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Copper and the herbicide atrazine impair the stress response of the freshwater fish *Prochilodus lineatus*

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ABSTRACT

In order to evaluate the effects of copper and atrazine on the stress response of the freshwater fish *Prochilodus lineatus*, juvenile fish were pre-exposed to copper (20 μg L^{-1}) or atrazine (10 μg L^{-1}) for 24 h and then submitted to air exposure for 3 min. Simultaneously fish kept in dechlorinated water for 24 h were subjected to air exposure and a non-stress group was not subjected to air stress or any contaminants. Animals were sampled immediately (t0) and after 1, 3 and 6 h of air exposure (t1, t3 and t6 respectively) for the analysis of plasma cortisol, glucose and Na^+, hepatic glycogen, branchial Na^+/K^+-ATPase (NKA), number of red blood cells per cubic millimeter of blood (RBC), hematocrit (Hct) and hemoglobin content (Hb). In fish pre-exposed to copper the stress response was inhibited, and at t1 and t3 both cortisol and glucose remained significantly lower compared to fish subjected to air stress only. In fish pre-exposed to atrazine there was no rise in cortisol, but there was an increase in plasma glucose, RBC, Hct and Hb at t0 and a return of these parameters to basal levels at t1, as they did not differ significantly in relation to non-stressed fish. Animals pre-exposed to either Cu or atrazine showed a significant reduction in NKA activity at t1 and t3, in relation to air stressed fish. These results clearly indicate that copper and atrazine impair cortisol stress response of *P. lineatus* and that fish subjected to a contaminant-induced stress, either by copper or atrazine, may not be able to respond to any additional stressors.

1. Introduction

Fish are exposed to different types of stressors in the wild, as well as in artificial conditions such as in aquaculture (Barton, 2002), and numerous physiological studies investigated the stress responses in fish (Jentoft et al., 2002; Teles et al., 2005; Miller et al., 2009). Stressors can produce effects that elicit a coordinated set of compensatory and/or adaptive physiological responses, enabling the animal to overcome the threat; these responses have been grouped broadly as primary, secondary and tertiary (Wendelaar Bonga, 1997; Barton, 2002). Primary responses involve initial neuroendocrine responses and include the release of catecholamines from chromaffin tissue and the stimulation of the hypothalamic–pituitary–interrenal (HPI) axis culminating in the release of corticosteroid hormones into circulation (Barton, 2002). In teleosts, cortisol is the primary circulating glucocorticoid and is released from the interrenal tissue, distributed in the head kidney region (Aluru and Vijayan, 2009). Although the cortisol response to stressors is well established in teleosts, its magnitude and duration is dependent upon the type, intensity and duration of the stressor as well as the history of the animal (Wendelaar Bonga, 1997; Barton, 2002; Iwama, 2006). Secondary responses include changes in plasma and tissue ion and metabolite levels and hematological features, all of which relate to physiological adjustments in metabolism, respiration, acid–base status, hydromineral balance, immune function and cellular responses (Mommsen et al., 1999). Additionally, tertiary responses occur, which refer to aspects of whole-animal performance such as changes in growth, condition, overall resistance to disease, metabolic scope for activity, behavior, and ultimately survival (Barton, 2002). The stress response is fundamentally a regulatory response to return the animal to "homeostatic norms" (Schreck, 2010) and due to this significant role, the impairment of the stress response may adversely affect fish health (Cericato et al., 2009).

Surface waters receive runoff and discharges from urban, agricultural and domestic sources that can lead to accumulation of contaminants containing mixtures of toxic compounds (Tilton et al., 2011). Water-borne contaminants are themselves stressful, but they can also impair the stress response of fish to a second stressor (Barton, 2002; Gagnon et al., 2006; Cericato et al., 2009). Copper is an essential metal for the function of most living organisms but in high concentrations is toxic to living cells (Zavitsanos et al., 2011). This metal can impair interrenal activity and cortisol secretion, affecting the ability of fish to cope with stressful situations (Nowak and Duda, 1996; Kime, 1999; Gagnon et al., 2006). Atrazine is a triazine herbicide extensively used and several studies have already shown that atrazine

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can also affect cortisol secretion in some fish species (Bisson and Hontela, 2002; Nieves-Puigdoller et al., 2007; Fortin et al., 2008).

