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Acute effects of nitrite on ion regulation in two neotropical fish species

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Abstract

To broaden the understanding of physiological responses of tropical fish to environmental stressors, the effects of nitrite on haematological parameters and plasma and red blood cell ion regulation were studied in two neotropical fish species, *Astyanax altiparanae* and *Prochilodus lineatus*. Both fish species were exposed to $NaNO_2$ (30 mg l^{-1}) over a 96-h period and blood samples were taken for ion and haematological analyses. The results revealed that nitrite leads to a decrease in *P. lineatus* blood haematocrit and haemoglobin content and an increase in blood methaemoglobin. *A. altiparanae* did not exhibit any significant difference in these haematological parameters. During the exposure to NO_2^- both fish species had significantly reduced plasma Na^+ concentration and red blood cell (RBC) K^+ concentration, but only *P. lineatus* showed an increase in extracellular K^+ concentration. When RBC volume was analyzed in vitro, after 2 min of exposure to $NaNO_2$, a 36% shrinkage was observed in *P. lineatus* cells, while only a 10% shrinkage was observed in *A. altiparanae* cells. These results suggest that for *P. lineatus*, nitrite entrance into the cell leads to methaemoglobin formation and K^+ efflux, causing red cell shrinkage and increased plasma K^+. However, *A. altiparanae* proved to be a species more resistant to nitrite, exhibiting fewer responses to this compound.

Keywords: Methaemoglobin; Potassium regulation; Red blood cell volume; Haematocrit; Nitrite; Neotropical fish; *Astyanax altiparanae*; *Prochilodus lineatus*

1. Introduction

Nitrite (NO_2^-) is a potential contaminant in aquatic environments that receive nitrogenous waste and also in recirculated aquaculture systems (Grosell and Jensen, 1999). Several studies have examined the toxicity and physiological effects of NO_2^- in fish (Bath and Eddy, 1980; Mensi et al., 1982; Williams and Eddy, 1987; Hilmy et al., 1987; Jensen, 1990; Nikinmaa and Jensen, 1992; Doblander and Lackner, 1996; Knudsen and Jensen, 1997). An elevated ambient nitrite concentration is problematic for freshwater fish, as nitrite is actively taken up across the gills in competition with chloride (Eddy and Williams, 1987). The principal effect of such nitrite loading is a progressive oxidation of haemoglobin to methaemoglobin, but several other physiological changes occur (Jensen, 1990). The interference with branchial ion exchange together with methaemoglobinemia and likely tissue hypoxia suggests that major changes may arise in blood O_2 transport and respiratory properties, and that perturbations of electrolyte and acid–base status may occur (Jensen et al., 1987). Knudsen and Jensen (1997) showed that nitrite interferes with K^+ homeostasis in carp leading to an extracellular hyperkalaemia. A recent

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study (Aggergaard and Jensen, 2001) has shown that rainbow trout exposed to nitrite present an increase in plasma K^+ and a decrease in plasma Cl^-. This rise in plasma K^+ is probably due to the release of K^+ from intracellular compartments (Knudsen and Jensen, 1997).

However, little is known about the physiological effects of nitrite on neotropical freshwater fish species. Bartlett et al. (1987) examined the influence of nitrite on methaemoglobin formation in 21 Amazonian fish species. They examined various aspects of the reaction by which nitrite converts haemoglobin to methaemoglobin in hemolysates and the reduction of methaemoglobin back to haemoglobin by the intact red cell. Moraes et al. (1998) studied the metabolic responses of *Hoplias malabaricus* to hypoxia caused by nitrite exposure. They analyzed the content of glycogen, glucose, lactate and pyruvate in different fish tissues. Except for these studies information about nitrite effects on ion regulation in freshwater neotropical fish is still lacking.

In order to broaden the understanding of the physiological responses of tropical fish to environmental stressors, the present study investigated the effects of nitrite on haematological parameters and plasma and red blood cell ion regulation of two Brazilian Characiformes: *Astyanax altiparanae* (Garutti and Britski, 2000) and *Prochilodus lineatus* (Valenciennes, 1847). These fish are common species of the Paraná Basin (southern Brazil) where they represent an important source of food. Both species might be exposed to environmental nitrite considering that *A. altiparanae* is normally found in streams adjacent to agricultural areas whereas *P. lineatus* is widely used in aquaculture.

2. Methods

2.1. Experimental animals

Experiments were conducted on juvenile *Prochilodus lineatus* (mass 24.24 ± 5.50 g, mean $\pm$ S.D., $n = 70$) and adult *Astyanax altiparanae* (mass 8.17 ± 3.98 g, mean $\pm$ S.D., $n = 52$). Fish were obtained from the Universidade Estadual de Londrina hatchery station. They were held in a 500 l tank, with aerated well water ($T = 21$ °C, pH = 7.5, hardness = 80 mg l^{-1} $CaCO_3$), with a 14:10 h light/dark cycle, for at least 7 days prior to experiments. Fish were fed at 48-h intervals on pellet food, except during, and on the day preceding the experiments.

