 2006; Langiano and Martinez, 2008; Ferreira et al., 2010; Modesto and Martinez, 2010 ; Salbego et al., 2010; Cattaneo et al., 2011; Menezes et al., 2011). It is important to note that concentrations tested in these studies were higher (ranging from 0.95 to 20 mg $L^{-1}$) than those employed in present study (0.13 and 0.70 mg $L^{-1}$), except for Menezes et al. (2011) who using an intermediate concentration (0.45 mg $L^{-1}$) were able to demonstrate significant changes in the response of biochemical parameters in the catfish *R. quelen* exposed to Roundup.

Based on the discussed above, it is possible that concentrations employed in the present study were not high enough to induce significant changes in the response of the biochemical parameters analyzed in *P. vivipara*. This suggests that this guppy is likely more tolerant to Roundup than the catfish *R. quelen*. In fact, the 96-h $LC_{50}$ value of Roundup for *R. quelen* is 10 mg $L^{-1}$ (Albinati et al., 2007), being lower than that

described for *G. yucatana* exposed to Rival (17.79 mg $L^{-1}$), a fish species belonging to the Poecillidae family like *P. vivipara*. Therefore, the lack of biochemical responses in *P. vivipara* after exposure to Roundup could be due to the comparatively low concentrations tested in the present study. Nevertheless, it is also possible that the herbicide was not effectively absorbed by guppies or they were able to detoxify it quickly before it could cause deleterious effects.

## 5. Conclusion

Findings reported in the present study show that exposure to Roundup reduces the sperm quality in the guppy *P. vivipara*, an effect likely associated with changes in plasma membrane integrity and mitochondrial functionality in spermatic cells. They suggest that exposure to environmentally realistic concentrations of this herbicide may negatively affect at long-term the reproduction of *P. vivipara*, with consequent changes in fish populations inhabiting environments contaminated with Roundup.

Finally, although both concentrations of Roundup tested did not affect the biochemical parameters analyzed in adults of *P. vivipara*, data from the present study indicated gender-related differences in the response of these parameters. These different biochemical patterns certainly deserve further future investigation, especially in the context of the increasing use of *P. vivipara* in ecotoxicological studies.

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## APPENDICES

Legend to Figures

Figure 1. Sperm quality in the guppy *Poecilia vivipara* kept under control conditions or exposed (96 h) to Roundup. Values are means ± SEM. (A) membrane integrity; (B) mitochondrial functionality; (C) DNA integrity; (D) motility; (E) motility period; (F) cell density. Different letters represent significant difference among treatments ($p<0.05$).

Figure 2. Spearman correlation analysis for sperm quality data obtained with the guppy *Poecilia vivipara* exposed (96 h) to Roundup in fresh water. Only results showing statistical significance ($p< 0.05$) are shown.

Figure 3. AChE activity in (A) muscle and (B) brain of the guppy *Poecilia vivipara* kept under control conditions or exposed (96 h) to Roundup in fresh water. Values are means ± SEM. Different lowercase and uppercase letters represent significant difference among treatments ($p<0.05$) in males and females, respectively. * denotes significant difference ($p<0.05$) between genders.

Figure 4. ACAP in (A) muscle, (B) gill and (C) liver of the guppy *Poecilia vivipara* kept under control conditions or exposed (96 h) to Roundup in fresh water. Values are means ± SEM. Different lowercase and uppercase letters represent significant difference among treatments ($p<0.05$) in males and females, respectively. * denotes significant difference between genders.

Figure 5. LPO in (A) muscle, (B) gill and (C) liver of the guppy *Poecilia vivipara* kept under control conditions or exposed (96 h) to Roundup in fresh water. Values are means ± SEM. Different lowercase and uppercase letters represent significant difference among treatments ($p<0.05$) in males and females, respectively. * denotes significant difference between genders.

