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Article

Changes in sperm motility of amazonian fish Tambaqui *Colossoma macropomum* (Cuvier 1816) (Characiformes: Serrasalmidae) exposed to two pesticides

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Abstract: The great biodiversity of neotropical fish species that have external fertilization as a reproductive strategy, like the tambaqui, requires more careful analyzes in toxicological tests of the various pesticides implemented in Brazilian agriculture over the last few years. In this context, the objective of the present work was to evaluate possible sperm alterations in tambaqui (*Colossoma macropomum*) semen exposed to two different pesticide residues. Seminal samples of sexually mature tambaqui males from a local fish farm were used. Semen was collected eight hours after hormone induction into graduated glass tubes. After initial assessment of the lack of prior activation, the experiment was carried out in a factorial scheme, testing two pesticides widely used in agricultural systems (glyphosate and fenitrothion). For each pesticide, five concentrations were tested (6, 12, 24, 120 and 240 mg/L), with motility analysis at times 0, 30 and 60 seconds after activation. As a control, activation with 0.9% NaCl solution and motility analysis at the same times described for pesticides were used. Results indicate that in natura samples exhibited initial motility of 89.2 ± 4.9% and mean duration of 100 seconds (up to 10% sperm motility). The reduction in sperm motility occurred significantly ($p < 0.05$) after 30 seconds in all concentrations tested, except for the concentration of 240 mg/L because no activation was observed. The tests described here demonstrate that tambaqui semen was sensitive to the process of exposure to pesticide residues, and can be used in biomonitoring analyzes of the aforementioned agricultural pesticides.
Keywords: *Agriculture; biomonitoring; ecotoxicology; amazonian fish; aquatic pollution; seminal quality.*

Alterações na motilidade espermática do peixe amazônico Tambaqui *Colossoma macropomum* (Cuvier 1816) (Characiformes: Serrasalmidae) exposto a dois pesticidas

Resumo: A grande biodiversidade das espécies de peixes neotropicais que possuem a fertilização externa como estratégia reprodutiva, a exemplo do tambaqui, exige análises mais criteriosas em testes toxicológicos dos diversos defensivos agrícolas implementados na agricultura brasileira ao longo dos últimos anos. Nesse contexto, o objetivo do presente trabalho foi avaliar possíveis alterações espermáticas no sêmen de tambaqui (*Colossoma macropomum*) exposto a resíduos de dois diferentes pesticidas. Foram utilizadas amostras seminais de machos de tambaqui sexualmente maduros provenientes de uma piscicultura local. O sêmen foi coletado oito horas pós indução hormonal em tubos de vidro graduados. Após avaliação inicial de inexistência de ativação prévia, foi realizado o experimento em esquema fatorial, sendo testados dois pesticidas muito utilizados em sistemas agrícolas (glifosato e fenitrotiona). Para cada pesticida foram testadas cinco concentrações (6, 12, 24, 120 e 240 mg/L), com análise da motilidade nos tempos 0, 30 e 60 segundos pós ativação. Como controle, foi utilizada a ativação com solução de NaCl a 0,9% e análise da motilidade nos mesmos tempos descritos para os pesticidas. Resultados indicam que as amostras *in natura* exibiram motilidade inicial de 89,2 ± 4,9% e tempo de duração médio de 100 segundos (até 10% de motilidade espermática). A redução da motilidade espermática ocorreu de forma significativa ($p < 0,05$) após 30 segundos em todas as concentrações testadas, exceto na concentração de 240 mg/L por não ter sido observada ativação. Os testes aqui descritos demonstram que o sêmen de tambaqui se mostrou sensível ao processo de exposição aos resíduos de pesticidas, podendo ser utilizado em análises de biomonitoramento dos referidos defensivos agrícolas.