2.2. Experimental protocol

After acclimation, short-term (96 h) static toxicity tests, without water renewal, were carried out to evaluate nitrite toxic effects. $NaNO_2$ was added to the water to a nominal concentration of 30 mg l^{-1}. Experiments were performed in 140 l tanks, containing from 6 to 10 fish each. Water was continuously aerated, light/dark regime was 14:10 h, water temperature was kept at 21 ± 1 °C, and pH 7.5. Terminal blood samples were taken from each fish after 6, 24, 48, 72 and 96 h of exposure to sodium nitrite. Control groups consisted of animals exposed only to the dilution water, some of them were sampled after 6 h (control 6 h) and some were sampled after 96 h (control 96 h) of exposure to the dilution water. Immediately after removal of the fish from the water, blood samples were taken from the caudal vein by means of heparinized plastic syringes.

2.3. Measurements

Water nitrite concentrations were determined from the reaction of nitrite with sulfanilamide yielding a diazo compound that couples with *N*-1-naphthyl-ethylenediamine-dihydrochloride to give a red azo dye (APHA, AWWA, WPC, 1981). Nitrite concentrations were calculated from the absorbance at 540 nm referring to parallel determinations of a series of standard nitrite solutions, using a spectrophotometer (Shimadzu UV 1203, Japan).

Haematocrit (Hct) values were determined by blood centrifugation (5 min, $5000 \times g$) in glass capillaries, using a microhaematocrit centrifuge (Luguimac S.R.L., Model LC 5, Argentina). Total haemoglobin (Hb) concentration of the blood was measured by the cyanomethaemoglobin method using a commercially available Kit (Analisa, Brazil). The fraction of methaemoglobin (metHb) in the blood was measured according to the method of Benesch et al. (1973). Blood samples were then centrifuged (5 min, $5000 \times g$) using a Centrimicro (Fanem, Model 243, Brazil) and plasma samples were stored frozen (-20 °C) until required for chemical analyses. Plasma sodium and potassium concentrations were measured by flame photome-

try (Analyser, Model 900, Brazil). All samples were analyzed in duplicate.

Red blood cells (RBC) from control and experimental animals were exposed to HNO_3 (0.75 N) for total ion extraction. After 20 min in acid the cells were centrifuged and the supernatant was reserved (−20 °C) for K^+ measurement by flame photometry. The precipitate was treated with NaOH (0.75 N) to solubilize protein (adapted from Rasmusson et al., 1993) and protein concentration was assayed according to Zaia et al. (1992) using ρ-benzoquinone as reagent. RBC potassium concentration was expressed as μmoles K^+. mg cell protein^{-1}.

Recently-collected red blood cells from fish not submitted to experimental protocol were used for in vitro tests. After blood centrifugation, cells were resuspended in freshwater fish buffer solution (in mM: 126.6 NaCl; 4.8 KCl; 1.2 $CaCl_2$; 2.5 $NaHCO_3$; 3.0 Na_2HPO_4; 2.9 NaH_2PO_4; pH 7.1) and allowed to rest at 4 °C for at least 1 h. Microsamples (10 μl) of RBC in saline solution were added to a polystyrene cuvette containing 1.5 ml of control saline (freshwater fish solution) or experimental saline (freshwater fish saline with $NaNO_3$ 30 mg l^{-1}). Cell volume was estimated by spectrophotometry at 400 nm (previously tested as optimum wavelength). This estimation is based on the optical density of RBC samples (as used by Flatman et al., 1996; Ortiz-Carranza et al., 1997). The method used here considers variation of transmittance (T) as indicative of changing volume: increased T (more light passing through the sample) indicated a reduction in RBC volume; decreased T (less light passing through the sample) indicated an increase in RBC volume. RBC samples from the same fish, diluted in control (cuvette A) and experimental saline (cuvette B), were run in the spectrophotometer at the same time, during 20 min. The transmittance obtained from each control sample was considered to be 1.0, and the experimental sample transmittance was expressed in relation to the control.

Cell volume measurements were also performed using RBCs submitted to $NaNO_2$ (30 mg l^{-1}) in saline solution plus $BaCl_2$ (1 mM), a non-specific K^+ channel blocker (Hille, 1992).

2.4. Statistical analyses

Data are presented as mean ± S.E.M. Differences between control 6 h and control 96 h, relative to the same blood parameter, were evaluated using Student's t-test. Considering that no significant difference was observed between the control values for all blood parameters analysed only one control group, formed by the sum of all control values was employed.

Differences among groups exposed to $NaNO_2$, for different time periods, including the control group, were tested for significance by one-way ANOVA and multiple range tests (Student–Newman–Keuls procedure) where appropriate. For RBC volume analyses Student's t-test was used to compare the first measurement (time zero) with all other measurements (time 2–20). A P-value below 0.05 was considered to indicate a significant difference between means in all statistical tests.