Figure 6. GST activity in (A) muscle, (B) gill and (C) liver of the guppy *Poecilia vivipara* kept under control conditions or exposed (96 h) to Roundup in fresh water. Values are means ± SEM. Different lowercase and uppercase letters represent difference among treatments ($p<0.05$) in males and females, respectively. * denotes significant difference between genders.

Figure 1

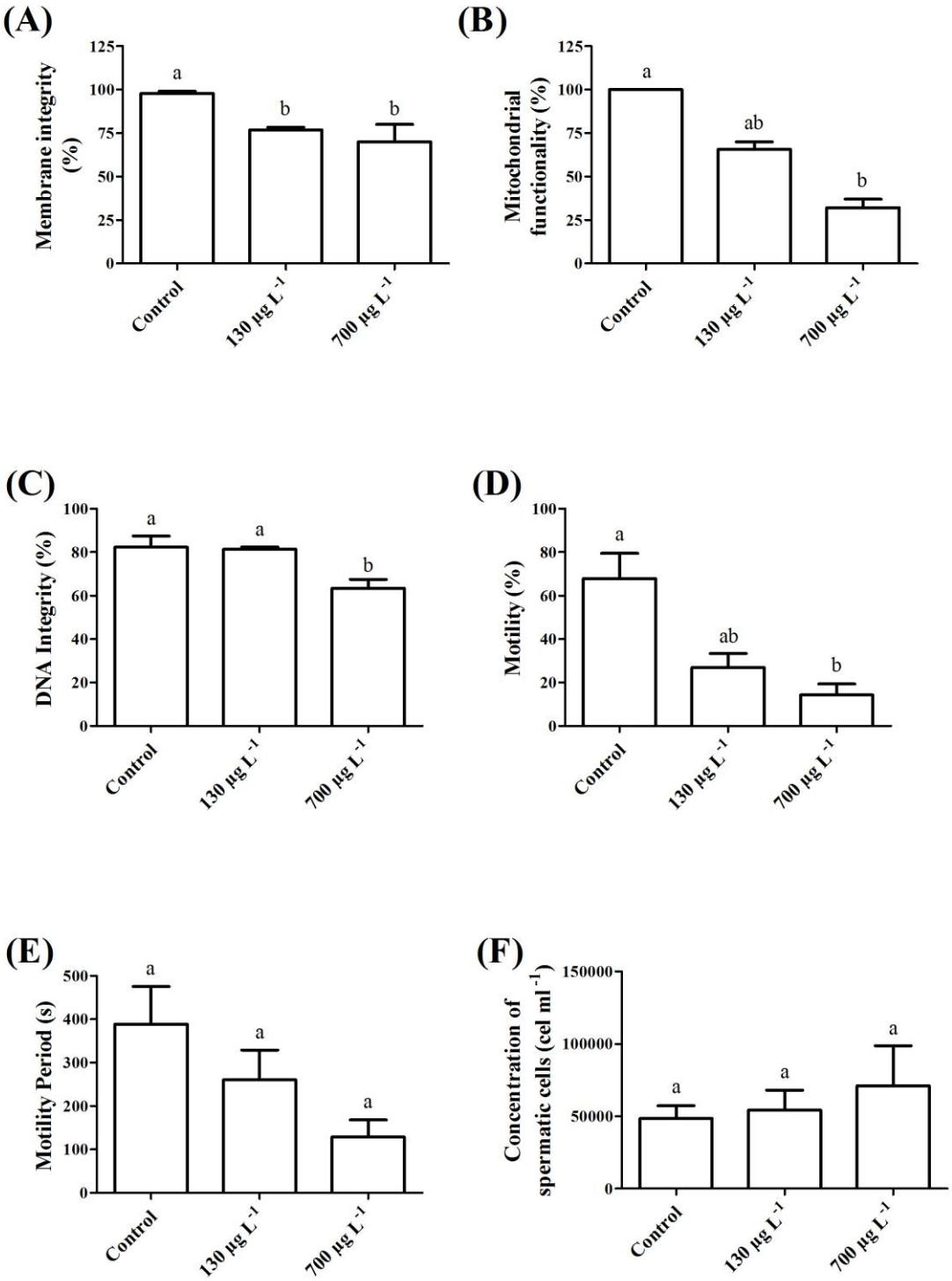

(A)
Membrane integrity (%)
125
100
75
50
25
0
a
b
b
Control
130 µg L-1
700 µg L-1
(B)
Mitochondrial functionality (%)
125
100
75
50
25
0
a
ab
b
Control
130 µg L-1
700 µg L-1
(C)
DNA Integrity (%)
100
80
60
40
20
0
a
a
b
Control
130 µg L-1
700 µg L-1
(D)
Motility (%)
100
80
60
40
20
0
a
ab
b
Control
130 µg L-1
700 µg L-1
(E)
Motility Period (s)
500
400
300
200
100
0
a
a
a
Control
130 µg L-1
700 µg L-1
(F)
Concentration of spermatic cells (cel ml-1)
150000
100000
50000
0
a
a
a
Control
130 µg L-1
700 µg L-1