In view of the above, the present study focused on the effects of copper and atrazine on the stress response of the Neotropical fish *Prochilodus lineatus* (Characiformes, Prochilodontidae). This species is a Neotropical freshwater fish widely distributed in the La Plata basin, where it represents 50–60% of the total ichthyofauna (Lombardi et al., 2010). It is raised in fish farming, mainly in southern Brazil (Voltolin et al., 2010), and it is used for human consumption. Several studies have shown that *P. lineatus* is sensitive to a variety of pollutants (Camargo et al., 2009; Cazenave et al., 2009; Modesto and Martinez, 2010; Simonato et al., 2011) but the interference that contaminants may have on its response to an additional stressor is not known.

2. Materials and methods

2.1. Acclimation of animals and experimental design

Juveniles of *P. lineatus*, weighing 15 ± 5 g and total length of 11.1 ± 1.6 cm (mean ± SD, n = 104) were obtained from the University Hatchery Station. Fish were acclimated during 5 days in 300 L tanks with dechlorinated water and constant aeration and a photoperiod of 12/12 h. The physical and chemical parameters of the water were continuously monitored throughout the acclimation period and the experiments, and remained constant (T = 22.8 ± 0.6 °C; pH = 7.5 ± 0.3; DO = 7.5 ± 0.9 mg O_2 L^{-1}; conductivity = 123.4 ± 9.7 μS cm^{-1}; hardness = 42.5 ± 6.0 mg $CaCO_3$ L^{-1}). During acclimation fish were fed with commercial fish food (42% protein — Cooperativa Agroindustrial Integrada, Brazil) at 48-hour intervals; feeding was suppressed 48 h before and during toxicity tests.

After acclimation fish were transferred to glass aquaria of 80 L (6 or 7 fish per aquarium) containing dechlorinated water with or without the addition of a contaminant, where they remained for a period of 24 h without water renewal. Fish were divided into four main groups: i) non-stress group = fish kept in dechlorinated water for 24 h, which were not subjected to air stress or contaminants, ii) air stress group = fish kept in dechlorinated water for 24 h and then subjected to air exposure, iii) copper + air stress group = fish exposed for 24 h in water containing copper (20 μg L^{-1}) and then subjected to air exposure, iv) atrazine + air stress group = fish exposed for 24 h in water containing atrazine (10 μg L^{-1}) and then subjected to air exposure. Fish from groups ii, iii and iv were subjected to an acute stress-handling stimulus by removing each fish individually from the tank with a small net and air exposing fish out of the water for 3 min. After air exposure, fish from each air stress group were subdivided into four groups, one of them was sampled immediately (t0 group); fish from the others three groups were transferred to different glass aquaria, containing only dechlorinated water, at the same density before the air stress, and they were sampled approximately after 1, 3 or 6 h of the air stress application (t1, t3 and t6). Fish from the non-stress group were also sampled before being transferred to another aquarium (t0) or after 1, 3 or 6 h of the transfer, but without any air exposure. In order to minimize the induction of stress during the transfer, fish from the non-stress group were removed very quickly from one aquarium to the other, with a small net, avoiding air exposure. Thus, for each one of the four treatments there were four different periods of sampling: zero (t0), one hour (t1), three hours (t3) and six hours (t6), summing up sixteen groups. The period of time after air stress was not precise, since the entire air stress protocol, considering 6 to 7 fish in each aquarium, lasted from 18 to 21 min and the time started to run when the first fish was transferred to another aquarium. Thus there was a 15 to 18 min delay (after the 1 hour-interval, 3 hour-interval or 6 hour-interval) at maximum, when the first fish that was air stressed was the last to be sampled.

Copper was added to the aquarium 3 h before the animals as copper chloride dihydrate (Acros Organics, USA) at a nominal concentration of 20 μg L^{-1}. This concentration is lower than the LC_{50} — 96 h (29 ± 3 μg Cu L^{-1}) determined for *P. lineatus* by Mazon and Fernandes (1999) using water with characteristics (pH = 7.3 and hardness = 24 mg $CaCO_3$ L^{-1}) similar to the water used in the present study. The herbicide atrazine (Sigma-Aldrich, USA) was added to the water 0.5 h before the fish, at a nominal concentration of 10 μg L^{-1}, which corresponds to 40% of the concentration indicated for agricultural use (Ventura et al., 2008).