3. Results

The addition of $NaNO_2$ to the water to a nominal concentration of 30 mg l^{-1} resulted in approximately 0.06 mM of nitrite in water (59.10 ± 7.57 μM, $n=21$), without any substantial alteration of this concentration during the experimental period (96 h).

Both blood haematocrit and total haemoglobin decreased significantly ($P<0.01$) in *P. lineatus* after 24 h of nitrite exposure and subsequently continued to decrease to values significantly lower than in control fish (Fig. 1a). These parameters were unchanged in *A. altiparanae* exposed to nitrite when compared to control values (Fig. 1b).

P. lineatus exposed to nitrite showed an increase in blood methaemoglobin that was significantly higher ($P<0.001$) than control values in all experimental periods (Fig. 2a). However, *A. altiparanae* showed a slight increase in blood methaemoglobin after 48 h of exposure to nitrite, but this increase was not significant relative to control values (Fig. 2b).

Plasma sodium concentration showed a declining trend during nitrite exposure (Fig. 3). In both fish species, plasma Na^+ decreased to values significantly lower ($P<0.01$) than control after 48, 72 and 96 h of exposure to $NaNO_2$.

After 6 h exposure of *P. lineatus* to NO_2^- a significant increase in extracellular K^+ ($P<0.01$) was observed (Fig. 4a). Plasma potassium concentration of *A. altiparanae* exposed to NO_2^- did not vary significantly (Fig. 4b). At 6 and 96 h *P. lineatus* RBC showed a significant reduction in [K^+] ($P<0.05$) of approximately 50% (Fig. 4a).

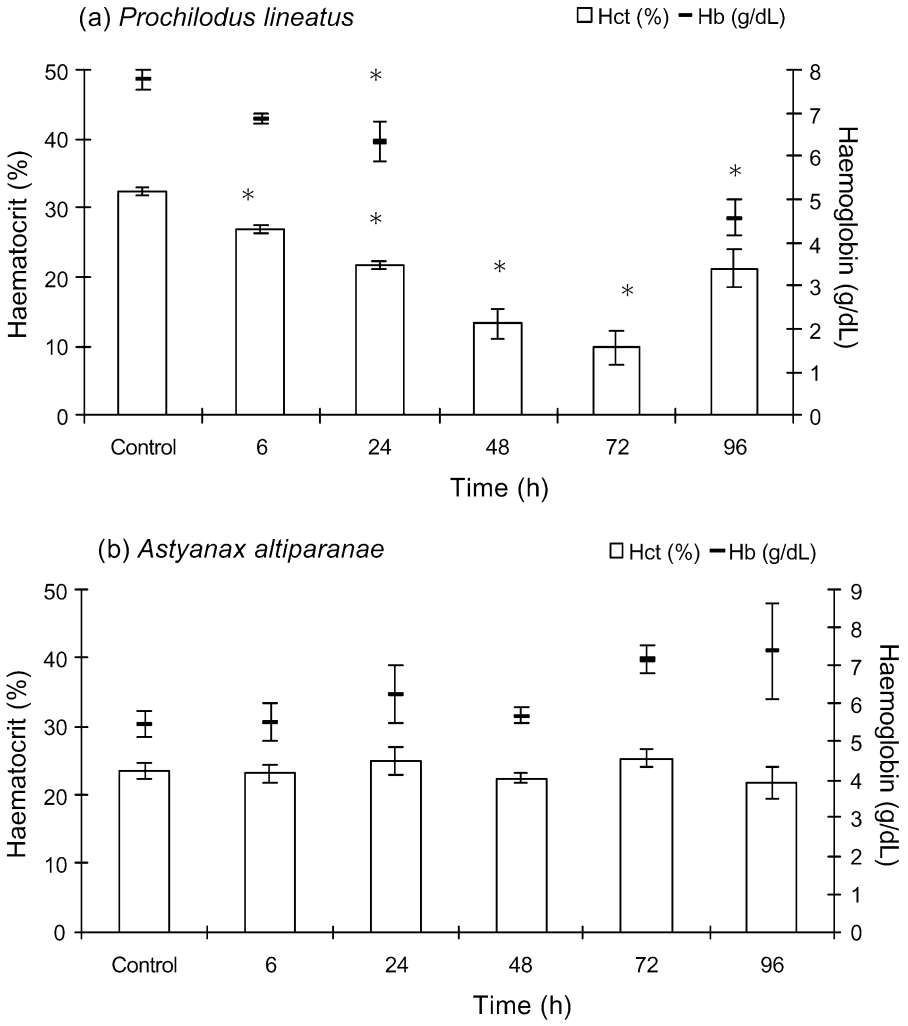

Fig. 1. Haematocrit (Hct) and total blood haemoglobin (Hb) in (a) *Prochilodus lineatus* and (b) *Astyanax altiparanae* during nitrite exposure. Data are expressed as mean ± S.E. (for *P. lineatus* Hct $n = 5–21$ and Hb $n = 5$; for *A. altiparanae* Hct $n = 5–19$ and Hb $n = 5–10$). * indicates values statistically different from respective controls ($P < 0.05$).