Figure 2

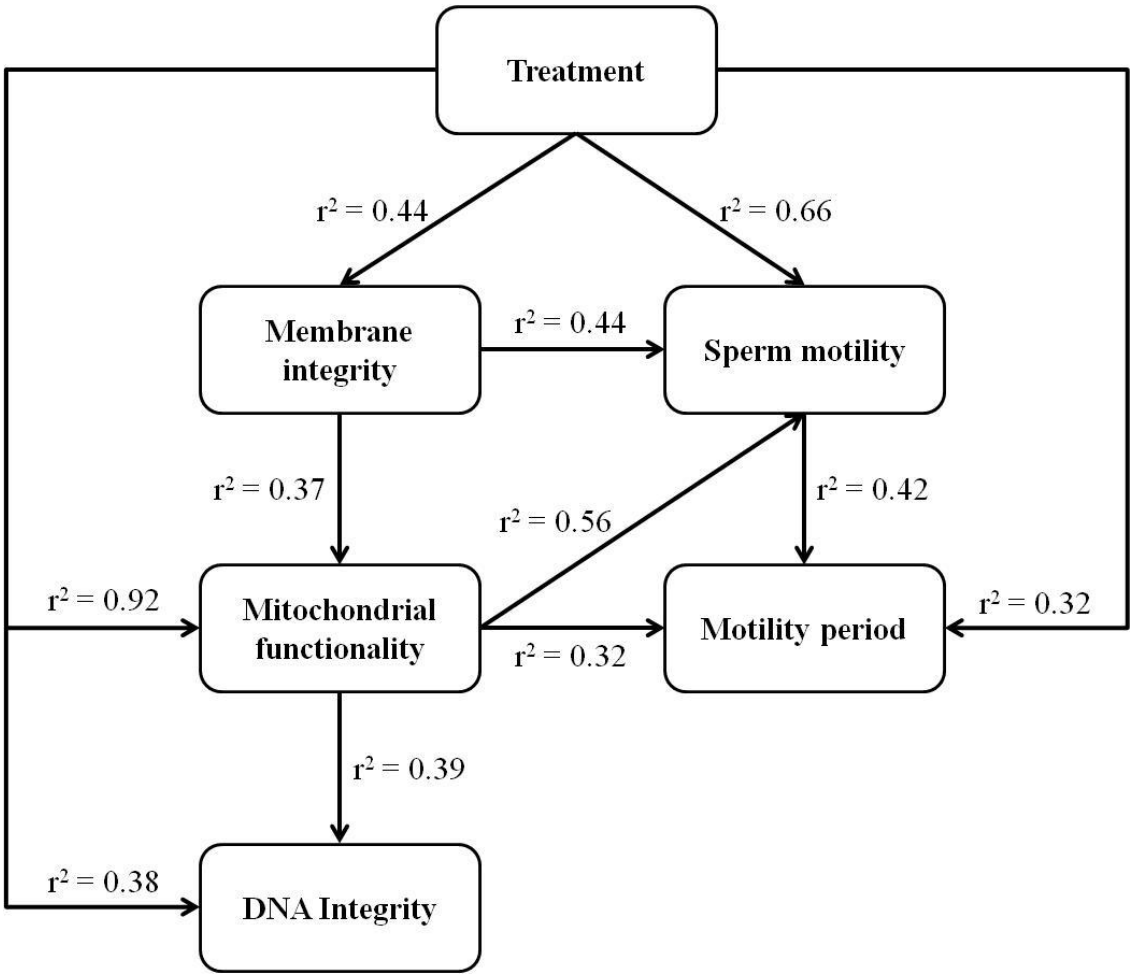
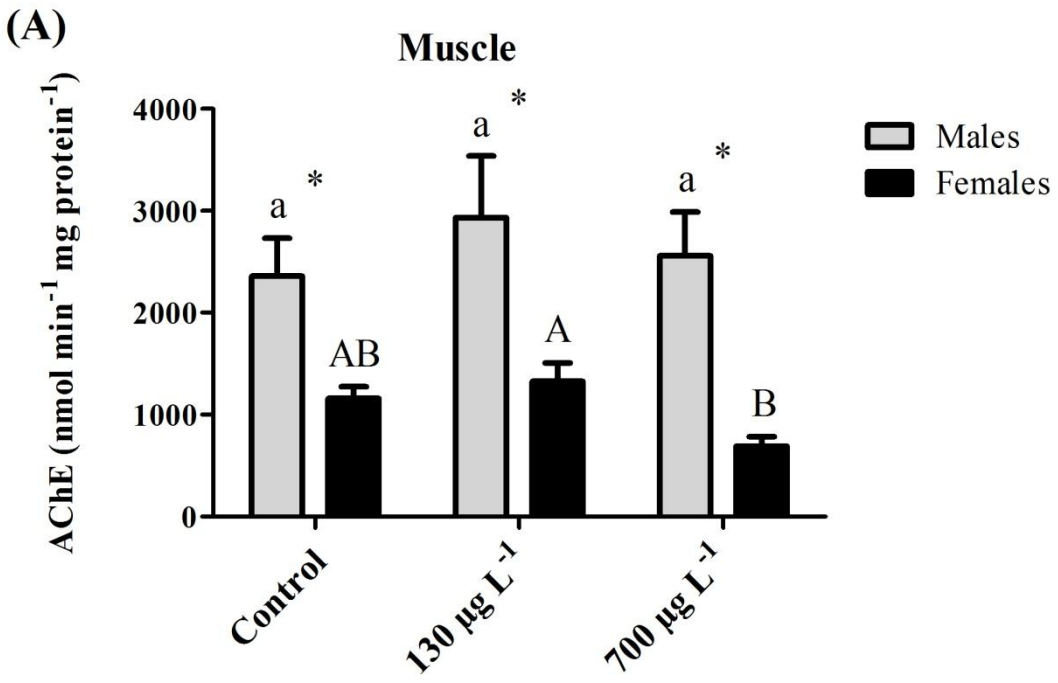

Treatment
r² = 0.44
r² = 0.66
Membrane integrity
r² = 0.44
Sperm motility
r² = 0.37
r² = 0.56
r² = 0.42
r² = 0.92
Mitochondrial functionality
r² = 0.32
Motility period
r² = 0.32
r² = 0.39
r² = 0.38
DNA Integrity

Figure 3


(A)
Muscle
AChE (nmol min-1 mg protein-1)
4000
3000
2000
1000
0
a
*
AB
a
*
A
a
*
B
Control
130 µg L-1
700 µg L-1
Males
Females

