

# Effects of acidic water, aluminum, and manganese on testicular steroidogenesis in *Astyanax altiparanae*

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**Abstract** Metals can influence the gonadal steroidogenesis and endocrine systems of fish, thereby affecting their reproduction. The effects of aluminum and manganese in acidic water were investigated on steroidogenesis in sexually mature male *Astyanax altiparanae*. Whether mature male fish recover from the effects of metals in metal-free water was also assessed. The fish were exposed to 0.5 mg L<sup>-1</sup> of isolated or combined aluminum and manganese in acidic pH (5.5) to keep the metals bioavailable. The fish underwent 96 h of acute exposure, and samples were taken 24 and 96 h after the beginning of the experiment. The fish were then maintained in metal-free water for 96 h. Plasma levels of testosterone, 11-ketotestosterone, 17 $\beta$ -estradiol, and cortisol were measured. Acidic water increased the plasma concentration of testosterone and 11-ketotestosterone. Aluminum increased the testosterone levels after 96 h of exposure. Manganese increased the 17 $\beta$ -estradiol levels after 24 h of exposure and maintained at high levels until the end of the experiment. With the exception of acidic pH, which increased cortisol levels after 24 h of exposure, no changes were observed in this corticosteroid during the acute experiment. Aluminum and manganese together also altered steroid levels but without a standard variation. The fish

recovered from the effects of most exposure conditions after 96 h in metal-free water. *A. altiparanae* could use reproductive tactics to trigger changes in testicular steroidogenesis by accelerating spermatogenesis and spermiogenesis, which may interfere with their reproductive dynamics.

**Keywords** Steroid hormones · Endocrine disruptors · Aluminum · Manganese · Acidic pH · *Astyanax altiparanae*

## Introduction

In polluted water, organisms may assimilate, absorb, accumulate, and/or transfer contaminants throughout the food chain, which may pose risks to ecological balance and human health (Seriani et al. 2015). Metals present in the water can act as endocrine disruptors (EDs), which can disturb the hypothalamic–pituitary–gonadal axis (Arukwe 2001; Iavicoli et al. 2009) and other endocrine organs in fish (Hontela and Lacroix 2006). These metals interfere with the endocrine system and alter processes, such as hormone synthesis and degradation, blocking hormone receptors or mimicking endogenous hormones at low concentrations (Matthiessen and Johnson 2007).

Aluminum (Al) can be toxic to fish in acidic waters. The concentration that triggers physiological disruptions varies according to the pH, aluminum speciation,

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and fish species (Gensemer and Playle 1999). Al can be found in the environment in ionic, polymerized, colloid, or particle forms. These forms are influenced by pH, which alters their bioavailability (Teien et al. 2007). Exposure to Al causes severe damage to the reproduction of teleosts, for example altering the production of hormones that modulate the hypothalamic–pituitary–gonadal axis (Hwang et al. 2000; Correia et al. 2010) and the metabolic processes related to reproduction (Vieira et al. 2013). Like Al, manganese (Mn) can adversely affect organisms at concentrations higher than  $0.05 \text{ mg L}^{-1}$  (USEPA 2009). Female rats exposed to Mn administered acutely into the third ventricle of the brain also show effects on their endocrine system, especially changes in the production and secretion of sex hormones (Pine et al. 2005). Manganese can alter ionoregulation and osmoregulation and cause metabolic disorders (Partridge and Lymbery 2009), lipid peroxidation, and oxidative stress in fish (Vieira et al. 2012).

In addition to their observed effects on sex steroids, metals are also known as EDs in other hormone classes, such as cortisol, acting on the hypothalamus–pituitary–interrenal axis of tilapia (Atli et al. 2015). The ability to activate a normal stress response and raise the serum cortisol response to a stressor is an integral part of the adaptive physiological response, and a disruption in the adaptive physiological response can reduce animal survival (Hontela and Lacroix, 2006).

In addition to metals, an acidic pH can itself alter the reproductive capacity of fish. For example, an acidic pH increased the plasma testosterone concentration and delayed the sexual differentiation of juvenile female Russian sturgeon (*Acipenser gueldenstaedtii* Brandt) (Zelennikov et al. 1999). Acidic rain has contributed to the reduction in the salmon population in southern Norway since the late nineteenth century (Sandoy and Langåker, 2001), and the combined effects of Al and acidic pH alter thyroid hormone levels in *Astyanax bimaculatus* (Vieira et al. 2013).

