

## Copper affect the multixenobiotic resistance mechanism (MXR) in embryo and larvae of *Rhamdia quelen*

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### Abstract

P-glycoproteins (P-gp) and Multidrug resistance protein (MRP) represent a family of ABC (ATP-binding cassette) transporters responsible for multixenobiotic resistance mechanism (MXR) in aquatic organisms. In the current study the modulation of P-gp and MRP proteins was evaluated in embryo and larvae of *Rhamdia quelen* fish species exposed to copper. Adult females were exposed by gavage during 60 days to copper (5 mg Cu kg<sup>-1</sup>) and eggs, embryos, and larvae from exposed and unexposed females were exposed to 30 mg Cu L<sup>-1</sup>. The activity of ABC transporters was accessed via calcein accumulation assay using the specific inhibitors: Verapamil (P-gp) and MK571 (MRP). P-gp activity was detected in all analyzed stages whereas MRP activity was observed after 36 and 96 hpf. Oocytes from females previously exposed and larvae stages (36 and 96 hpf) accumulated less calcein than no exposed oocytes, showing higher ABC transporters activity. In individuals exposed to copper, a higher inhibitory effect was observed 1 hpf. The modulation of ABC transporter proteins is time dependent throughout the development, and the initial stages are more sensible to copper. These findings highlight the MXR mechanism as a biomarker of pollutant exposure in early stages of development of *R. quelen*.

Keywords: MXR mechanism, *Rhamdia quelen*, inorganic copper, biomarkers, early development stages.

### INTRODUCTION

The super-family of transport proteins ABC (ATP-binding cassette) are highly conserved transmembrane proteins (Dean & Annilo, 2005), and some of its members are related with those of the aquatic organisms known as multixenobiotic defense mechanism (MXR). The MXR is like the multidrug resistance mechanism (MDR), found in tumor cell lines of mammals as the result of low intracellular accumulation of anti-cancer drugs (Locher, 2016).

ABC transporters act as efflux pumps reducing the intracellular accumulation of endogenous metabolites and xenobiotic compounds, hence protecting exposed organisms from possible toxic effects of chemicals (Litman *et al.*, 2001). Moreover, it has been shown that some compounds can

decrease or inhibit the activity of the transport both *in vitro* (Oosterhuis *et al.*, 2008) and *in vivo*, leading to the increase in the intracellular concentration of xenobiotics potentiating the effect of these toxic compounds (Bošnjak *et al.*, 2009). Chemicals that inhibit the transporters associated with the MXR are described as chemosensitizers agents classified into two categories: (1) competitive inhibitors with high affinity that prevent the binding and consequently the active transport of other substrates; and (2) non-competitive, blocking the ATPase activity of transporters (Faria *et al.*, 2011).

The MXR was described in a diversity of organisms and is related with P-glycoprotein (P-gp, ABCB) and multidrug resistance protein (MRP, ABCC). According to Zaja *et al.* (2008) and Lončar *et al.* (2010) the activity of these proteins presents a relevant role in ecotoxicological context. The P-gp

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is primarily involved in the efflux of various unmodified xenobiotics (phase 0 of cellular detoxification) (Szakács *et al.*, 2008) while the MRP substrates are mainly phase I and II detoxification metabolites conjugated with glutathione (GSH) or glucuronic acid (GA) sulfate. According to Xu *et al.* (2005) the MRP acts as phase III of cellular detoxification mechanism, as described for metals such as arsenic, mercury and cadmium (Kala *et al.*, 2000; Long *et al.*, 2011; Zalups & Bridges, 2010).

Few studies have investigated the modulation of these transporters in early stage of development in fish and use them as biomarkers of chemical exposure (Costa *et al.*, 2012; Fischer *et al.*, 2013; Long *et al.*, 2011). The early stage of development is particularly sensitive to pollutants exposure (de Andrade Brito *et al.*, 2018) but the majority of the studies are devoted to invertebrates (Faria *et al.*, 2011; Roepke *et al.*, 2006).

Fish embryo and larvae are more sensitive to environmental impacts, including toxic substances compared to the juveniles and adults (Barreto *et al.*, 2020; de Andrade Brito *et al.*, 2018). The intoxication sensitivity observed in early developmental stages of fish showed interesting endpoints that strongly suggest their use as biomarkers of exposure to single or complex mixtures of pollutants. The toxicity of copper to early developmental stages of fish were described in *D. rerio* embryos resulting in mortality, hatching inhibition, impairment of larval development and lateral line dysfunction (Cunha *et al.*, 2016; Fischer *et al.*, 2013). Despite of that, no data are available about the effects of low doses of copper in tropical fish and after parental administration.