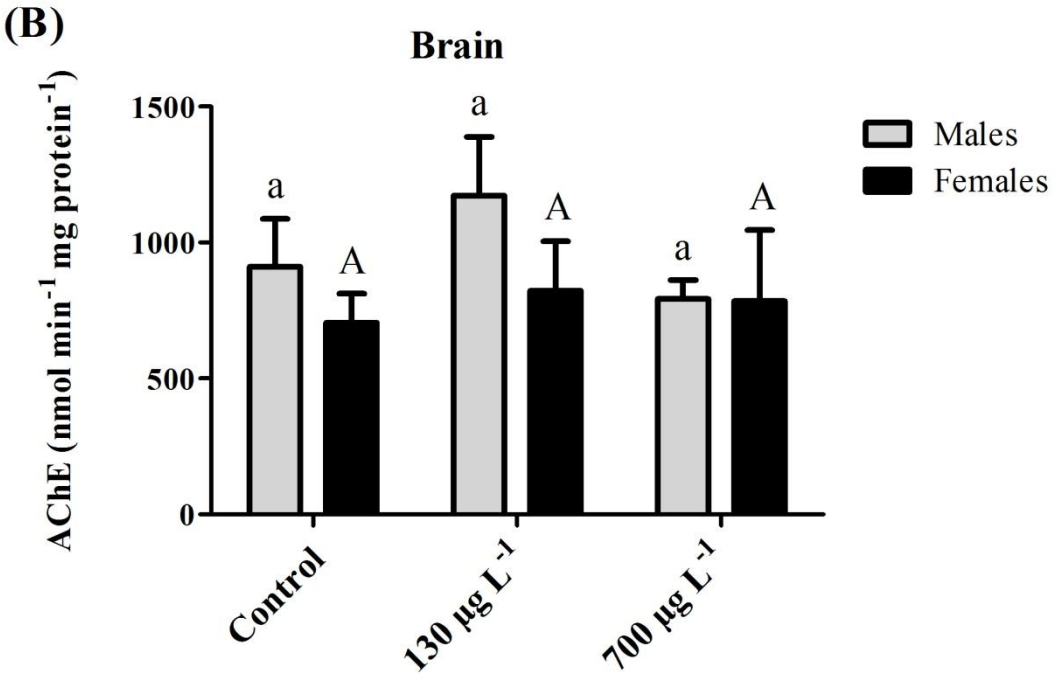

(B)
Brain
AChE (nmol min-1 mg protein-1)
1500
1000
500
0
a
A
a
A
a
A
Control
130 µg L-1
700 µg L-1
Males
Females