Red blood cells of *A. altiparanae* presented a significant decrease in $[K^+]$ ($P < 0.05$) only after 24 h of exposure to NO_2^- (Fig. 4b).

When RBC volume was analyzed in vitro a 36% shrinkage was observed in *P. lineatus* cells, after 2 min of exposure to $NaNO_2$, which did not recover ($P < 0.001$; Fig. 5a). However, when $BaCl_2$ (1 mM) was added to saline solution this effect was blocked ($P > 0.05$; Fig. 5a). In *A. altiparanae* cells exposed to nitrite a 10% shrinkage was observed ($P < 0.001$; Fig. 5b) and the addition of $BaCl_2$ promoted a 17% volume increase ($P < 0.001$; Fig. 5b).

4. Discussion

The first studies concerning the effect of nitrite on fish physiology have already reported a net nitrite uptake by the gills (Bath and Eddy, 1980; Williams and Eddy, 1987). When NO_2^- reaches the blood it crosses the erythrocyte membrane and oxidizes haemoglobin to methaemoglobin. The toxicity of nitrite may result from a combination of effects rather than from a simple effect, such as methaemoglobinaemia, in particular. Recent studies have shown that nitrite interferes with K^+ homeostasis (Knudsen and Jensen, 1997).

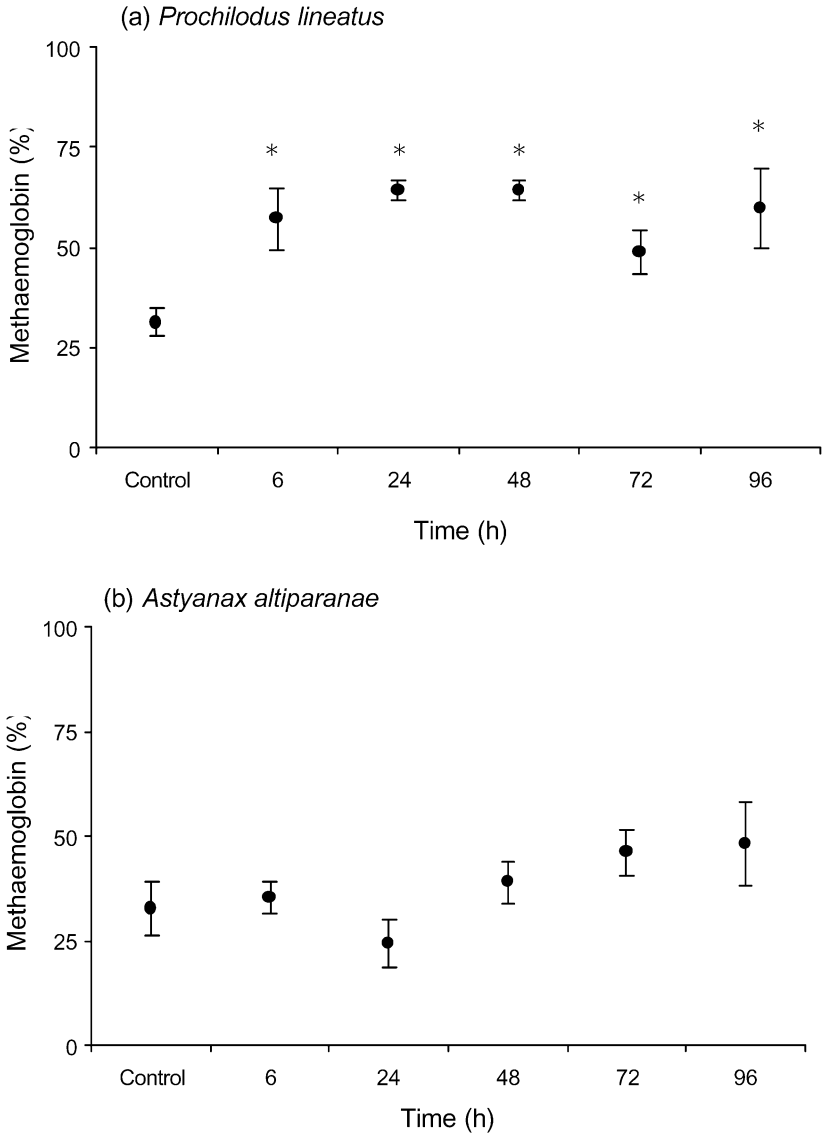

Fig. 2. Methaemoglobin content (as a percentage of total haemoglobin) in (a) *Prochilodus lineatus* and (b) *Astyanax altiparanae* during nitrite exposure. Data are expressed as mean ± S.E. (for *P. lineatus* $n = 4–11$; for *A. altiparanae* $n = 3–11$). * indicates values statistically different from respective controls ($P < 0.05$).