The current study was carried out to investigate the influence of copper on the activity of ABC transporters (P-gp and MRP) during embryonic and larval development of the tropical fish species *Rhamdia quelen* after sub-chronic exposure of adult females.

## MATERIALS AND METHODS

### Adult animals' exposure

The experiment was conducted in the Laboratory of Reproductive Technology for Farmed Aquatic Animal (LATRAAC), at the Institute for Research in Environmental Aquaculture (InPAA), Western Paraná State University (UNIOESTE), Brazil. Twenty females of catfish *Rhamdia quelen* (weight 235.32±60.67 g) were exposed to copper (CuSO<sub>4</sub>+5H<sub>2</sub>O) (5 mg kg<sup>-1</sup> fish). The metal was administered through *oral gavage*, one dose every 10 days for 60 days. This group is referred as "Cu". A "C" group (n = 20) received the gavages containing only NaCl 0.9%, medium used for copper solution preparation. For gavage procedures, fishes were previously anaesthetized with tricaine methane sulfonate (MS-222, 200mg L<sup>-1</sup>).

### Gametes collection and fertilization

Six females from each group (C and Cu), exhibiting a rounded abdomen, reddish urogenital papilla, and uniform

oocyte color and size (Sanches *et al.*, 2011) were used to obtain the oocytes. Spawning was induced by intramuscular injection of two doses of carp pituitary extract (CPE). The first injection comprised 0.5 mg CPE kg<sup>-1</sup> and twelve hours after a second dose of 5 mg CPE kg<sup>-1</sup> was injected (Bombardelli *et al.*, 2006). The oocytes were collected (10 hours at 24 °C after the second dose of the hormone injection) in petri dishes through abdominal ventral massage in the cephalocaudal direction.

A pool of semen from 6 individuals without copper exposure was used to fertilize the oocytes. Two hundred microliters of the semen pool were added to 3 mL of oocytes from each female (C and Cu groups). Gametes were activated by adding 20 mL of water as described by Bombardelli *et al.* (2006). The fertilization was performed in duplicate for each treatment.

For the collection of embryos and larvae during the embryonic development, two water recirculation systems with controlled temperature (27 °C) were used. One system consisted of experimental incubators (2.5 L) where 30 mg L<sup>-1</sup> of copper was added (group named "Copper") Bombardelli *et al.*, 2016). The other system was copper free (group named "Control").

### Experimental groups

The four experimental groups are described in the Table 1.

### ABC transporters activity

The transport activity was analyzed in different stages from each group, using 6 samples of each of the following periods: oocytes (300 µL = approximately 200 oocytes), 11 hours post fertilization (hpf) eggs (400 µL = approximately 200 eggs), 36 hpf embryos (100 individuals), and 96 hpf larvae (100 individuals).

The activity of P-gp and/or MRPs were analyzed using the calcein-acetoxymethyl ester (CAL-AM) substrate in the absence and presence of specific inhibitors verapamil (P-gp) and MK571 (MRP) Di and Kerns, 2016; Pessatti and Fontana, 2013). CAL-AM is a nonfluorescent substrate of ABC protein transporters, and its accumulation is inversely proportional to transporters activity. Inside the cell CAL-AM is converted into calcein (fluorescent) by sterases. The fluorophore is retained in the cell, not being a substrate for the ABC protein transporters. Thus, high fluorescence is indicative of low activity and low fluorescence means a high transport activity (Essodaigui *et al.*, 1998).

Table 1. Organization of the tested groups.

| Group name            | Adult female                    | egg, embryo, and larva      |
|-----------------------|---------------------------------|-----------------------------|
|                       | (5 mg Cu kg <sup>-1</sup> fish) | (30 mg Cu L <sup>-1</sup> ) |
| C <sup>Control</sup>  | -                               | -                           |
| Cu <sup>Control</sup> | +                               | -                           |
| C <sup>Copper</sup>   | -                               | +                           |
| Cu <sup>Copper</sup>  | +                               | +                           |

