Semina: Ciências Agrárias
ISSN: 1676-546X
semina.agrarias@uel.br
Universidade Estadual de Londrina
Brasil

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Semina: Ciências Agrárias, vol. 37, núm. 3, mayo-junio, 2016, pp. 1667-1677
Universidade Estadual de Londrina
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DOI: 10.5433/1679-0359.2016v37n3p1667

Sperm motility, fertilization, and larval development of silver catfish (*Rhamdia quelen*) in copper-contaminated water

Motilidade espermática, fertilização e desenvolvimento inicial de jundiá (*Rhamdia quelen)* em água contaminada com cobre

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Abstract

The objective of this study was to evaluate the effect of copper-contaminated water on sperm motility, fertilization, and embryonic and larval development of silver catfish (*Rhamdia quelen*). A randomized experimental design with five treatments and four replicates was used. Two experiments were carried out: (1) controlled fertilization was performed under different levels of copper contamination and egg hatching was performed in clean water; and (2) copper-contaminated water was used for both fertilization and hatching assays. The time of sperm motility and sperm motility rates linearly decreased with increasing copper concentration in the water. Fertilization and hatching rates were also affected when the concentrations of copper in the water were above 0.0979 mg Cu^{+2} L^{-1} and 0.0331 mg Cu^{+2} L^{-1}, respectively. Gamete exposure to levels between 15 mg Cu^{+2} L^{-1} and 60 mg Cu^{+2} L^{-1} for short periods of time negatively affected sperm motility, oocyte fertilization, and egg hatching rates. In addition, when gametes and embryos were exposed at levels above 0.03 mg Cu^{+2} L^{-1} during long periods of time, egg hatching rates were reduced, and at levels between 0.05 mg Cu^{+2} L^{-1} and 0.20 mg Cu^{+2} L^{-1} the number of abnormal larvae increased.

Key words: Embryo. Fish. Heavy metal. Reproduction. Spermatozoa.

Resumo

O objetivo deste estudo foi avaliar o efeito da água contaminada de cobre na motilidade espermática, fertilização e desenvolvimento embrionário e larval de jundiá (*Rhamdia quelen*). O delineamento experimental foi inteiramente casualizado, com cinco tratamentos e quatro repetições. Dois ensaios foram conduzidos: (1) a fertilização controlada foi realizada com diferentes níveis de água contaminada com cobre e a incubação do ovo realizada em água limpa; e (2) a contaminação da água foi utilizada tanto na fertilização e quanto na incubação dos ovos. O tempo de motilidade de espermatozóides e as taxas de motilidade do esperma foram reduzidas com o aumento da concentração de cobre na água. As taxas de fertilização e eclosão também foram afetados quando as concentrações de cobre na água

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Recebido para publicação 16/06/15 Aprovado em 23/09/15

estavam acima de 0,0979 mg Cu^{+2} L^{-1} e 0,0331 mg Cu^{+2} L^{-1}, respectivamente. A exposição dos gametas por um curto período de tempo em níveis entre 15 ou 60 mg de Cu^{+2} L^{-1} promoveu danos sobre a motilidade dos espermatozóides, a fertilização de oócitos e a eclosão dos ovos. Além disso, quando os gametas e embriões foram expostos a níveis acima de 0,03 mg de Cu^{+2} L^{-1} por um longo período de tempo, a eclosão dos ovos foi reduzida, e em níveis de 0,05 a 0.20mg Cu^{+2} L^{-1} o anormalidade larval aumentou.
Palavras-chave: Embrião. Espermatozóides. Metais pesados. Peixe. Reprodução.

Introduction

Reports on the lethal effect of contaminated water on aquatic organisms resulting from human activities such as the release of pesticides and heavy metals, have aroused concern (SCOTT; SLOMAN, 2004). Even sublethal concentrations may have medium- and long-term consequences such as reduced reproduction in fish. In the water, gametes are exposed to pollutants (ABASCAL et al., 2007) that can modify their features (DIETRICH et al., 2012), reducing or preventing natural or artificial propagation (WITECK et al., 2011).