Figure 4

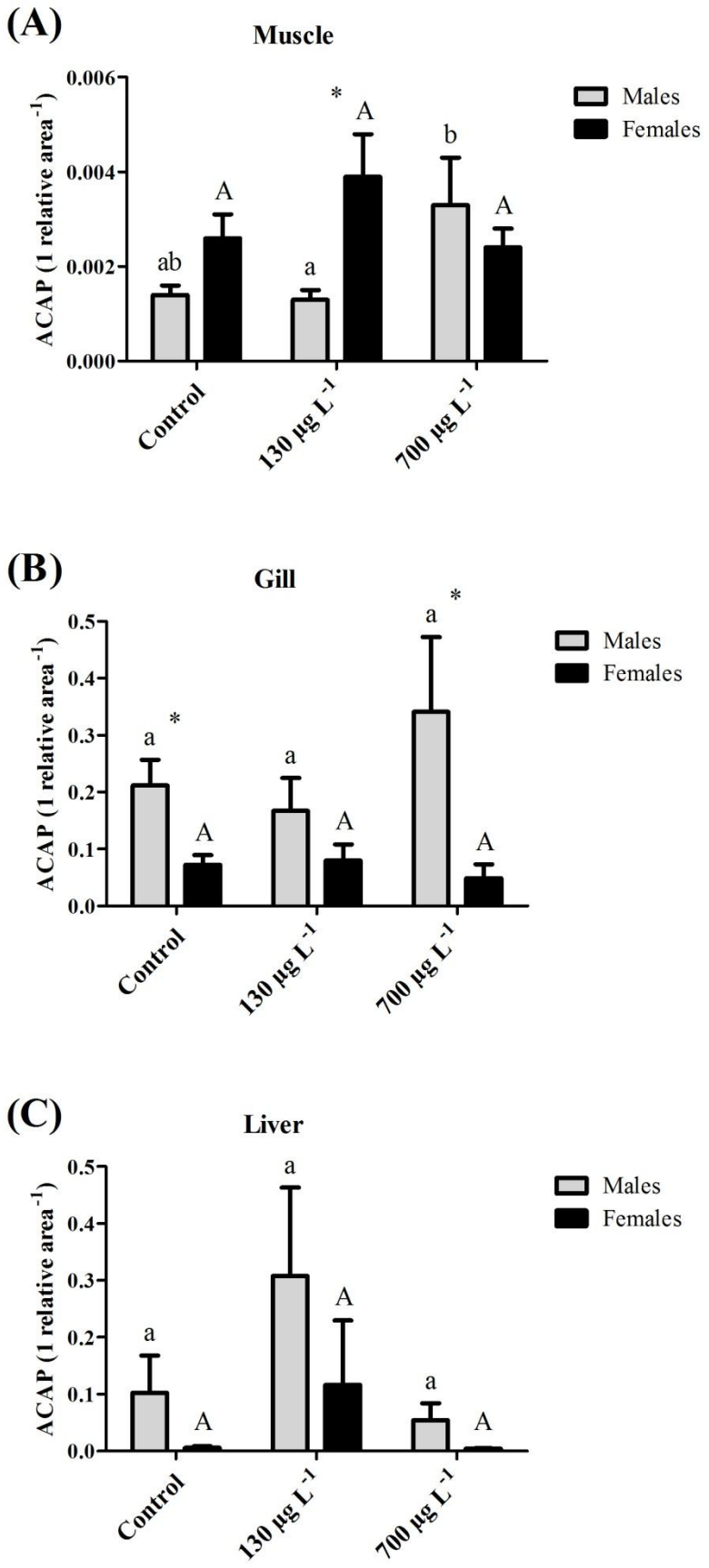

(A)
Muscle
ACAP (1 relative area$^{-1}$)
0.006
0.004
0.002
0.000
Males
Females
ab
A
a
*
A
b
A
Control
130 µg L$^{-1}$
700 µg L$^{-1}$
(B)
Gill
ACAP (1 relative area$^{-1}$)
0.5
0.4
0.3
0.2
0.1
0.0
Males
Females
a
*
A
a
A
a
*
A
Control
130 µg L$^{-1}$
700 µg L$^{-1}$
(C)
Liver
ACAP (1 relative area$^{-1}$)
0.5
0.4
0.3
0.2
0.1
0.0
Males
Females
a
A
a
A
a
A
Control
130 µg L$^{-1}$
700 µg L$^{-1}$