The assays were carried out in transparent 24-well plates. Oocytes, embryos, and larvae were incubated for 30 minutes in the presence or absence of inhibitors: verapamil (10  $\mu$ M) or MK571 (0.25  $\mu$ M). After this period CAL-AM (1  $\mu$ M) was added for 60 min. Plates were kept protected from light and under mild agitation during the incubation period. Then, samples were collected in vials tubes (1.5 mL) and kept at -80 °C. Subsequently the samples were lysed (300  $\mu$ L of Triton X-100 2% / PBS) and centrifuged (10 min at 2500 x g). One aliquot of the supernatant was separated for protein quantification (Bradford, 1976) and 200  $\mu$ L was used to determine fluorescence. Calcein fluorescence was measured using Tecan Genius microplate reader with filters as described: Excitation  $\lambda$  = 485 nm, and Emission  $\lambda$  = 530 nm. The protein concentration in the homogenates was determined using Bradford's method (Bradford, 1976) with bovine serum albumin as the standard.

### Fertilization and survival rates

The fertilization rate was verified 11 hpf by counting, in a 1 mL sample of translucent and with visible embryonic development (fertilized eggs), and opaque or white eggs (non-fertilized eggs) under a stereomicroscope. The rate was calculated from triplicate of 200 eggs counting.

Four hundred hatched eggs from each group were separated and maintained until 96 hpf to calculate the survival rate.

### Statistical procedures

Data were evaluated using the GraphPad Prism 3 program. First, the Shapiro-Wilk test was performed to verify the normality of the data. For data presenting normal distribution, the ANOVA test was applied, followed by the pos hoc Tukey test for comparison between groups and genders, while for data without normal distribution the Kruskal-Wallis test was applied. The differences found between the groups were accepted as significant only for values of  $p < 0.05$ .

## RESULTS

### ABC transporters activity

Oocytes from copper exposed females ( $Cu^{Control}$ ) accumulated 50.5 % less calcein than oocytes from control females ( $C^{Control}$ ) (Figure 1A). In the presence of the P-gp specific inhibitor Verapamil the calcein concentration increased 77 % in  $C^{Control}$  and 110 % in  $Cu^{Control}$  oocytes, while the MRP inhibitor MRK 571 did not modified calcein accumulation in oocytes (Figure 1A).

Copper increased calcein accumulation in 11 hpf eggs from control and exposed females (Figure 1B). Verapamil led to an increase in calcein accumulation in eggs not exposed to copper from both groups of females ( $C^{Control}$  and  $Cu^{Control}$ ), and MK 571 did not show inhibitory effect (higher accumulation of calcein) in all groups (Figure 1B).

In individuals 36 hpf (larvae) (Figure 1C) the values of calcein accumulated did not changed in all groups ( $C^{control}$ ,  $C^{copper}$ ,  $Cu^{control}$ ,  $Cu^{copper}$ ) in the absence of inhibitors. The presence of Verapamil did not increase calcein accumulation only in  $C^{copper}$  group. The incubation with MK 571 increased the calcein accumulation in all groups but no difference was observed between  $C^{control}$ ,  $Cu^{control}$  and  $C^{copper}$ ,  $Cu^{copper}$ .

After 96 hpf (Figure 1D) the larvae from the unexposed female eggs in the absence of copper ( $C^{control}$ ) accumulated more ca ul lcein in relation to  $C^{copper}$ ,  $Cu^{control}$  and  $Cu^{copper}$  groups and without inhibitors (Vrp and MK 571). The presence of verapamil increased the accumulation of calcein in all groups ( $C^{control}$ ,  $C^{copper}$ ,  $Cu^{control}$ ,  $Cu^{copper}$ ). The MK 571 inhibitor showed its inhibitory effect (higher accumulation) in all groups, and was more effective than Verapamil (Figure 1D).

### Fertilization and survival rates

The fertilization rate was checked 11 hpf (Figure 2A), when the blastopore closes. Eggs exposed to copper from females not exposed to copper ( $C^{copper}$ ) presented a reduced fertilization rate (16.73 %) compared to eggs not exposed from females not exposed ( $C^{control}$ ). The decrease rate of fertilization was about 14.2 %, when comparing eggs exposed to eggs not exposed to copper, both from females exposed to copper ( $Cu^{Copper}$  X  $Cu^{Control}$ ) (Figure 2A). The eggs exposure to copper ( $C^{copper}$ ) decreased survival (41.9%) compared to control group eggs ( $C^{control}$ ) (Figure 2B).