There have been several reports on the influence of heavy metals on the quality of gametes (LAHNSTEINER et al., 2004), fertilization of oocytes (RURANGWA et al., 1998), sperm motility, egg hatching (WITECK et al., 2011), and quality of larvae (SARNOWSKI, 2004). Among several pollutants, copper is a potential pollutant of water bodies because it derives from agricultural, industrial, and domestic effluents (MILESI et al., 2008). Considering the complex interactions between pollutants and aquatic biological systems, it is important to continue studying the effects of heavy metals on the reproductive processes of freshwater fish and their potential impact on natural populations and aquaculture production.

The present study was carried out to evaluate the effect of copper-contaminated water on sperm motility, controlled fertilization, and embryonic and larval development of the silver catfish (*Rhamdia quelen*). *R. quelen* is an important species to fisheries and aquaculture in South America, and is widely used as a biological model in reproductive research (KOAKOSKI et al., 2012).

Materials and Methods

Two experiments were carried out to evaluate the effects of copper-contaminated water on sperm and oocytes during fertilization and on egg incubation. In the first, oocyte fertilization and egg incubation were performed in uncontaminated water, whereas in the second, copper-contaminated water was used.

Males and females of silver catfish (*Rhamdia quelen*) were kept in a 200 m^2 earthen pond with water supply to compensate for water loss through evaporation and infiltration. Fish were fed commercial feed containing 32% crude protein.

Experiment 1: Gametes exposure and fertilization of oocyte in copper-contaminated water and egg incubation in uncontaminated water

Seven females (263.00 ± 74.37 g) and five males (173.60 ± 22.30 g) were used in the experiment. Selected females exhibited a rounded abdomen, reddish urogenital papilla, and uniform oocyte color and size (SANCHES et al., 2011a). Males releasing sperm after gentle pressure on the abdomen were selected (SANCHES et al., 2011a).

The selected breeders were individually weighed, marked, and separated according to sex in two tanks equipped with aeration and constant water renewal. Hormonal induction to synchronize ovulation or spermiation was performed by injecting carp pituitary extract (CPE) intramuscularly into the dorsal region (BOMBARDELLI et al., 2006). In females, hormonal manipulation was applied in two doses. The first one comprised 0.5 mg CPE. kg^{-1} and the second, applied 12 hours later, had a

concentration of 5.0 mg CPE.kg^{-1} (SANCHES et al., 2011a). Males underwent hormonal manipulation with a single dose of 3 mg CPE.kg^{-1}.

Water physico-chemical parameters were monitored every 2 hours. Dissolved oxygen (Hanna digital oximeter F-HI 9147), pH (Hanna digital pH meter F-HI 8424), and electrical conductivity (Hanna, HI 99301) were 4.25 ± 0.077 mg L^{-1}, 7.76 ± 0.45, and 0.07 ± 0.04 mS.cm^{-1} respectively. Temperature was maintained at 27.35 ± 1.91 ºC.

Gametes were collected at 240 accumulated thermal units after the last hormonal application (corresponding to 8 h and 46 min at a water temperature of 27.35 ºC). Individual fish were caught, dried with a paper towel, and subjected to abdominal massage in the cephalocaudal direction (WITECK et al., 2011). The first batches of oocytes and drops of sperm were discarded in order to avoid possible contamination with urine or feces (BROOKS et al., 1997). Remaining oocytes were collected in a Petri dish (ambient temperature of 25 ºC). Approximately 5 mL of sperm was collected in a test tube (~0.1 mL) (ambient temperature of 25 ºC). Sperm was stored at 12 ºC during the period of time required to conduct manipulation experiments (TESSARO et al., 2012). Oocytes and sperm from each breeder were separately mixed and stored, according to Sanches et al. (2011a).

Sperm parameters were assessed by measuring time of sperm motility (ROMAGOSA et al., 2010), sperm survival rate (TESSARO et al., 2012), and sperm concentration (SANCHES et al., 2011b). Time of sperm motility and fertilization assays were performed using a randomized experimental design comprising five treatments with four replicates each. Based on pilot experiments, different levels of copper-contaminated water (0.0, 15.0, 30.0, 45.0, and 60.0 mg Cu^{+2} L^{-1}) were considered. Contaminated water samples were used for gamete activation and to conduct controlled fertilization. Eggs were incubated in uncontaminated water. The experimental units (in duplicate) for fertilization assays consisted of 2.5-L incubators with a water recirculation system, containing 2,387 ± 45 eggs. Incubators were kept under controlled temperature (25 ± 1 ºC). For sperm motility assays, a sample from the sperm pool was diluted in copper-contaminated water.