2.2. Sampling

The animals were always sampled at noon and to reduce the induction of stress during sampling fish were removed very quickly from the aquaria with a small net and they were immediately anesthetized with benzocaine (0.12 g L^{-1}) for blood sampling. After that fish were killed by medullar section to remove the gills and liver. These procedures followed the standard protocols approved by the Committee for Animal Experimentation of Londrina State University. Blood was collected through the caudal vein with a heparinized plastic syringe. The blood samples were stored in plastic tubes for the hematological analysis and the cells were pelleted by centrifugation (1870 *g*, 10 min). Gill arches were washed with saline (NaCl 154 mM and sucrose 25 mM) and stored in SEI buffer (sucrose 300 mM; EDTA 0.1 mM; imidazole 30 mM; β-mercaptoethanol 0.035%; pH 7.4) in an ultrafreezer (−70 °C) for the determination of Na^+/K^+-ATPase (NKA) activity. The liver was removed and stored dry in plastic vials at −70 °C for the analysis of glycogen content.

2.3. Determination of physiological parameters

An aliquot of the blood was used for the determination of hematocrit, by microcentrifugation in capillary tubes, and hemoglobin, by the cyanmethemoglobin method in a spectrophotometer at 540 nm. The number of red blood cells per cubic millimeter of blood (RBC) was counted on an improved Neubauer hemocytometer using blood samples fixed in formol citrate.

Plasma cortisol was determined by an immunoenzymatic assay (commercial kit from Diagnostic Systems, Laboratories, USA) on microplates at 450 nm. The glucose concentration was determined by the glucose oxidase method in a spectrophotometer at 505 nm (commercial kit from Labtest, Brazil). The determination of liver glycogen was performed according to the method described by Bidinotto et al. (1997).

Plasma aliquots were used to determine the concentration of sodium (Na^+) ions using a flame photometer (Analyzer, Brazil). The enzyme activity of NKA was determined in the branchial filaments homogenized with SEI buffer (10× the volume) and centrifuged (7800 *g*, 15 min, 4 °C). The supernatant was used to determine Na^+/K^+ ATPase activity, according to the method described by Quabius et al. (1997) and adapted for a microplate reader by Nolan (2000). The assay consists of determining the phosphate released by the samples incubated in buffer (NaCl 100 mM, $MgCl_2$ 8 mM, imidazole 30 mM, EDTA 0.1 mM, ATP 3 mM, pH 7.6) containing KCl (5 mM) or ouabain (2.5 mM). A solution of 0.65 mM phosphate (Sigma) was used as standard and the samples were analyzed in triplicate at 620 nm in a microplate reader. Na^+/K^+ ATPase activity was expressed as μmol Pi(mg protein h)$^{-1}$. Protein concentration was determined according to the method described by Lowry et al. (1951), using bovine serum albumin (BSA) as standard in a spectrophotometer at 700 nm.

2.4. Statistical analysis

The results obtained for each experimental group (non-stress, air stress, copper + air stress and atrazine + air stress) at each sampling

time (t0, t1, t2, t3 and t6) were compared to each other, using parametric (one-way ANOVA) or non-parametric (Kruskal–Wallis) analysis of variance, depending on the distribution of the data and the homogeneity of the variances. Where appropriate a multiple-range test (Student Newman Keuls test) was used to identify the differences. Values of $P \leq 0.05$ were considered significant.

3. Results

3.1. The stress response

Plasma cortisol increased significantly in *P. lineatus* subjected to air stress after 1 (667%) and 3 h (997%) of the application of the stress, in comparison to non-stressed fish (Fig. 1). Plasma glucose showed a significant increase immediately after air stress (t0) and remained increased after 1 and 3 h (Fig. 2A). On the other hand, fish from the air stress group showed a significant decrease in liver glycogen, at all experimental times, in comparison to non-stress group (Fig. 2B).

Plasma Na^+ decreased significantly in fish sampled immediately after the application of the air stress (t0) in comparison to non-stressed fish (Fig. 3A). In contrast, branchial NKA activity showed a significant increase after 1 and 3 h of the stress (Fig. 3B).