## DISCUSSION

Most studies about ABC transporters have been developed in somatic cells in terms of functionality of these proteins (Fischer *et al.*, 2011; Diaz de Cerio *et al.*, 2012), but the characterization of the mechanism is not yet completely elucidated in fish. Particularly the studies are scarcer during the early stages of development where the fish is supposed more sensitive to chemicals exposure (Barreto *et al.*, 2020). In the present study the activity of proteins ABC transporters in different stages of development of *H. quelen*, oocytes, embryos and larvae were investigated after incubation of two well-known modulators compounds Verapamil and MK571 (Pessatti & Fontana *et al.*, 2013) also described by Cunha *et al.* (2016) in *D. rerio*.

In *Rhamdia quelen* the oocytes efflux activity was most effectively blocked (higher accumulation) by the presence of verapamil (P-gp inhibitor) but no apparent activity by blocking using the MK571 inhibitor (MRPs) was found. This finding support that the ABC (P-gp) transcripts were found in the initial stages of development in the species, indicating a gene maternally transferred to the embryo where the efflux activity can be already observed even 1 hpf (Fischer *et al.*, 2013). Similarly, MRPs transcripts (*abcc1*, *abcc5*) were also maternally transferred in zebrafish eggs (Long *et al.*, 2011; Fischer *et al.*, 2013). Although the presence of eggs transcripts also was described by Faria *et al.* (2011) this event was only observed in larvae of *D. rerio* after 1-day hatching.

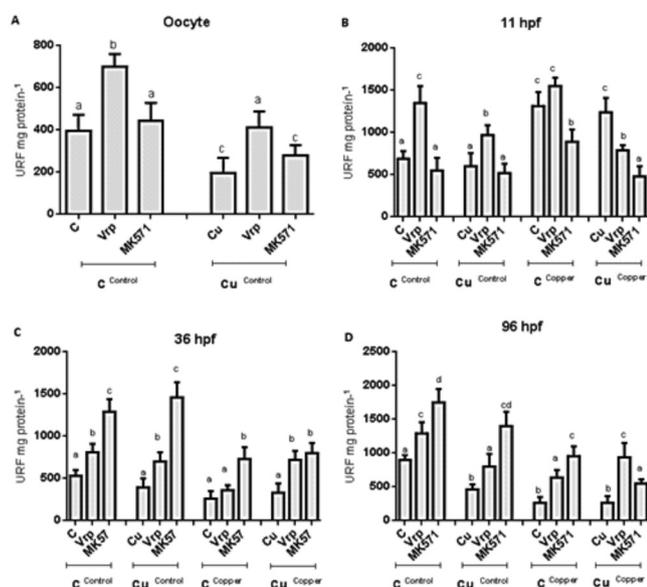


Figure 1. Calcein accumulation in *Rhamdia quelen* oocytes, embryos, and larvae after inorganic copper exposure. A) Oocytes. B) Embryos 11 hpf. C) Larvae 36 hpf. D) Larvae 96 hpf. Different letters mean different values among groups ( $p < 0.05$ ).

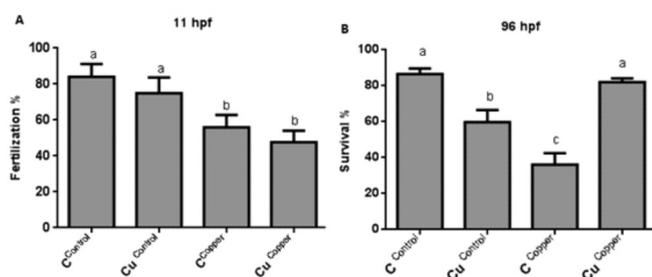


Figure 2. Fertilization (A) and survival (B) rates *Rhamdia quelen* eggs after exposure to inorganic copper. Different letters mean different values among groups ( $p < 0.05$ ).

Quantitative *real-time PCR* (qRT-PCR) analyze shows that the *abcc1* and *abcb1* genes are rarely transcript and their levels only increase 90-120 minutes after fertilization in *Strongylocentrotus purpuratus* (sea urchin) embryos. Instead, the efflux activity increases by 25 min post-fertilization and the increase was kept in the presence of inhibitors of protein synthesis and mRNA (Hamdoun *et al.*, 2004). These data indicate that the regulation of efflux activity in the initial stages is post-translational.