Knowledge has progressed in various related aspects, including the effects of metals, such as Al and Mn, on the reproductive physiology (primarily endocrine and metabolism) of female teleosts in native species of Brazil, such as the lambaris *Astyanax altiparanae* or *Astyanax bimaculatus* (Correia et al. 2012; Vieira et al. 2013) and *Astyanax fasciatus*

(Narcizo et al. 2010). The *Astyanax* genus comprises approximately one hundred species that are widespread throughout Brazilian hydrographic basins (Domingues et al. 2007), including those in southeastern Brazil, a region where the accumulation of Al in the soil and river waters is common (CETESB 2012). Moreover, *A. altiparanae* shows great phenotypic plasticity and could thus be useful as a bio-indicator (Siqueira-Silva et al. 2015).

The State of São Paulo's tropical soils present naturally high concentrations of the metals Al, Mn, and iron. Thus, soils constitute a significant source of these metals to water bodies through particulate entrainment due to erosion, which is caused by several factors, such as heavy rain and a lack of riparian vegetation (CETESB 2012). Camargo et al. (2009) reported the water quality of the State of São Paulo in 2007 and identified that 35 % of the surface waters destined for public consumption contain high levels of dissolved Al ( $100\text{--}5700 \text{ mg L}^{-1}$ ). Al and Mn are usually found at that concentration in the Paraíba do Sul River Basin (i.e., the region of the origin of the animals) and above the maximum threshold ( $0.1 \text{ mg L}^{-1}$ ) allowed by Brazilian regulations (CONAMA 2005).

Our study assessed the impact of the exposure to isolated or combined Al and Mn together with the effect of acidic pH on the plasma concentration of steroids in mature male *A. altiparanae* during the breeding season. Considering the metabolic and endocrine effects of metals on *Astyanax* (Correia et al. 2012; Vieira et al. 2013), we hypothesized that they can also be considered EDs in males. Hence, the reproductive potential of males would be compromised as a result of related changes in spermatogenesis.

## Materials and methods

### Experimental conditions and water chemistry

In the present study, two filters, a mechanical polypropylene filter (Health<sup>®</sup>, São José do Rio Preto, SP Brazil) and a carbon-activated filter (Aleas), were used to remove solid particles and reduce the organic matter and remove any chemical contaminants from the water. The experimental water was continuously aerated. The water temperature during the experiment

was monitored daily with a multiparameter probe (Pro Plus, YSI), which was also used to determine the nitrate ( $\text{NO}_3^-$ ) and ammonium ( $\text{NH}_4^+$ ) concentrations. The pH was monitored (maintained at different values according to the experimental group) with a pH meter (Gehaka). Additionally, the Al and Mn concentrations were evaluated prior to and after water renewal with an atomic absorption spectrophotometer (GBC, Avanta AAS-932 Plus, IL, USA) with a detection limit of  $0.01 \text{ mg L}^{-1}$  for both metals. The water samplings for metal determination were performed at each water renewal prior to and after this procedure. These samples were filtered with a  $0.45\text{-}\mu\text{m}$  syringe filter (Minisart<sup>®</sup>—Sartorius, Goettingen, Germany), stored in plastic recipients, and fixed in  $\text{HNO}_3$ .

The Al solution was obtained using aluminum sulfate [ $\text{Al}_2(\text{SO}_4)_3$ ; Sigma-Aldrich] from a stock solution prepared with Milli-Q water that was acidified to pH 2.5 with 65 %  $\text{HNO}_3$  (Suprapur, Merck).  $\text{MnSO}_4$  sulfate was used (Sigma-Aldrich) in the Mn stock solution, which was also prepared with Milli-Q water that was acidified to pH 2.5 with 65 %  $\text{HNO}_3$  (Suprapur, Merck).

#### Characteristics of *A. altiparanae*

The Hydrobiology and Aquaculture Unit of the Energetic Company of São Paulo State (CESP; Paraibuna City, São Paulo, Brazil) donated 140 *Astyanax altiparanae* mature males (Garutti and Britski 2000). The sex of the animals was determined using anal fin characteristics, which present a rough texture in the males of this species. The fish were 2 years old with an average body weight of  $24.29 \pm 1.17 \text{ g}$  and a mean total length of  $11.78 \pm 0.18 \text{ cm}$ . Prior to the experiment, the fish underwent a 7-day acclimation period in the animal facilities for ectotherms of the Physiology Department (Biosciences Institute, University of São Paulo). The fish were fed with extruded feed with 32 % crude protein once per day; feeding was suspended prior to starting the experiment and withheld throughout the experiment.