Figure 5

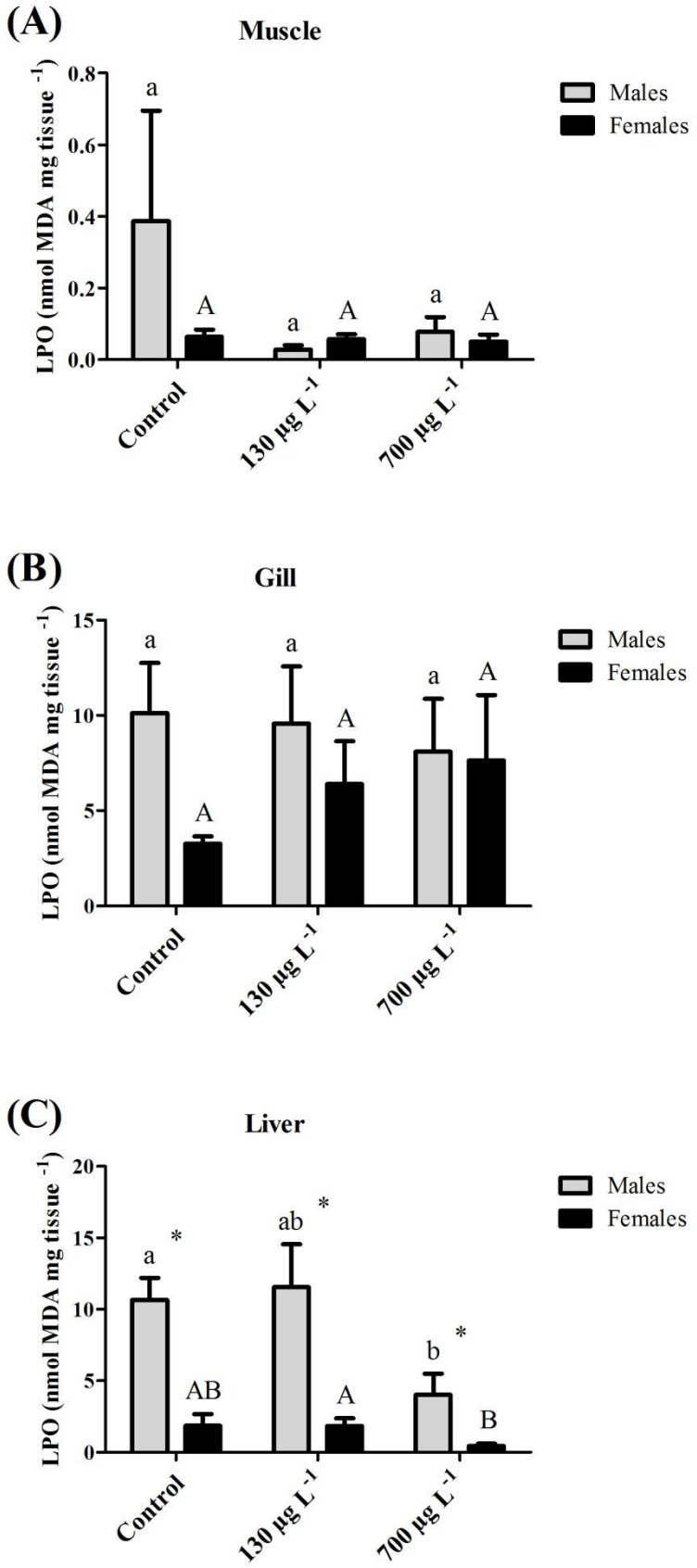