Among the hematological parameters analyzed only the number of red blood cells per cubic millimeter (RBC) changed significantly after the application of air stress, and fish showed a significant increase on RBC at t0 when compared to non-stressed fish (Fig. 4A). The values of hemoglobin and hematocrit remained unaltered in comparison to non-stress groups, throughout all the experimental times (Figs. 4B and 5). Taken together these results (increased RBC without any change in hematocrit) indicate that there was a decrease in mean RBC volume at t0.

3.2. Effects of Cu on the stress response

In fish pre-exposed to copper and then subjected to air stress the values of plasma cortisol and glucose and liver glycogen were significantly lower than in fish submitted only to air stress (Figs. 1 and 2). Plasma cortisol was significantly lower than in air stressed fish at t1, t3 and t6, and also significantly lower than in non-stressed fish at t6 (Fig. 1). Plasma glucose was significantly lower than in air stressed fish at t0, t1 and t3 (Fig. 2A). Liver glycogen was significantly lower in relation to air stressed fish at t0 and t1, and at all sampling times

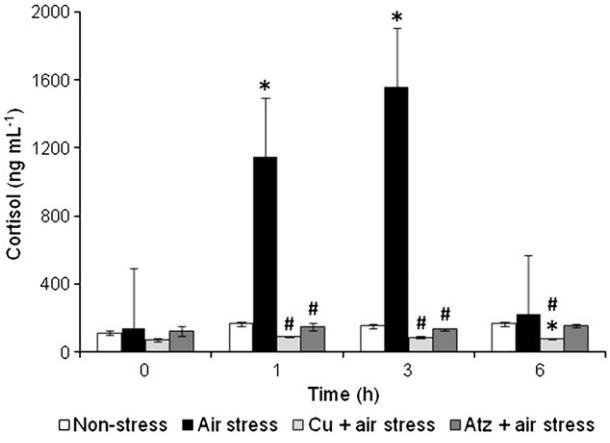

Fig. 1. Plasma cortisol in *Prochilodus lineatus* sampled immediately after (t0) or after 1, 3 and 6 h of the air stress. The bars indicate mean and the vertical lines the SE (number of animals: 4 to 6). *Significant difference in relation to non-stress group. #Significant difference in relation to the air stress group. Comparisons are valid for the same time period ($P \leq 0.05$).

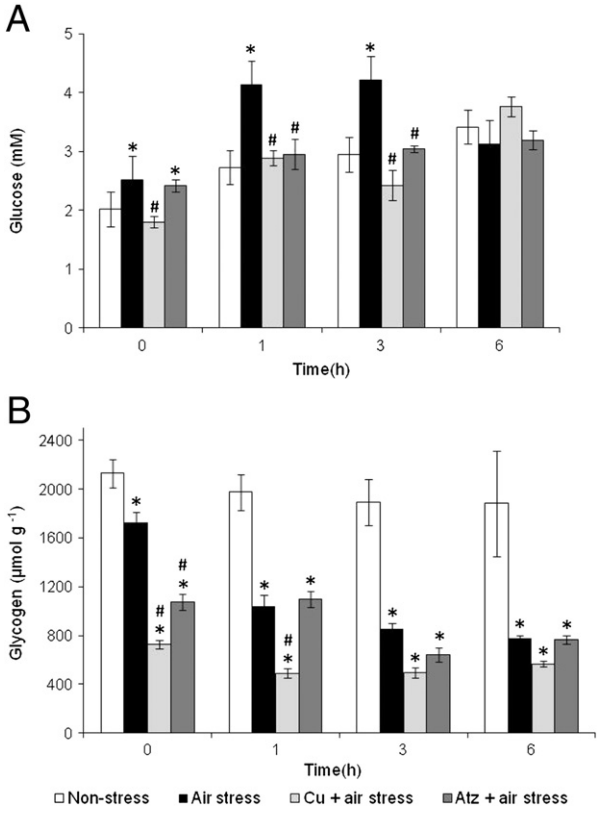

Fig. 2. Plasma glucose (A) and hepatic content of glycogen (B) in *Prochilodus lineatus* sampled immediately after (t0) or after 1, 3 and 6 h of the air stress. The bars indicate mean and the vertical lines the SE (number of animals: 4 to 6). *Significant difference in relation to non-stress group. #Significant difference in relation to the air stress group. Comparisons are valid for the same time period ($P \leq 0.05$).

liver glycogen was lower than in fish from the non-stress groups (Fig. 2B).