Pilot experiments used the same experimental set up and protocols but a smaller number of incubators and water volume. In these assays, different concentrations of copper were added to the water to determine embryonic mortality. These results were used to choose the lowest copper concentration and plan the experimental treatments. Pilot assays were repeated as many times as necessary until the lethal concentration could be determined.

For the controlled fertilization assays, 100 µL of sperm (BOMBARDELLI et al., 2006) was added to 2 mL of oocytes (2,387 ± 45 oocytes). Gametes were activated by adding 20 mL of copper-contaminated water, using Copper II Nitrate Trihydrate P.A. (Vetec Chemicals®). Immediately after water contamination, gametes and water were kept in gentle motion for 60 seconds. Subsequently, eggs were washed three times and incubated in uncontaminated water.

Electrical conductivity, pH, and dissolved oxygen of the water where eggs were incubated were measured before fertilization (time = 0 hours) and eight hours later (time = 8 hours). Fertilization rates were determined eight hours after activation of gametes (HILBIG et al., 2008) by counting three samples of 183 ± 45 eggs from each experimental unit. Twenty-four hours after gamete activation (when most eggs had hatched), 100 larvae from each experimental unit were collected and reared for 21 days in uncontaminated water to determine survival rates. The remaining larvae in the incubators were fixed in 4% buffered formalin solution and counted to estimate egg hatching rates. Fixed larvae were observed under a stereomicroscope (×10) in order to determine the percentage of normal larvae (SANCHES et

al., 2011a). Larval development was classified as normal or abnormal according to Jezierska et al. (2000).

Larvae used in the rearing experiments were kept in incubators for 4 days to develop their swimming abilities. They were then transferred to 20 PVC aquaria of 30 L each, at a density of 3.33 larvae L^{-1}. Aquaria with a recirculation system and a total volume of 1.5 m^3, were kept under controlled temperature conditions (25.0 ± 0.1 °C). Electrical conductivity, pH, and dissolved oxygen were measured weekly.

Experiment 2: Gametes exposure, fertilization of oocyte and egg incubation in copper-contaminated water

Eight females (255.0 ± 57.32 g) and six males (216.7 ± 58.54 g) were selected to produce oocytes and sperm, as described previously. Controlled fertilization and incubation protocols, as well as the experimental set up were carried out as in experiment 1. Based on pilot assays (as described above), treatments consisted in using different concentrations of copper-contaminated water (0.00, 0.05, 0.10, 0.15, and 0.20 mg Cu^{+2} L^{-1}). Each treatment was performed in a separate recirculation system and the copper-contaminated water from each treatment was used for controlled fertilization and incubation assays. The experimental units of the fertilization assays contained 2,660 ± 20 eggs and fertilization rates were measured by counting three samples of 212 ± 28 eggs from each experimental unit. Gametes and embryos were kept in copper-contaminated water until egg hatching.

The analysis of sperm parameters was done as described before, except concerning the determination of sperm motility. Motility was assessed by subjective estimation under a light microscope (×400; adapted from VIVEIROS et al., 2012; HERNANDEZ CUADRADO et al., 2014). For this, 80 µL of sperm was diluted in 800 µL of copper-contaminated water.

Fertilization and egg hatching rates, larval development, and larvae survival after 21 days of rearing were determined as described in experiment 1. Water physico-chemical parameters in the incubation assays were measured just before fertilization (time = 0 hours) and eight hours later (time = 8 hours), at the beginning of egg hatching, and at the end of egg hatching.

Statistics analysis

One-way analysis of variance (ANOVA) and linear regression analysis (5% significance level) were used to analyze the results. The assumptions of normality and homogeneity of variances were checked. Statistical analysis was performed using STATISTICA 7.0®.