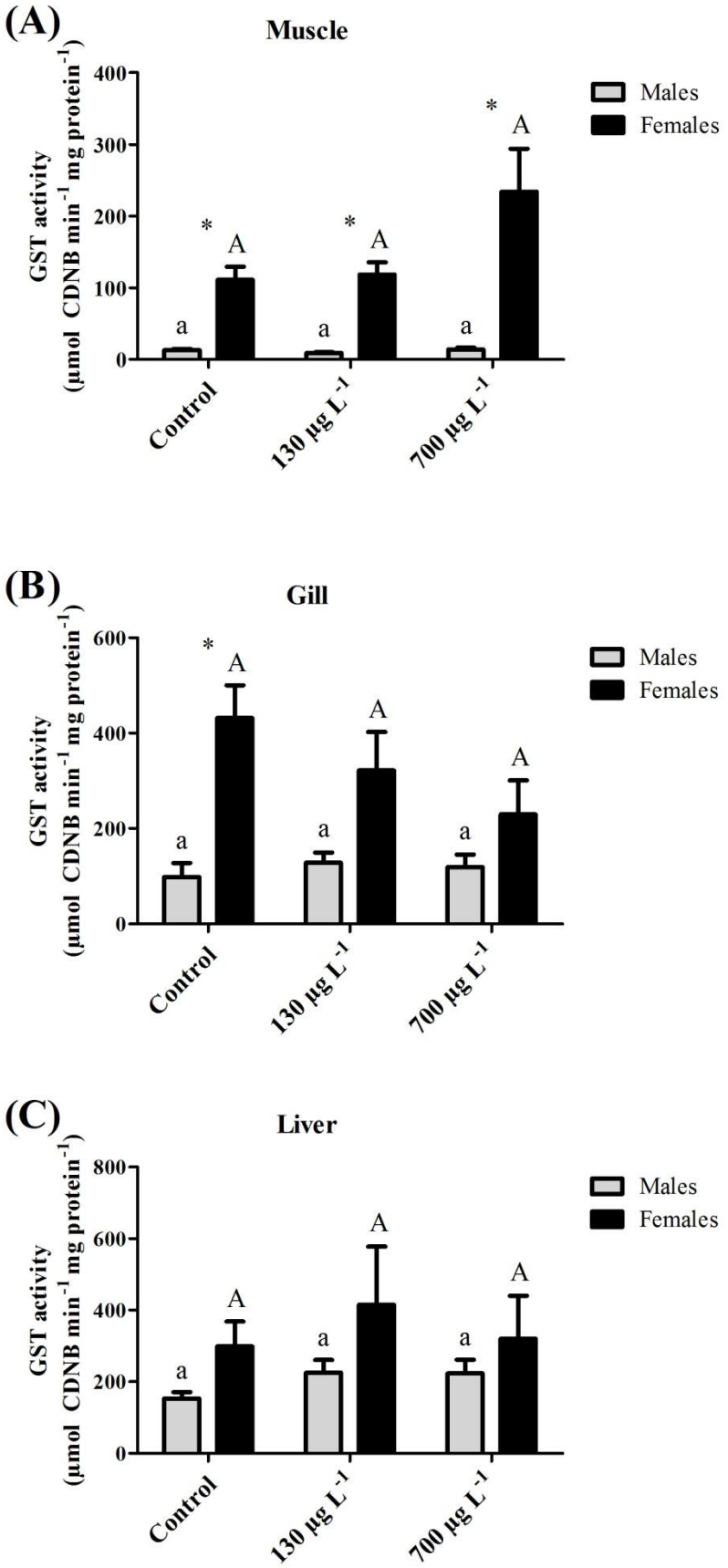