No significant variation in plasma Na^+ was observed in fish pre-exposed to copper and submitted to air stress in comparison to fish subjected only to the air stress (Fig. 3A). On the other hand, the activity of NKA was significantly lower at t1 and t3, and higher at t6, in comparison to air stressed fish (Fig. 3B).

RBC values were lower in fish pre-exposed to Cu and sampled immediately after and 6 h after the application of the air stress, in comparison to respective air stress groups (Fig. 4A). Immediately after the air stress fish pre-exposed to copper also showed a significant increase in hematocrit when compared both to the non-stressed and the air stressed groups (Fig. 4B). Hb content was significantly lower in fish pre-exposed to Cu and sampled 1 h after the air stress in comparison both to non-stressed and to air stressed fish (Fig. 5).

3.3. Effects of atrazine on the stress response

Plasma cortisol and glucose in *P. lineatus* pre-exposed to atrazine were significantly lower after 1 and 3 h of the application of the air stress when compared with fish exposed only to air stress (Figs. 1 and 2A). Liver glycogen was significantly lower in relation to air stressed fish at t0, and significantly lower than the non-stressed groups at all sampling times (Fig. 2B).

Fish pre-exposed to atrazine and subjected to air stress did not show any variation in plasma Na^+ in comparison to the respective air stress groups (Fig. 3A). However, the activity of NKA was significant lower at t1 and t3 in than in air stressed fish (Fig. 3B).

Values significantly higher of RBC, Hb content and hematocrit were observed in fish pre-exposed to atrazine immediately after the application of the acute stress, in comparison both to the stressed

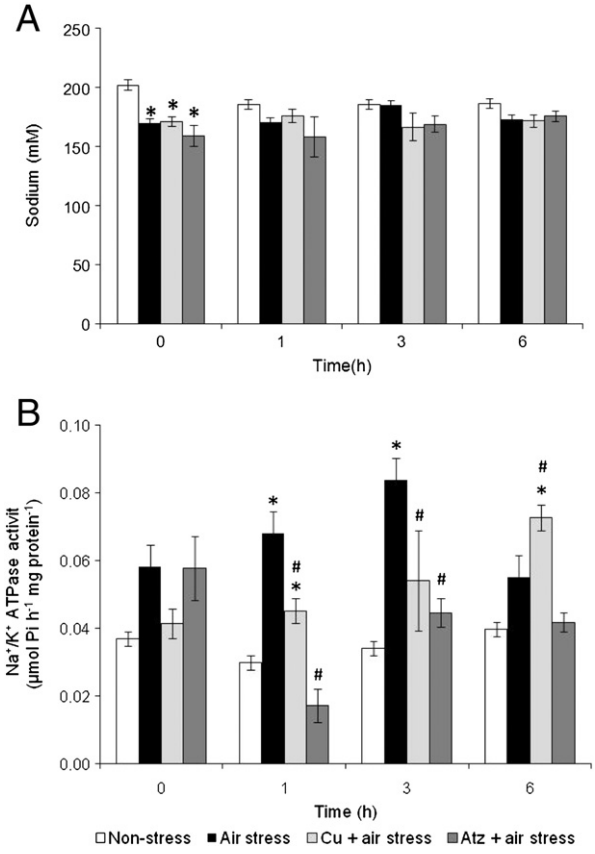

Fig. 3. Plasma sodium (A) and branchial Na^+/K^+ ATPase activity (B) in *Prochilodus lineatus* sampled immediately after (t0) or after 1, 3 and 6 h of the air stress. The bars indicate mean and the vertical lines the SE (number of animals: 4 to 6). *Significant difference in relation to non-stress group. #Significant difference in relation to the air stress group. Comparisons are valid for the same time period ($P \leq 0.05$).