#### Experimental design

Five experimental groups ( $n = 120$ ) with two replicates ( $n = 12$  in each tank) were used in the present work: (1) a control at neutral pH (CTR-n), (2) an acidic

pH group (pH-ac), (3) an Al group + acidic pH (Al), (4) an Mn group + acidic pH (Mn), and (5) an Al + Mn group + acidic pH (Al–Mn). The metals were stored at a nominal concentration of  $0.5 \text{ mg L}^{-1}$  in groups 3, 4, and 5. The animals were housed in  $10 (40 \times 50 \times 70 \text{ cm})$  glass tanks with 132 L water per tank and a density of  $2.20 \text{ g L}^{-1}$  of fish (12 animals per tank). The water was renewed daily (90 %). The photoperiod used in the experiment was L:D 13:11 (Porto-Foresti et al. 2010) with artificial lighting from the facility.

The experiment was performed in November 2012 and was divided into two stages. In the first stage, the acute exposure to metals was carried out for 96 h, and this was divided into two different exposure times of 24 and 96 h. This period was established after previous experiments in the laboratory (Vieira et al. 2013). The second stage, also named the recovery period, also lasted 96 h. In the second stage, the fish returned to the initial conditions in metal-free water (pH close to neutral and without metal addition) and were fed the same diet used during the acclimation period. The fish were sampled after 24 and 96 h under these recovery conditions. The tests were semi-static with partial water renewal (90 % of the total volume) at 24-h intervals.

#### Blood sampling

In the present study, four sampling events were performed, all in the morning. The fish were placed in smaller containers and anesthetized with benzocaine (ethyl-*p*-aminobenzoate) solubilized in ethanol at a ratio of 1 g/10 L of water. After anesthesia, each fish was placed in a vessel dissection device; blood was collected from the caudal vasculature using 1-ml heparinized syringes (Hepamax). The blood was centrifuged for 5 min at  $655.2 \times g$  at room temperature, and plasma was distributed in aliquots and frozen at  $-80 \text{ }^\circ\text{C}$  until use (approximately 6 months).

#### Histological analysis of testes

To confirm the maturation stage, the testes were removed from the fish, fixed for 20–24 h in Bouin's solution, and dehydrated with an increasing series of ethanol dilutions. Next, the testes were infiltrated with historesin (Historesin Leica);  $3\text{-}\mu\text{m}$  sections were obtained and stained with Schiff periodic acid (PAS)/

hematoxylin/Metaniil Yellow (Quintero-Hunter et al. 1991). Then, the prepared sections were analyzed and documented in a computerized system for image capturing (transmitted light microscope—Leica DM 1000; camera—Leica DFC 295, and image capture program—Leica Application Suite Professional, LAS V3.6) (data not presented).

### Steroid analysis

The gonadal steroids 17 $\beta$  estradiol (E<sub>2</sub>), testosterone (T), and 11-ketotestosterone (11-KT) were measured in duplicate in blood plasma using ELISA commercial kits (Cayman Chemical Company, MI, USA). Cortisol (C) levels were measured using the same method with commercial kits by International IBL (Hamburg, Germany). An ether extraction protocol was used to separate protein and lipid fractions. The plasma proteins were precipitated with ethyl ether at a 1:3 ratio. After centrifugation, the supernatant was transferred, dried with nitrogen, and stored at 4 °C until use. The absorbance measurements were then read in plates with a Spectra Max 250 spectrophotometer (Molecular Devices), and steroid concentrations were calculated comparing the optic density (at 405 nm for E<sub>2</sub>, T and 11-KT and 450 nm for C) of the samples with the specific standard curve.

### Statistical analysis

The data were expressed as the mean  $\pm$  SE of the mean (SEM). For statistical analysis, the hormone concentrations were transformed to log<sub>10</sub> to normalize the data; the graphs showed values in their original concentrations. To assess the pH and metal effects, acute exposure and recovery experiments were performed separately at each exposure time. For those experiments, one-way ANOVA followed by the Holm–Sidak post hoc test was performed using the control group as a reference. The data were analyzed using the Sigma Stat 3.5 program for windows with a significance level of  $P < 0.05$ .