Results

Electrical conductivity, dissolved oxygen, pH, and temperature of the water used in egg incubation assays remained stable in both experiments (Table 1 and Table 2). Females produced 1,990 ± 20 oocytes mL^{-1} and 1,330 ± 10 oocytes.mL^{-1}, whereas males produced 7.57 × 10^{10} sperm cells.mL^{-1} and 4.88 × 10^{10} sperm cells.mL^{-1}, in the first and second experiments, respectively. Sperm survival rates were 92.6% and 93.5%, and sperm motility was 29.33 ± 1.91 seconds and 22.30 ± 1.02 seconds, in the first and second experiments, respectively.

Table 1. Physicochemical parameters of the water used in the incubation systems to first experiment. Time zero (0) hour corresponds to the moment of fertilization, and time eight (8) hours corresponds to eight hours after the fertilization (n=4 experimental unit).

Parameters	Time 0 hour	Time 8 hours
Electrical Conductivity (mS cm^{-1})	0.08±0.01	0.09±0.00
Dissolved oxygen (mg L^{-1})	4.50±0.34	5.10±0.56
pH	7.55±0.10	7.73±0.09
Temperature (ºC)	24.70±0.20	25.00±0.30

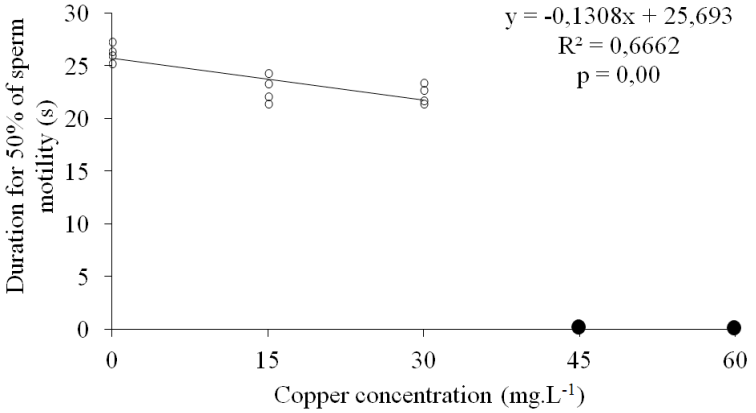
Table 2. Physicochemical parameters of the water used in the egg incubation systems to second experiment. Time zero (0) hour corresponds to the moment of fertilization, time eight (8) hours corresponds to eight hours after the fertilization. Beginning and end of hatching correspond to the moment of beginning and end of egg hatching (n=4 experimental unit).

Parameter	0 hours	8 hours	Beginning hatching	End hatching
Electrical Conductivity (mS cm^{-1})	0.04±0.00	0.04±0.00	0.04±0.00	0.04±0.00
Dissolved oxygen (mg L^{-1})	5.64±0.55	5.15±1.01	4.94±0.54	5.70±0.73
pH	7.92±0.08	7.85±0.91	8.23±0.10	8.17±0.09
Temperature (ºC)	24.20±0.17	25.42±0.16	25.12±0.11	26.28±0.24

Experiment 1: Sperm parameters, fertilization and hatching rates, and larval normality and survival

The time of sperm motility was inversely proportional to the increase of copper concentration in the water ($p < 0.05$). Motility was drastically compromised at concentrations of 45 mg Cu^{+2} L^{-1} and 60 mg Cu^{+2} L^{-1} (Figure 1) due to sperm agglutination. Agglutination indexes were not quantified.

Figure 1. Time of spermatozoa motility in semen of jundiá (*Rhamdia quelen*) diluted in water containing different concentrations of copper. Open circles represent valid data submitted to statistical analysis (The mean from an experimental unit resulted by the evaluation of three repetition; n=4 experimental unit). Full circles represents non valid data due occurrence of null results on all evaluations. The evaluations were carried out at 24.70±0.20 ºC (n=4).



Fertilization and egg hatching rates also linearly decreased as water copper concentration increased ($p < 0.05$) (Figure 2).