With the blocked efflux by MK571 showing that the ABC proteins activity can be reversed in 36 hpf larvae also kept 96 hpf (last analyzed stage), the current data suggest a temporal distribution of this mechanism of transport in early stages of development, as this finding was confirmed by Verapamil blocking. The most apparent modulation of MRP-1 in the final stages of development (96 hpf) may be related to the protein physiological function in the tissues, as intestine, kidney and liver completely developed at this stage. Cunha *et al.* (2016) found in *D. rerio* the *abcc* gene being detected mainly at 72 hpf where the organogenesis is already completed for this species.

According to the literature, the time profile of the transporter activation seems to present an interspecific characteristic. Hamdoun *et al.* (2002) using calcein - AM and the MK571 inhibitor described a shortly transporter activity after fertilization in sea urchin embryos. Similar condition was described by McFadzen *et al.* (2000) in fertilized *Mytilus edulis* embryos where the Rhodamine B accumulated in the presence of a Pgp inhibitor (Verapamil), while Toomey and Epel (1993) did not find the presence of this protein in urchin sea larvae (*Anamesus lytechinus*) or even the efflux capacity.

In the current study after chronic exposure of females to copper, the lowest accumulation of calcein in oocytes collected in relation to non-exposed female oocytes, could be explained by the increased activity of proteins ABC transporters (Pgp and MRPs). This finding is due to the presence of mRNA from females and the consequent expression of ABC transporter proteins. Others have described the increase of ABC transporter proteins after exposure to metals as coral (Cu), copepods (Cu and Cd), Antarctic fish *Trematomus Bernacchi* and oyster *C. virginica* exposed to Cd (Ivanina & Sokolova, 2008; Venn *et al.*, 2009; Zucchi *et al.*, 2010; Della Torre *et al.*, 2012; Jeong *et al.*, 2014). However, embryos exposed to copper after 11 hpf without the influence of maternal exposure showed the efflux activity of calcein - AM inhibition. Wu *et al.* (2015) reported an increase in calcein accumulated in *Lytechinus pictus* embryos (sea urchin) in blastula stage also by exposure to copper, corroborating the results in the present study. The literature regarding the influence of pollutants on ABC transporters in early stages of development is still scarce. The evidence of copper exposure may disturb the transporter mechanisms on earlier stages of development in fish is an important highlight in environmental toxicology. In general, during the early stages fish are very sensible to chemical exposure and the consequence for adult population is unpredictable (Brito *et al.*, 2018; Barreto *et al.*, 2020).

After the larvae formation in *Rhamdia quelen* (36 and 96 hpf) the inhibition of transporter proteins might be reversed as evidenced by calcein accumulation decreasing. This is quite different if embryos are exposed and not females when, a higher accumulation is observed. During these stages the activity of MRP is induced as demonstrated by the lower accumulation of calcein, but these findings do not interfere with the larvae survival rate.

The copper induction transporter proteins and the role of P-gp in the detoxification was confirmed by Li *et al.* (1997). The increase in copper concentration in gill and mantle on the oyster *Crassostrea angulata* was significantly higher after the interference of the *abcb1* gene. Wu *et al.* (2015) described an inhibition of MRP activity significantly increasing the copper concentration showing that ABC transporters can dislocate copper to outside of the cell while Cui *et al.* (2007) described the participation of different transporters proteins

involved in metal detoxification. This mechanism may utilize the ABC transporter proteins to remove glutathione (GSH) complexes with metals (Cole and Deeley, 2006), or by other metabolites generated in the cell due to exposure to toxic agents that activates this mechanism of transport. Some authors as Vollrath *et al.* (2006) have suggested that the induction of ABC genes can be related to the ability of metals to produce ROS and consequently increasing its transport from cell. The ROS production would be responsible for the activation of nuclear factor Nrf2 which binds to sequences ARE (antioxidant response element) and regulates a battery of antioxidant genes (Kang *et al.*, 2005; Adachi *et al.*, 2007).

The current study is in agreement with other where the MXR activity is quite high in embryo and larval stages of different aquatic animals. The data suggest that the earlier stages may be more sensible to pollutant exposure by ABC transporters proteins inhibition. This evidence allows the development of studies where the synergism found in complex mixtures (greater environmental relevance) can act as chemosensitizers (blockers). The importance of characterizing the modulation of proteins in different stages of development is due to the proposed use of the MXR activity as a biomarker. As discussed in the present study, there is a temporal modulation of ABC transporter proteins throughout the development. These findings if properly applied, could reveal the role of transporter proteins in the earlier stage of development in fish as an important biomarker in aquatic toxicology studies and fish cultivation.

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