## Results

### Water chemistry

After assessing the main physicochemical parameters, the following values were obtained: Ca<sup>2+</sup> = 8.88 mg

L<sup>-1</sup>, Fe = 0.09 mg L<sup>-1</sup>, SO<sub>4</sub> = 12 mg L<sup>-1</sup>, PO<sub>4</sub> = 0.46 mg L<sup>-1</sup>, total nitrogen compounds = 0.40 mg L<sup>-1</sup>, alkalinity = 15 mg CaCO<sub>3</sub> L<sup>-1</sup>, organic matter = 19 %, chlorides = 4.90 mg L<sup>-1</sup>, conductivity (25 °C) = 65.50  $\mu$ S cm<sup>-1</sup>, organic carbon C = 1.3 mg L<sup>-1</sup>, inorganic carbon = 1.2 mg C L<sup>-1</sup>, total hardness = 26 mg CaCO<sub>3</sub> L<sup>-1</sup>, and turbidity = 1 NVT. All variables showed suitable values and allowed a survival rate of 100 % during the experimental period. Additionally, other parameters were monitored throughout the experiment (Table 1) to ensure the necessary conditions for fish maintenance.

### Hormonal analysis

#### *Plasma concentration of 11-ketotestosterone*

During the experiment, there was a trend toward increasing 11-KT levels even among fish exposed to CTR-n. The exposure to acidic pH triggered an increase in the 11-KT levels during the first 24 h compared with fish exposed to CTR-n, whereas the fish exposed to Al–Mn had reduced 11KT plasma levels ( $P < 0.001$ ; Fig. 1a). The exposure to Al also resulted in increased 11-KT levels but only after 96 h of exposure ( $P = 0.001$ ; Fig. 1a).

During the recovery period, the fish of the pH-ac, Al, and Al–Mn groups showed recovery after 24 h in clean water with no significant differences compared with the CTR-n group (Fig. 1b). The fish of the Mn group had decreased 11-KT values compared with the CTR-n group after 24 h in clean water ( $P = 0.002$ ) and completely recovered only after 96 h (Fig. 2b). There was also an increase in the 11-KT levels after 96 h in clean water in the fish in the pH-ac group ( $P < 0.001$ ). The fish in the Al–Mn group showed a decrease in 11-KT after 96 h ( $P < 0.001$ ) compared with the CTR-n group (Fig. 1b).

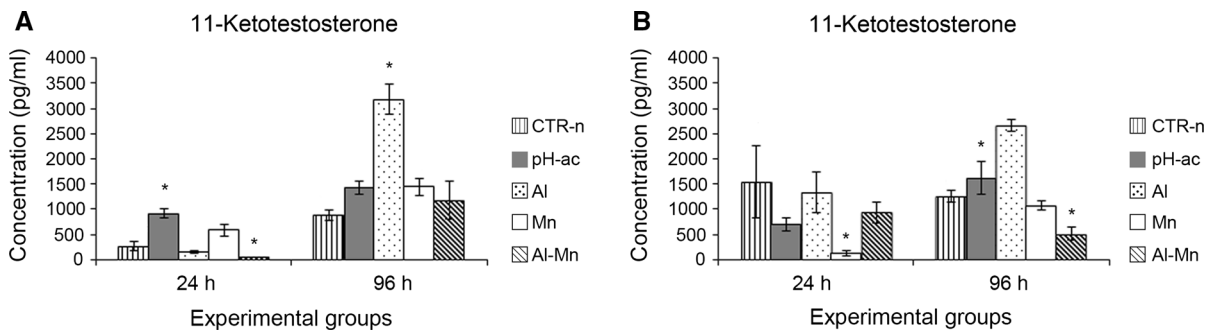
#### *Plasma concentration of testosterone*

Within 24 h, the fish in the pH-ac, Al, and Al–Mn groups had increased T levels ( $P < 0.001$ ; Fig. 2a). However, even when maintained under experimental conditions, the values returned to control levels after 96 h. The only exception was the Al group, in which the T levels continued to increase until 96 h ( $P = 0.033$ ; Fig. 2a).