Palavras-chave: *Agricultura; biomonitoramento; ecotoxicologia; peixe amazônico; poluição aquática; qualidade seminal.*

Introduction

In recent decades, the world has been facing a serious problem: the "Biodiversity Crisis". As the human population grows exponentially, increasing demand for natural resources, species are becoming extinct both locally and globally, especially in tropical zones of the world, at rates much higher than natural extinction rates. This is caused directly due to human actions, such as pollution, destruction of natural habitats, modification of natural habitats, deforestation, agricultural expansion, overfishing and overhunting, introduction of exotic species, fragmentation of habitats, among others (Wilson 1985, Savage 1995, Primack & Rodrigues 2001, Brooks et al. 2022, Singh 2002, Brook et al. 2006, Pimm et al. 2006, Laurance 2006, Wheeler 2008, Costa et al. 2012, Pimm et al. 2014, Ceballos et al. 2015). When we compare biodiversity and health of freshwater environments with terrestrial or marine, the scenario is even worse: the so-called "Freshwater Biodiversity Crisis". Although the threats are the same as those already mentioned, the proportional area of freshwater environments is much smaller when compared to terrestrial or marine environments, representing less than 1% of the planet's surface, but comprising a very rich biodiversity. In addition, several human activities are dependent on freshwater, and humanity directly depends on this resource as well (Dudgeon et al. 2006, Darwall et al. 2018, Harrison et al. 2018, Latrubesse et al. 2019, Reid et al. 2019).

Even though the "Biodiversity Crisis" has become an increasingly serious problem, especially the "Freshwater Biodiversity Crisis", due to agricultural expansion, Brazil has arisen as one of the countries that most employ pesticides in the past decades, more expressively from 2002 onwards, showing that we are failing to face and deal appropriately with the "Biodiversity Crisis". This fact raises concerns about the increased use of these substances, mainly due to the possibility of contaminating man and animals (Rembischevsk & Caldas 2018). A fact that hinders the conservation of Brazilian fauna. In this context, the determination of lethal and sublethal doses of pesticides in living organisms should be analyzed in toxicity tests (Ragassi et al. 2017).

In an attempt to monitor the environmental changes caused by the indiscriminate discharge of toxic substances with xenobiotic potential, researchers report the need for ecotoxicological studies as a way to assess the possible aggressions of these substances that are released into the natural environment, such as agricultural pesticides, and their interaction with ecosystems and their biodiversity (Montanha & Pimpão 2012). According to Torres et al. (2017), such indiscriminate release of polluting agents in aquatic environments has become a limiting factor for the continued supply of fish consumer markets, whether from fishing or even aquaculture. In addition to the direct risks to human health, it is possible that fish are being contaminated by toxic products that reach aquatic environments, which may represent an additional risk for their consumers (Waichman 2008).

The tambaqui *Colossoma macropomum* (Cuvier 1816) is the most farmed native fish species in the country (PEIXEBR 2022), as it presents a good adaptation to climate conditions, which are considered ideal for round fish species. It is important to emphasize that the cultivation of native species to supply the market is important to reduce the negative pressures that fishing can exert on the populations of these species, and to prevent the local extinction of species as well. *Colossoma macropomum* presents external fecundation and fertilization, as well as annual reproduction and total spawn, with the river flooding period being the main spawning season for this fish (Vieira et al. 1999). In teleosts with external fertilization such as round fish, when spawning occurs, the gametes are released into the environment for fertilization to occur (Witeck et al. 2011). At that moment, gametes are exposed to various contaminants present in the water, including heavy metals mercury, zinc, lead, copper, and cadmium (Kime & Nash 1999), as well as pesticides leached from the soil by the rain or even by the inadequate disposal of containers and waste (Ferreira 2016), which end up acting as endocrine disruptors in fish (Uren-Webster et al. 2014). Furthermore, these trace elements, at certain levels, can impair sperm motility and oocyte fertilization (Kime 1995), thus causing often irreversible damage to the maintenance of natural stocks renewal and reproductive cycles, and, consequently, to the maintenance of the variability and diversity of fish.

In the past few years, several studies involving the characterization of sperm and embryos from a number of native fish species have generated successful sperm analysis protocols in conjunction with the cryopreservation technique, conservation of cells at low temperatures. This allows the availability throughout the year of biological samples for many species of environmental interest (Viveiros et al. 2009, Carneiro et al. 2012, Salmito-Vanderley et al. 2016), whose usefulness in ecotoxicological tests can now be analyzed. In this context, the objective of the present work was to evaluate possible sperm alterations in the semen of tambaqui *C. macropomum* exposed to two different pesticides, to assess whether these pesticides cause any negative effects that interfere with the reproduction of the species, and, consequently, affecting the maintenance of natural stocks renewal.

Material and Methods

Our study was conducted during the period between January and May of 2021, in the facilities of a fish farm located in the municipality of Santa Inês, State of Maranhão, northeastern Brazil (latitude 03°40'00" south and longitude 45°22'48" west), at approximately 250 km from the capital city of São Luís. Experiments were conducted with the approval of the Ethics Committee for the Use of Animals at the Universidade Estadual do Maranhão (CEUA/UEMA) under license 01200.002200/2015-06(449).