Nitrite enters the erythrocytes and leads to metHb formation (Hilmy et al., 1987; Jensen 1990; Knudsen and Jensen, 1997; May et al., 2000), at a rate that is proportional to the RBC K^+ leakage (Jensen, 1990; Nikinmaa and Jensen, 1992; Knudsen and Jensen, 1997; Adragna and Lauf, 1998). Some authors have shown the important role of haemoglobin quaternary structures on the regulation of RBC membrane transport systems (Jensen, 1990; Nikinmaa and Jensen, 1992; Adragna and Lauf, 1998). When oxygenated, haemoglobin assumes the R-form and when deoxygenated, the T-form, methaemoglobin also attains an R-like quaternary structure, but this is probably different from the oxyhaemoglobin form. These different conformations may somehow interact with the erythrocyte membrane and activate or inhibit some transport systems (Jensen, 1990; Adragna and Lauf, 1998). Nikinmaa and Jensen (1992) have already shown that nitrite-induced methaemoglo-

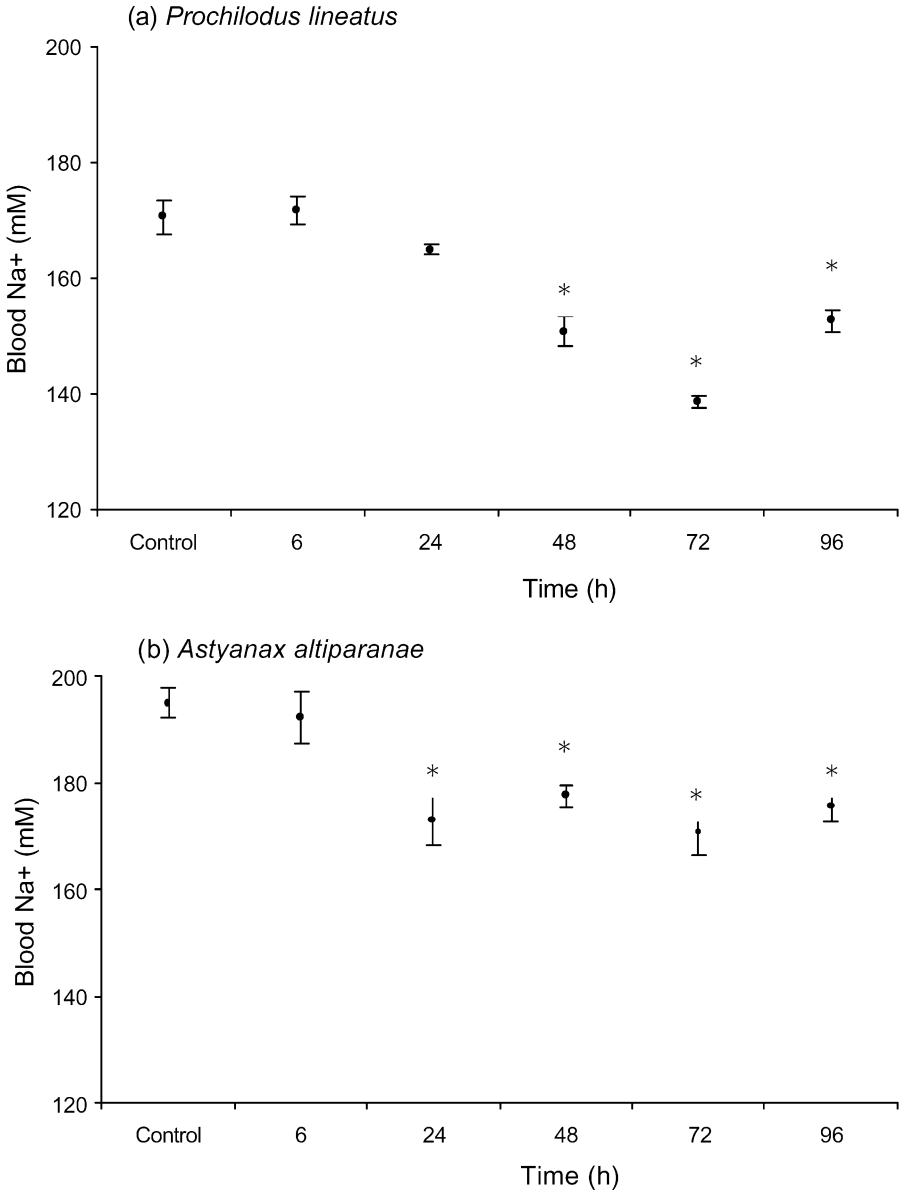

Fig. 3. Sodium plasma concentration in (a) *Prochilodus lineatus* and (b) *Astyanax altiparanae* during nitrite exposure. Data are expressed as mean ± S.E. (for *P. lineatus* $n = 5–12$; for *A. altiparanae* $n = 5–19$). * indicates values statistically different from respective controls ($P < 0.05$).

bin has an inhibitory effect on the Na^+/H^+ exchanger. It was also already observed that metHb interactions with the cell membrane have effects on other ion pathways involved in RBC K^+ efflux. Knudsen and Jensen (1997) showed that the nitrite-induced efflux of K^+ from the red cells results from stimulation of a chloride-dependent K^+ efflux mechanism: potassium–chloride cotransport.