Copper-contaminated water did not influence larval development ($p > 0.05$): 87.8 ± 1.3% to 90.5 ± 1.1% of the larvae were normal (Table 3). Larval development was not analyzed at 60 mg Cu^{+2} L^{-1} due to the reduced number of hatched eggs in this treatment. Similarly, copper-contaminated water did not affect survival rates of larvae reared for 21 days in uncontaminated water ($p > 0.05$) (Table 3).

Figure 2. A) Controlled fertilization rates of oocytes, The fertilization mean from an experimental unit resulted by the evaluation of 183±45 eggs; n=4 experimental unit; and B) Eggs hatch rates of silver catfish (*Rhamdia quelen*) submitted to gamete activation in copper-contaminated water and controlled incubation in uncontaminated water. The hatching mean from an experimental unit resulted by the evaluation of 2,387±45 eggs; n=4 experimental unit. The evaluations were carried out at 25.00±0.30 ºC (n=4).

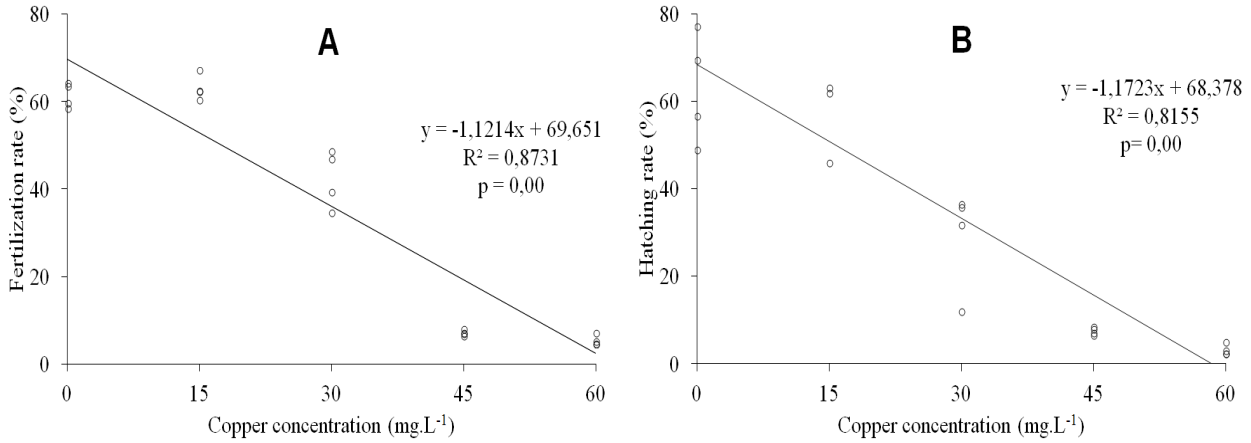


Table 3. Percentage of normal larvae immediately after egg hatching and percentage of survival of the progenies reared for 21 days in uncontaminated water. Progenies of silver catfish (*Rhamdia quelen*) derived from oocytes fertilized in water contaminated with different concentrations of copper, and eggs incubated in uncontaminated water.

Parameters	Concentration of copper in water (mg L^{-1})					
	0	15	30	45	60	p
Normal larvae (%)[1]	89.3±2.7	90.5±1.1	90.0±1.6	87.8±1.3	---	0.21
Survival (%)[2]	32.5±32.0	18.3±16.0	25.0±12.9	13.3±14.1	15.3±5.5	0.58

[1](The mean resulted by evaluation of 300 larvae, from an experimental unit; n=4 experimental unit). [2](The mean resulted by evaluation of 100 larvae, from an experimental unit; n=4 experimental unit).

Experiment 2: Sperm parameters, fertilization and hatching rate, larval normality and survival

Results showed that even at low copper concentrations, the use of contaminated water during gamete activation caused damage to sperm motility. The time of sperm motility and sperm motility was inversely proportional to the increase in water copper concentration ($p < 0.05$) (Figure 3).

After exposure of gametes and embryos to copper-contaminated water, fertilization and egg hatching rates exhibited a linear response plateau ($p < 0.05$). Model results suggest that problems related to fertilization and hatching may occur already at contamination levels of 0.0979 mg Cu^{+2} L^{-1} and 0.0331 mg Cu^{+2} L^{-1}, respectively (Figure 4). At these contamination levels, estimated fertilization and hatching rates were 53.78% and 41.29% of the total amount of the oocytes and eggs, respectively (Figure 4).