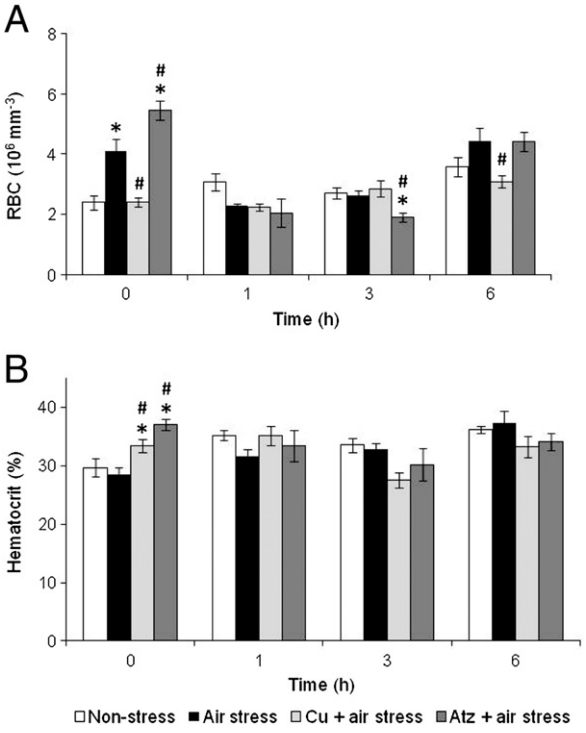

Fig. 4. Number of red blood cells per cubic millimeter of blood (A) and hematocrit (B) in *Prochilodus lineatus* sampled immediately after (t0) or after 1, 3 and 6 h of the air stress. The bars indicate mean and the vertical lines the SE (number of animals: 4 to 7). *Significant difference in relation to non-stress group. #Significant difference in relation to the air stress group. Comparisons are valid for the same time period ($P \leq 0.05$).

and non-stressed groups (Figs. 4 and 5). However, after 3 h of the application of the air stress RBC and Hb values decreased significantly, in relation to the air stress groups (Figs. 4A and 5).

4. Discussion

It is well known that acute stressor exposure causes transient elevation in plasma cortisol levels, which are quickly re-established to resting levels during recovery from stress in fish (Mommsen et al., 1999; Aluru and Vijayan, 2009). In *P. lineatus*, this increase was observed after 1 and 3 h from the application of the air stress, together with an increased glucose and a decreased hepatic glycogen. Cortisol is commonly used as an indicator of stress (Cericato et al., 2008; Langiano and Martinez, 2008; Simonato et al., 2008; Camargo et al., 2009) and it has broad metabolic effect (Pankhurst, 2010). As a strategy to handle the prolonged energy demand during stress conditions, cortisol regulates glucose mobilization by inducing liver glycogenolysis and gluconeogenesis (Vijayan et al., 1997), leading to plasma glucose increase and a decrease in glycogen content (Hontela, 1998).

In fact, we observed the release of glucose immediately after air stress together with a reduction in liver glycogen. This glucose concentration at t0 may be attributed to the activation of the hypothalamic–sympathetic–chromaffin axis (HSC) and the subsequent release of catecholamines by chromaffin cells (Barton, 2002). Adrenaline, which normally increases immediately after stress, promotes a rapid breakdown of liver glycogen and therefore a higher concentration of glucose in the blood (Pankhurst, 2010). Also, adrenaline is probably related to the increased RBC at t0, as it induces immature erythrocyte release into circulation by spleen contraction. This increase in the number of immature erythrocytes can be envisioned as a pre-adaptation to hypoxia-induced acute stress events (Valenzuela et al., 2005).

In this study we observed a decrease on plasma Na^+ in all groups of fish sampled immediately after air stress. This sodium decrease is probably associated with passive ion losses due to increased permeability of tight junctions, which control the diffusion rate through the paracellular pathways of the branchial epithelium (Wendelaar Bonga, 1997). In order to compensate sodium losses, fish exposed only to air stress showed increased activity of NKA at t1 and t3, concomitant with increased cortisol. Studies carried out with the African catfish, *Clarias gariepinus* (Babitha and Peter, 2010) and with the rainbow trout, *Oncorhynchus mykiss* (Shrimpton and McCormick, 1999), among others, have already shown that cortisol increases the activity of NKA contributing to hydromineral regulation in freshwater fish.