The decrease in RBC K^+ observed in this work in both fish species might be a consequence of metHb formation, as a reflection of NO_2^- entry into the animal and then into the cell. The studied species showed different time courses in the response to NO_2^-. *P. lineatus* seems to be more sensitive than *A. altiparanae*, presenting significant metHb variations and an earlier RBC K^+ leakage. This significant cellular K^+ lost in *P. lineatus* was

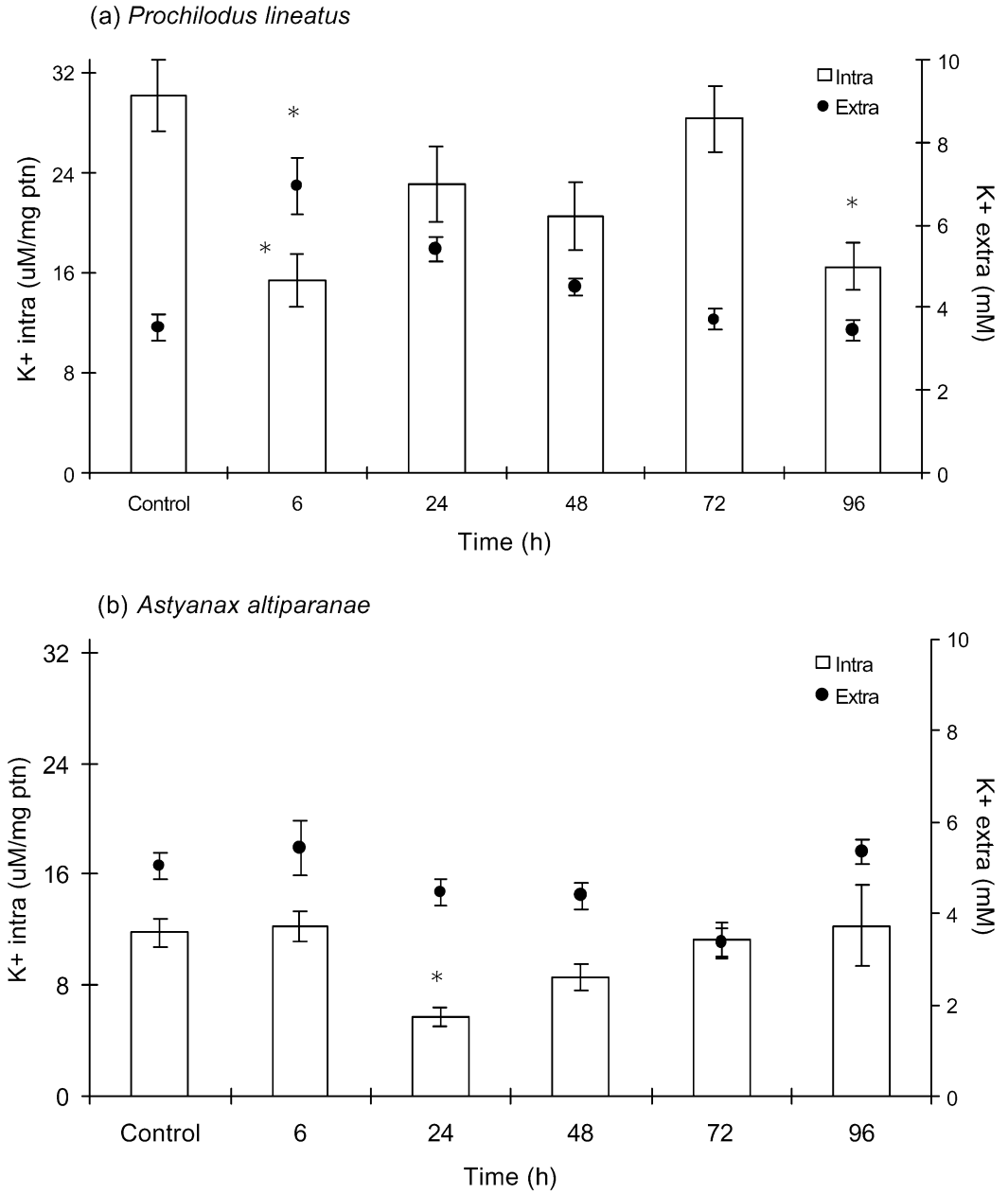

Fig. 4. Extracellular and intracellular K^+ concentration in (a) *Prochilodus lineatus* and (b) *Astyanax altiparanae* during nitrite exposure. Data are expressed as mean ± S.E. (for *P. lineatus* K^+ extra $n = 5–12$ and K^+ intra $n = 5–15$; for *A. altiparanae* K^+ extra $n = 4–19$ and K^+ intra $n = 4–16$). * indicates values statistically different from respective controls ($P < 0.05$).

observed at 6 and 96 h of exposure to NO_2^-For this fish a metHb increase was observed after 6 h, which was maintained in all experimental periods. The fact that a metHb increase does not correspond to a the significant RBC K^+ efflux at 24, 48 and 72 h, does not mean that both parameters are not related, but may indicate that these cells are working to regulate intracellular K^+ levels.