Figure 3. A) Time of spermatozoa motility, and B) Sperm motility rate in silver catfish (*Rhamdia quelen*) sperm diluted in water containing different levels of copper. The mean from an experimental unit resulted by the evaluation of three repetition; n=4 experimental unit. The evaluations were carried out at 24.20±0.17 ºC (n=4).

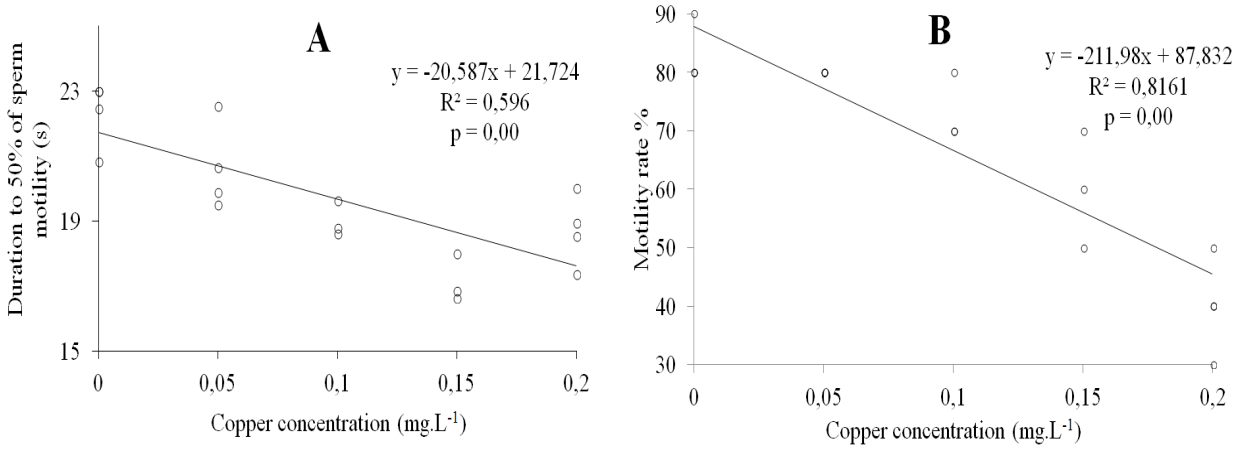
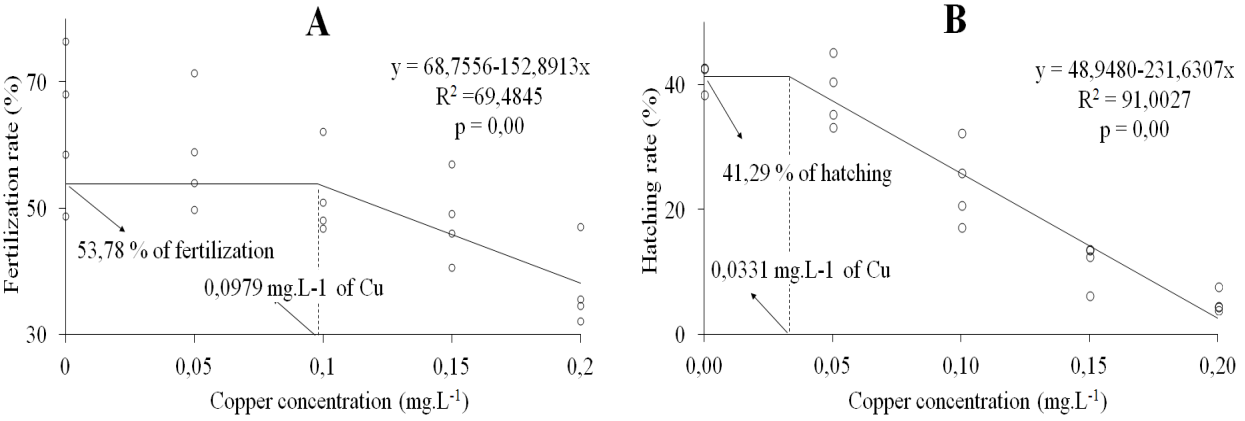