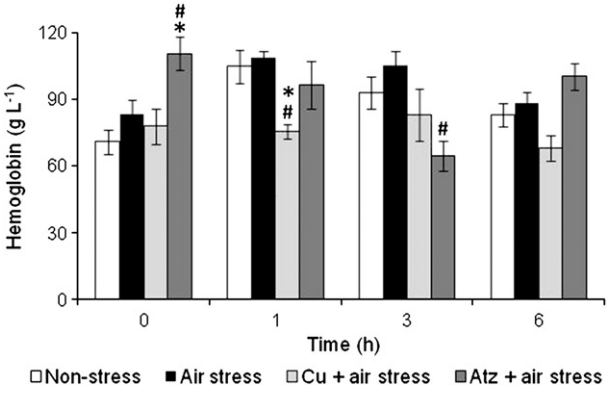

Fig. 5. Hemoglobin content in *Prochilodus lineatus* sampled immediately after (t0) or after 1, 3 and 6 h of the air stress. The bars indicate mean and the vertical lines the SE (number of animals: 4 to 7). *Significant difference in relation to non-stress group. #Significant difference in relation to the air stress group. Comparisons are valid for the same time period ($P \leq 0.05$).

Cortisol has been shown to direct its ionoregulatory function by regulating the activity and the expression of gill NKA (Dang et al., 2000; McCormick et al., 2008; Babitha and Peter, 2010).

When *P. lineatus* was pre-exposed to copper the stress response was inhibited as there was no increase in plasma cortisol and glucose in any of the periods after the application of the air stress, and both cortisol and glucose concentrations remained similar to the values determined in fish not submitted to any stress. Gagnon et al. (2006) also observed that air exposure-induced increase in plasma cortisol was lower in *O. mykiss* after 30-day exposure to 30 and 80 μg L^{-1} of Cu. These authors suggested that Cu, at high concentrations, disrupts cortisol secretion through a direct toxic effect on interrenal cells. These results reinforce the idea that metals, such as copper, can act as endocrine disruptor in aquatic organisms (Handy, 2003), inhibiting the stress response in fish and thus affecting their ability to cope with additional stressors (Kime, 1999).

The reduced NKA activity shown by fish pre-exposed to copper after 1 and 3 h of the stress application, in relation to air stressed fish, might be related both to the inhibition of cortisol release as well as to a direct copper effect on NKA. Several studies have already shown that copper exposure leads to a decrease of this enzyme. Tilapia, *Oreochromis mossambicus*, (Li et al., 1998; Dang et al., 2000) and the rainbow trout (Grosell and Wood, 2002) exposed to copper, with different experimental designs showed a decrease on this enzyme activity. Cu has been demonstrated to disrupt ionoregulation by inhibiting NKA in the gill during waterborne Cu exposures in freshwater (Blanchard and Grosell, 2006).

P. lineatus pre-exposed to Cu did not show the RBC increase as the air stressed fish did, immediately after stress application (t0), indicating that Cu pre-exposure impaired the release of immature red blood cells into circulation. On the other hand, the higher hematocrit values observed at t0 suggest the swelling of erythrocytes, probably caused by the release of adrenaline, which promotes the activation of Na^+/H^+ erythrocytes leading to cell swelling (Nikinmaa, 1992).

The results obtained immediately after stress application in fish pre-exposed to atrazine indicated that there was an adrenergic activation and in response there was a release of glucose and immature erythrocytes into the bloodstream, leading to an increased plasma glucose, RBC, hemoglobin and hematocrit. There was no release of cortisol and plasma glucose returned to basal levels as the two parameters did not differ significantly in relation to non-stressed fish. The lack of cortisol response after an additional stress was also observed in silver catfish fingerlings (*Rhamdia quelen*) exposed to five pesticides, including the herbicide atrazine (Cericato et al., 2008). These authors hypothesized that the mechanism involved in the attenuation of the cortisol response to stress provoked by atrazine might be related to interrenal toxicity; however in a more recent study, the same authors (Cericato et al., 2009) showed that the weakened cortisol response to acute stress might be related to a regulation of cortisol secretion in higher levels within the HPI axis (i.e., in the hypothalamus and/or in the pituitary gland). The reduced NKA activity shown by fish pre-exposed to atrazine after 1 and 3 h of the stress application, in relation to air stressed fish, reflects the inhibition of cortisol release.

In conclusion, these results clearly indicate that copper and atrazine impair cortisol stress response of *P. lineatus* and that fish subjected to a contaminant-induced stress, either by copper or atrazine, may not be able to respond to any additional stressors.

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