Changes in extracellular K^+ in *P. lineatus* support the idea of RBC K^+ loss: an increase in metHb and a decrease in cellular K^+ were followed by a significant increase in extracellular

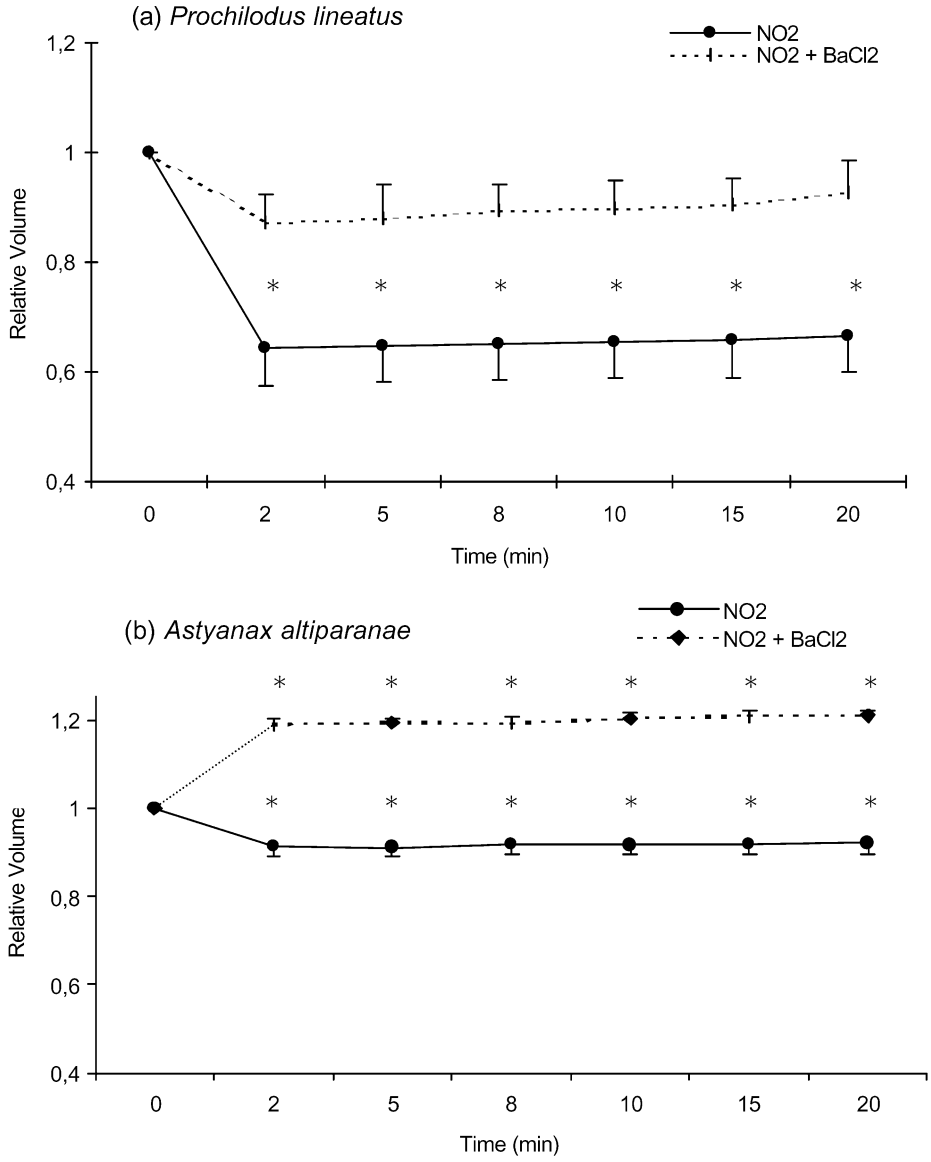

Fig. 5. Time-dependent changes in relative volume of red blood cells exposed to nitrite, with or without $BaCl_2$. (a) *Prochilodus lineatus* ($n=5$–7) and (b) *Astyanax altiparanae* ($n=5$–8). Data are expressed as mean ± S.E. * indicates values statistically different from time zero ($P<0.05$).

K^+ at 6 h of exposure to NO_2^-. After this time, a clear tendency towards extracellular K^+ recovery could be noted, indicating extracellular K^+ regulation, probably by the gills. Knudsen and Jensen (1997) observed the same correlation between intra and extracellular K^+ when carp were exposed to NO_2^- for 48 h.