Figure 4. A) Controlled fertilization rates of oocytes, and B) Eggs hatch rates of silver catfish (*Rhamdia quelen*) submitted to gamete activation and controlled incubation of the eggs in water contaminated with different levels of copper. The fertilization mean from an experimental unit resulted by the evaluation of 212±28 eggs; n=4 experimental unit. The hatching mean from an experimental unit resulted by the evaluation of 2,660±20 eggs; n=4 experimental unit. The evaluations of fertilization and hatching rates were carried out at 25.42±0.16 ºC (n=4) and 26.28±0.24ºC (n=4), respectively.



By maintaining gametes and embryos in contaminated water until hatching, the percentage of normal larvae decreased with an increase in the water copper concentration ($p < 0.05$) (Figure 5). However, when gametes and embryos were reared for 21 days in uncontaminated water, no difference in survival rates was observed between treatments (Table 4).

Figure 5. Percentage of normal larvae derived from silver catfish (*Rhamdia quelen*) progenies exposed to water containing different concentrations of copper from fertilization until the moment of egg hatching. The mean resulted by evaluation of 300 larvae, from an experimental unit; n=4 experimental unit.

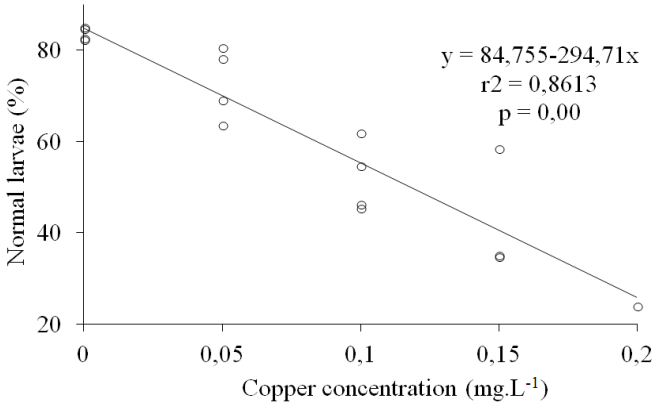


Table 4. Percentage of survival of the progenies reared for 21 days in uncontaminated water. Progenies of silver catfish (*Rhamdia quelen*) derived from fertilized oocytes and eggs incubated in water contaminated with different concentrations of copper.

Parameter	Concentration of copper in water (mg L^{-1})					
	0.00	0.05	0.10	0.15	0.20	p
Survival(%)[1]	55.5±28.5	47.5±19.0	71.5±6.6	69.5±10.7	68.5±6.9	0.23

[1](The mean resulted by evaluation of 100 larvae, from an experimental unit; n=4 experimental unit).

Discussion

During both experiments, water electrical conductivity, dissolved oxygen, pH, and temperature remained at levels considered suitable for this species (BALDISSEROTTO; RADÜNZ NETO, 2004). The values of sperm concentration found in this study were similar to those described by Borges et al. (2005) ($6.6 \times 10^{10} \pm 3.6 \times 10^{10}$ cells mL^{-1}) and Sanches et al. (2011a) (4.38×10^{10} cells mL^{-1}). The production of oocytes was also within the normal range reported for this species (GOMES et al., 2000).

The time of sperm motility, of sperm diluted in uncontaminated water, was similar to the one determined by Hilbig et al. (2008) (23.9 ± 2.7 seconds to 26.2 ± 3.4 seconds) and Sanches et al. (2011a) (22.19 ± 1.03 seconds). Sperm survival rates were similar to those described by Tessaro et al. (2012) (62.57 ± 3.62 to 93.52 ± 0.85 seconds). Sperm dilution in copper-contaminated water at concentrations above 45 mg L^{-1} had a negative effect on sperm motility, possibly due to the ability of heavy metals to bind to flagellum proteins affecting sperm motility (DIETRICH et al., 2010). Furthermore, at these concentrations, agglutination of sperm cells occurs, contributing to the inhibition of sperm motility. The loss of sperm motility may be associated with alterations in cell structure induced by pollutants, such as changes in the midpiece of the flagellum, an area that contains mitochondria and is involved in the production of energy. In the gold fish *Carassius auratus*, sperm exposure to 100 mg L^{-1} of mercury chloride reduced flagellum length, whereas exposure to 0.01 mg L^{-1} for 24 hours increased head length, width and area (VAN LOOK; KIME, 2003).