Seminal samples were collected from six males of the tambaqui species *C. macropomum*, selected from the breeding stock of the fish farm and which presented semen release when submitted to gentle pressure in the abdominal region. Then, the fish were placed in masonry tanks, with constant water circulation for subsequent hormonal induction, using 2.0 mg of raw carp pituitary extract – CPE per kg of live fish (Pinheiro et al. 2016).

After 8 hours of hormonal induction, the fish were restrained and their urogenital region was cleaned and dried with a paper towel to avoid contamination (by water, mucus, or urine). Then, semen collection was performed in graduated glass test tubes. Samples were kept in an isothermal box at a temperature ranging from 4 to 6 °C.

Samples were identified and analyzed individually at the fish farm breeding laboratory for absence of sperm motility by observation under an light microscope at 400× magnification. After confirmation that sperm were immobile, 2 µL aliquots of semen from each sample were activated with 100 µL of 0.9% NaCl saline solution for initial characterization, observing the staining, viscosity, initial subjective sperm motility rate and motility duration time, also under an optical microscope with 400× magnification. Samples with subjective motility above 80% were included in the experiment (Santos 2013).

Milt samples of each male were exposed to two pesticides (glyphosate 480 g/L – ISORGAN; and fenitrothion, SUMITHION 500 g/L) at five concentrations (6, 12, 24, 120 e 240 mg/L), with dilution in 0.9% NaCl saline solution. Exposition began with the direct activation of 2 µL aliquots of semen with 100 µL of each of the pesticide dilutions. There was an immediate subjective analysis of sperm motility at times 0 (after homogenization), 30 and 60 seconds after activation/exposure, with the aid of a light microscope with a magnification of 400×, by the same evaluator. As a control treatment, we conducted the analysis of sperm motility after activation with a 0.9% NaCl solution (Santos 2013) with the same analysis times described for pesticide residues.

The chosen experimental design was a factorial test. A statistical analysis was performed through the assessment of means and standard deviations, from which the analysis of variance test (ANOVA) was obtained. When there was observable difference between treatments, the Skot-knott test was applied, at a significance level of 5% in the statistical program SISVAR 5.7.

Results

During the initial observation period, semen samples from all individuals presented white color and high viscosity. *In natura* samples exhibited an initial motility of 89.2 ± 4.9% and a mean duration of 100 seconds (up to 10% of sperm motility). The activations were carried out at environmental temperature (27–29 °C) and the solutions containing

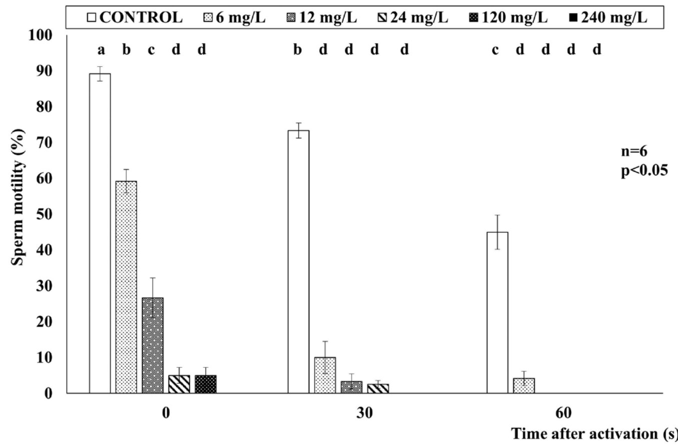

Figure 1. Percent sperm motility (mean ± standard error) of Tambaqui semen exposed to different concentrations of glyphosate.