A. altiparanae exhibited a decrease in RBC K^+ only when submitted to NO_2^- for 24 h. However, this intracellular K^+ decrease was not followed by an extracellular K^+ increase. Also, it was not possible to correlate this RBC K^+ lost with an increased metHb occurrence at this exposure period. This apparent resistance of *A. altiparanae* to NO_2^-, when compared to *P. lineatus,* could be related to different levels of metHb reductase, an enzyme that reduces methaemoglobin back to haemoglobin. Other possible explanation for differential effects of nitrite in the two species would be related to differences in the epithelial permea-

bility or in the permeability of red blood cell membrane to nitrite. Also, it is important to point out that in this study two different fish species were employed, which present differences in size (*A. altiparanae* is approx. half the size of *P. lineatus*) and stage of maturity (adult *A. altiparanae* and juvenile *P. lineatus*). Hilmy et al. (1987) studying the resistance to NO_2^- in different sizes of *Clarias lazera*, suggested that small fish could be more resistant to NO_2^-. The fact that *A. altiparanae* appears to be more resistant to NO_2^- might be related to its smaller size, but also to its maturity stage and some other intrinsic species characteristics.

In addition to the high interespecific variability concerning K^+ RBC content, the differences observed in intracellular K^+ between both fish species might be related to the protein assay employed. The method with ρ-benzoquinone as reagent, which was developed to measure blood protein concentration (Zaia et al., 1992) showed to have low sensitivity to detect total cell protein and may have produced an overestimation of *P. lineatus* K^+ RBC. Even with this methodological limitation the results of this study show an NO_2^- effect on RBC K^+ content.

Results of this investigation indicated that acute exposure of *P. lineatus* to nitrite resulted in a decrease in haemoglobin content and haematocrit values. Jensen et al. (1987) and Hilmy et al. (1987) also reported a significant decrease in these parameters after the exposure of adult *Cyprinus carpio* and juvenile *Clarias lazera*, respectively, to nitrite. This haematocrit reduction is probably an indication of red blood cell volume decrease, as a result of K^+ leakage. Taking into account that intracellular haemoglobin concentrations can increase to values that surpass the solubility limit in consequence of red cell shrinkage, it is possible that intracellular Hb crystals are formed (Jensen et al., 1987). This degeneration of red cell structural and functional properties may cause an increased removal of these cells from circulation and contribute to the decrease in total blood Hb, seen in nitrite-exposed fish (Jensen, 1996).

In order to validate the decrease in haematocrit as a result of RBC shrinkage, an in vitro evaluation of the erythrocyte volume submitted to NO_2^- was performed. The cells of both species shrunk and, as for the other parameters analysed, *P. lineatus* erythrocytes showed higher sensitivity to NO_2^- than the cells of *A. altiparanae.* These results corroborate in vivo observations, which showed a decrease in haematocrit for *P. lineatus* that was not detected in *A. altiparanae.*

When *P. lineatus* RBCs were exposed to nitrite plus $BaCl_2$ no change in cellular volume was observed. This result suggests that $BaCl_2$ blocks K^+ efflux by a conductive pathway and consequently there is no water flow outwards from the cell leading to a volume reduction. However, the in vitro experiments with *A. altiparanae* cells in the presence of $BaCl_2$ showed an increase in cell volume. According to Hille (1992), Ba^{2+} is highly permeant via Ca^{2+} channel and this may explain this RBC increased volume: Ba^{+2} entrance into the cell followed by water. Nevertheless more in vitro studies to evaluate nitrite effects on fish RBC volume are required, including the use of more specific K^+ channel and K^+ transporter blockers as well as intracellular K^+ measurements.

In contrast to the major changes in potassium balance when fish are exposed to nitrite, other cations, such as sodium, are either unchanged or show only small changes (Jensen, 1996). In this work, a significant decline in plasma sodium during nitrite treatment was observed in both fish species. According to Jensen (1996) sodium decrease may be due to an expansion in extracellular volume. The observed decrease in haematocrit of *P. lineatus* exposed to nitrite might support this idea. However, *A. altiparanae* did not show any reduction in haematocrit during nitrite exposure. Since a sodium decrease might be occurring in parallel with a plasma hyperkalemia, Jensen et al. (1987) suggested that NO_2^- could interfere in Na^+–K^+–ATPase resulting in tissue K^+ efflux and Na^+ uptake. In this study sodium decrease was followed by a K^+ increase only in *P. lineatus* while *A. altiparanae* exhibited a Na^+ decrease without changing K^+. Nikinmaa and Jensen (1992) suggested that the pronounced influence of nitrite-induced methaemoglobinemia on adrenergic proton efflux results from an inhibition of the red cell sodium/proton exchanger by the R-like haemoglobin conformations. This inhibition could partially explain the decreased plasma sodium concentration observed in *P. lineatus*. However, it would not be a possible explanation for sodium reduction measured in *A. altiparanae* since this latter fish species did not present nitrite-induced methaemoglobinemia. Another possible explanation for this sodium reduction, considering that nitrite inhibits carbonic anhydrase in trout gills

(Gaino et al., 1984), is that this inhibition also reduces the production of H^+, counterions for Na^+ uptake, reducing the Na^+ influx rate (Harris and Coley, 1991).

In summary, this study shows that for *P. lineatus*, nitrite entrance into the cell, leads to methaemoglobin formation and K^+ efflux, causing red cell shrinkage and increased plasma K^+. On the other hand, *A. altiparanae* proved to be a species more resistant to nitrite, exhibiting fewer responses to this compound.

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