The heavy metals may also inhibit the activity of cytochrome-related proteins and enzymes

responsible for redox functions (SORRENTINO et al., 2005). Copper, specifically, is able to interfere with osmoregulation (BIANCHINI et al., 2004) and oxygen consumption rate, through alterations in enzymes involved in metabolic functions, such as in the sodium pump (MCGEER et al., 2000; HANDY, 2003). Problems related to the effect of copper on Na^+ and K^+ ion transport, and on the ATPase enzyme activity were also observed in the common carp *Cyprinus carpio* (DE BOECK et al., 2003) and the Mozambique tilapia *Oreochromis mossambicus* (LI et al., 1998).

The negative effects of heavy metals on sperm motility have also been observed in other species. Despite the fact that Abascal et al. (2007) did not verify loss of sperm motility sea bass

(*Dicentrarchus labrax*) sperm after 20 seconds exposure to 100 ppm of copper, water contaminated with 0.01 ppm of mercury chloride significantly damaged sperm cells. Mode of action and toxicity of heavy metals and other pollutants are probably species specific.

Oocytes and embryos are also negatively affected by heavy metals and other pollutants, which may interfere with fertilization (WITECK et al., 2011) and embryonic development. Concentrations of 100 mg L^{-1} of mercury or cadmium completely inhibited sperm motility in the rainbow trout *Oncorhynchus mykiss*, and the exposure of its eggs to 10 mg L^{-1} of these metals for 4 hours reduced fertility rates (DIETRICH et al., 2010).

The results of the present study suggest that copper-contaminated water has toxic effects on gametes, embryos, and larvae of the silver catfish (*Rhamdia quelen*). Sperm quality was clearly affected by metal contamination, since sperm motility decreased linearly with an increase in metal concentration in water. In addition, fertilization and hatching rates were also affected when copper concentrations in water were above 0.0979 mg Cu^{+2} L^{-1} and 0.0331 mg Cu^{+2} L^{-1}, respectively.

The damage caused by this pollutant is certainly cumulative and depends on several factors, such as concentration and time of exposure. This was confirmed by the observation that, under brief copper exposure, larval development was normal, whereas the amount of normal larvae decreased proportionally to copper increase in water used to hatch the embryos.

Hilbig et al. (2008) observed that eggs of *R. quelen* were also sensitive to lead-contaminated water at concentrations above 0.25 mg PB L^{-1}, whereas cadmium-contaminated water was seen to cause toxicity at levels above 40.42 mg Cd L^{-1} (WITECK et al., 2011).

The negative effects of pollutants on fertilization and embryonic development may also be related to the effect of pollutants on the micropyle, preventing the entry of the sperm cell in the oocyte (KIME, 1995). The toxicity of heavy metals present in activating solutions also cause negative effects on the sperm (RURANGWA et al., 2004), particularly on the maintenance of cell integrity. Furthermore, exposure to heavy metals for long periods of time may damage the cell membranes of gametes and embryos (BROOKS et al., 1997), or affect physiological processes important for ontogenesis.

Despite evidence of the damage caused by exposure of *R. quelen* gametes, embryos, and larvae to copper-contaminated water, little is known about the mechanisms that control these processes. In general, low levels of copper-contaminated water affect the larvae. This may be due either to the direct effect of metals on gametes and embryos, or indirectly to the alteration or inhibition of important physiological processes for cellular energy production or ontogenesis.

Conclusion

Exposure of *R. quelen* gametes to copper-contaminated water can negatively influence the natural or artificial propagation of this species.

Exposure to copper negatively affected sperm motility, oocyte fertilization, and egg hatching. In addition, it affected larval development by increasing larval abnormality.

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