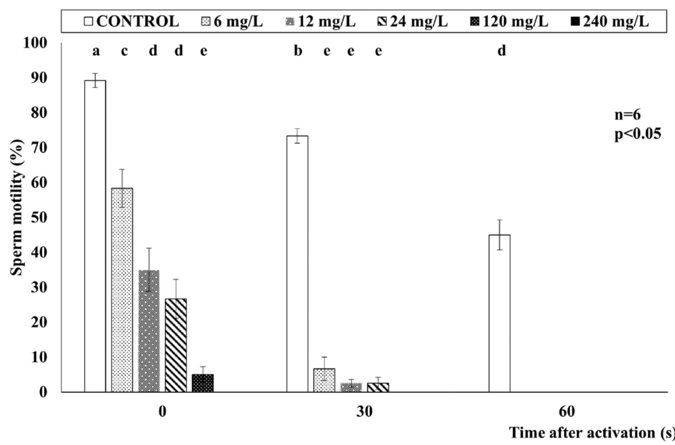

Figure 2. Percent sperm motility (mean ± standard error) of Tambaqui semen exposed to different concentrations of fenitrothion.

pesticide residues showed a ph ranging from 5.2 to 5.5 for Glyphosate, and from 5 to 5.7 for fenitrothion, based on the value of 5.9 for NaCl.

From the tested treatments with direct exposure of semen to pesticide residues, it was possible to observe the effect of exposing tambaqui semen to residues of glyphosate (Figure 1) and fenitrothion (Figure 2) soon after exposure. All treatments showed a significant reduction ($p < 0.05$) when compared to sperm activated only with 0.9% NaCl, with more deleterious effects at concentrations above 24 mg/L glyphosate, and sperm agglutination at a concentration of 240 mg/L, it is not possible to attribute sperm motility rate subjectively.

Discussion

Glyphosate is an herbicide belonging to the organochlorine group and is classified as an extremely toxic product (Class I) (ANVISA 2021), widely used mainly in plant cultures, such as rice, potatoes, bananas, onions, corn, pastures, soybeans, ornamental shrubs, and flowers, at different stages of development. Changes in body structure, early hatching of eggs, embryo mortality and larval depigmentation are indicative of the toxic effects of these substances (Sanchez 2015).

Lopes et al. (2014), in an experiment conducted with fish *Danio rerio* (Hamilton 1822), observed that sperm exposed for 24 and

96 hours to 5 and 10 mg/L of glyphosate exhibited a decrease in motility and in its duration. Moreover, according to these authors, individuals that were subjected to higher concentrations for a prolonged period showed functional and membrane changes in sperm mitochondria, as well as a reduction in DNA integrity, indicating that glyphosate is a highly dangerous agent for the reproduction of this species, and even harming others.

The tambaqui species is considered a biological model due to its resilience to environmental changes (Val & Oliveira 2021). It has been pointed out as a bioindicator of environmental pollution and used in several toxicological studies, such as the assessment of genotoxic and hematological parameters (Carvalho-Neta et al. 2015), and branchial lesions and erythrocytic abnormalities (Castro et al. 2019), both using individuals from an environmental protection area from the Upaon-Açu island, State of Maranhão, northeastern Brazil. Regarding agricultural defensives specifically, studies carried out with tambaqui by Cunha et al. (2018) indicated alteration and damage of nuclear erythrocytes in the gills and liver of tambaqui exposed to pesticides such as Deltamethrin.

As observed for glyphosate, sperm motility rates were significantly reduced ($p < 0.05$) after direct activation in all tested concentrations of fenitrothion (Figure 2), with no sperm motility being verified at a concentration of 240 mg/L right after direct activation (time 0). Fenitrothion, also belonging to the group of organophosphates, is a class II insecticide (moderate toxicity) widely used in pest control (Milanez et al. 2007). In Brazil, it has been used in agriculture since 1959, authorized in cotton, onion, chrysanthemum, apple, and soy crops, to control ants (ANVISA 2021). In aquaculture, it is used to control insect larvae, just as already reported in Bangladesh, India, to combat the tiger beetle (Rahma et al. 2020). Even considering their history of use in Brazilian agriculture and livestock, there are few organophosphates and pesticides in general that are authorized by the national legislation for fish farming (Tavechio et al. 2009), with no formal authorization or indication of the use of fenitrothion in aquaculture being recorded.

Agricultural pesticides, mainly pyrethroids and organophosphates, have been largely used in Brazil since the 1990s, with the main objective of providing increases in agricultural production by combating pests that, if not controlled, can exterminate the entire crop in a short period of time (Moraes 2019). However, while these substances bring benefits to agriculture, the number of studies that prove the ecotoxicological effects of pesticides on human and animal health, especially those living in aquatic environments, is undeniable (Montanha & Pimpão 2012, Santana & Cavalcante 2016, Ribeiro & Américo-Pinheiro 2018).