(A)
Muscle
LPO (nmol MDA mg tissue -1)
0.8
0.6
0.4
0.2
0.0
a
A
a
A
a
A
Males
Females
Control
130 µg L -1
700 µg L -1
(B)
Gill
LPO (nmol MDA mg tissue -1)
15
10
5
0
a
A
a
A
a
A
Males
Females
Control
130 µg L -1
700 µg L -1
(C)
Liver
LPO (nmol MDA mg tissue -1)
20
15
10
5
0
a *
AB
ab *
A
b *
B
Males
Females
Control
130 µg L -1
700 µg L -1

Figure 6


(A)
Muscle
GST activity
(µmol CDNB min-1 mg protein-1)
400
300
200
100
0
Males
Females
*
A
a
Control
130 µg L-1
700 µg L-1
(B)
Gill
600
400
200
0
(C)
Liver
800
600
400
200
0

# III. CONCLUSÃO GERAL

Os resultados do presente estudo mostraram que a exposição ao Roundup reduz a qualidade do sêmen do barrigudinho *P. vivipara*, um efeito possivelmente associado ao dano na integridade da membrana plasmática bem como na funcionalidade mitocondrial das células espermáticas. O que sugere que a exposição a concentrações ambientalmente realísticas deste herbicida pode afetar negativamente a reprodução de *P. vivipara* à longo prazo e, consequentemente, promover alterações em populações que habitam ambientes contaminados com Roundup.

Por fim, apesar de ambas as concentrações de Roundup testadas não afetarem os parâmetros bioquímicos analisados em adultos de *P. vivipara*, os resultados do presente estudo indicaram diferenças relacionadas ao sexo na resposta destes parâmetros. Estes diferentes padrões bioquímicos certamente merecem ser melhor investigados, especialmente no contexto de aumentar a utilização de *P. vivipara* em estudos ecotoxicológicos.

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