Several species of Neotropical fish employ external fecundation and fertilization as a reproductive strategy, with total discharge of male and female gametes in the water (Witeck et al. 2011), a fact that provides a large exposure of gametes to the contaminated environment (Rodrigues et al. 2019). As a result, the entire fertilization process, from sperm motility to embryonic development, can be directly affected by excess contaminants, such as pesticide residues leached from the soil by rain, or even by inadequate disposal of containers and residues (Kime & Nash 1999, Ferreira 2016). These contaminants greatly influence the reproduction and renewal of fish species stocks, which can lead to an environmental imbalance and a reduction in species diversity (Mondal et al. 2015).

Amazonian fish known as round fish, such as the tambaqui *Colossoma macropomum*, and the pirapitinga *Piaractus brachypomus* (Cuvier 1818), as well as their hybrids, have become the most important native species of Brazilian fish farming, especially in the North and Northeast regions of the country (Muniz et al. 2008, PEIXEBR 2021), due to their good adaptation to the climatic conditions found in those regions, which are considered ideal for these species. They are species that have external fecundation and fertilization, with annual and total reproduction, with the river flooding period being the main spawning season for this group of fish (Vieira et al. 1999). In captivity, they are reproduced through the hormonal induction technique, using raw Carp Pituitary Extract – CPE (Maria et al. 2011), in addition to synthetic hormones.

Normally, the action of organophosphates occurs through the irreversible inhibition of enzyme acetylcholinesterase (AChE), responsible for the degradation of acetylcholine, the main neurotransmitter in the central nervous system of insects (Barboza et al. 2018). In this sense, organophosphates are widely used in fish farms to control fish parasites, as well as to combat insect larvae of order Odonata, which have the habit of preying on fish larvae and causing financial damage to producers (Fortunato et al. 2020). Despite being efficient in combating and chemically controlling dragonfly nymphs and other insects, organophosphates have been proven to be considerably toxic (Queiroz 2017).

Organophosphates combined with pyrethroids are already used both in agriculture and in livestock, as the mixture of both promotes synergism in their actions (Trevis et al. 2010). In a study to assess the toxic effect of the mixture between organophosphates and pyrethroids in *Pimephales promelas* larvae, it was observed that the combination resulted in high toxicity (Wheelock et al. 2005), demonstrating the importance of conducting studies aimed at evaluating the application of the mixture of these two pesticides in aquatic environments.

In a recent study published by Santos et al. (2021), when evaluating the toxic effects of pesticides on the reproductive processes of freshwater fish based on articles published from 2000 to 2019, they observed that insecticides were present in 78% of the studies, mainly Endosulfan (35%) and Cypermethrin (13%), which are classified respectively as organochlorine and pyrethroid. The authors also highlighted that the most reported routes of action in the studies (57.5%) were reproductive endocrine disorders, with changes ranging such as decreased fertility due to histological damage to testicles and ovaries; impairment of the vitellogenesis process and interruption in the steroidogenesis process; delay in gonad maturation evidenced by alterations in the Gonadosomatic Index; alteration in reproductive and parental behavior; compromised olfactory response and consequent disorder in reproductive migrations; as well as disturbances in the coordination of courtship behavior of male and female fish and spawning time (Jaensson et al. 2007, Singh & Singh 2008, Marcon et al 2015, Sumon et al. 2019).

Our study is the first one reporting the use of sperm cells from native Neotropical fish (tambaqui) directly exposed to pesticides in ecotoxicological tests. Tests demonstrated that these organisms are highly susceptible to changes that can be caused by contact with pesticide residues, such as glyphosate and fenitrothion. Therefore, the presence of pesticides in freshwater environments can interfere in the reproduction of tambaqui fishes, and consequently, in the renewal of this fish species stocks. However, the determination of a protocol for analysis must be conducted, as to standardize the techniques and to express a result that can be taken into consideration by competent government bodies that mediate requests for the authorization of use of new chemical substances as agricultural pesticides in Brazil. In addition, we believe that the results here obtained would be extrapolated to other native species that have a similar reproductive cycle or biology, helping us to understand how contaminants generated by agricultural production can affect the reproduction of these species, and how can we adopt conservation actions to prevent this.

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Raimunda Nonata Fortes Carvalho-Neta: Substantial contribution in the concept and design of the study; Contribution to critical revision, adding intellectual content.

Conflicts of Interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

Data Availability

The data used in our analysis is available at Biota Neotropica Dataverse https://doi.org/10.48331/scielodata.PIL6JB

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