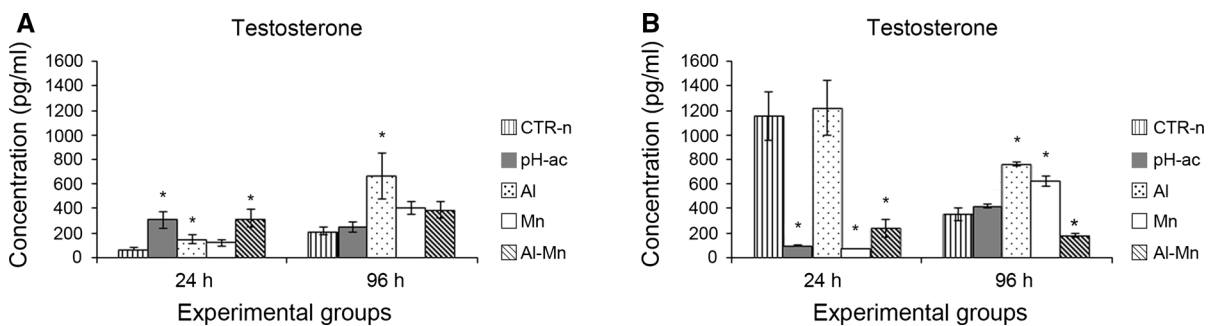
**Table 1** Physicochemical parameters of the water during the experiment (Mean  $\pm$  SEM)

| Parameters   | CTR-n            | pH-ac            | Al               | Mn               | Al-Mn            |
|--|------------------|------------------|------------------|------------------|------------------|
| pH   | 7.20 $\pm$ 0.07  | 5.84 $\pm$ 0.18  | 5.64 $\pm$ 0.11  | 5.86 $\pm$ 0.17  | 5.71 $\pm$ 0.13  |
| Dissolved O <sub>2</sub> (mg L <sup>-1</sup> )     | 7.39 $\pm$ 0.15  | 7.51 $\pm$ 0.14  | 7.43 $\pm$ 0.21  | 7.45 $\pm$ 0.12  | 7.43 $\pm$ 0.18  |
| Temperature (°C)                                   | 24.82 $\pm$ 0.14 | 24.84 $\pm$ 0.14 | 24.98 $\pm$ 0.11 | 24.80 $\pm$ 0.13 | 24.83 $\pm$ 0.12 |
| NH <sub>4</sub> <sup>+</sup> (mg L <sup>-1</sup> ) | 0.72 $\pm$ 0.14  | 0.81 $\pm$ 0.17  | 1.18 $\pm$ 0.23  | 0.76 $\pm$ 0.2   | 1.10 $\pm$ 0.4   |
| NO <sub>3</sub> <sup>-</sup> (mg L <sup>-1</sup> ) | 0.39 $\pm$ 0.03  | 0.42 $\pm$ 0.03  | 0.42 $\pm$ 0.06  | 0.45 $\pm$ 0.06  | 0.50 $\pm$ 0.08  |
| Total Al (mg L <sup>-1</sup> )                     | ND               | ND               | 0.60 $\pm$ 0.03  | ND               | 0.55 $\pm$ 0.05  |
| Dissolved Al (mg L <sup>-1</sup> )                 | ND               | ND               | 0.36 $\pm$ 0.04  | ND               | 0.38 $\pm$ 0.06  |
| Total Mn (mg L <sup>-1</sup> )                     | ND               | ND               | ND               | 0.52 $\pm$ 0.05  | 0.57 $\pm$ 0.02  |
| Dissolved Mn (mg L <sup>-1</sup> )                 | ND               | ND               | ND               | 0.31 $\pm$ 0.06  | 0.51 $\pm$ 0.04  |

ND not detected, CTR-n control at neutral pH



**Fig. 1** Plasma concentration of 11-ketotestosterone in male *Astyanax altiparanae* after acute exposure (a 24 and 96 h) and recovery (b 24 and 96 h) ( $n = 12$ /tank). \*Significant difference compared with the control (CTR-n) group within the same experimental period



**Fig. 2** Plasma concentration of testosterone in male *Astyanax altiparanae* after acute exposure (a 24 and 96 h) and recovery (b 24 and 96 h) ( $n = 12$ /tank). \*Significant difference compared with the control (CTR-n) group within the same experimental period

During the recovery period, T levels tend to increase in all groups. The fish in the pH-ac group showed lower T levels after 24 h in clean water ( $P = 0.017$ ); however, the values returned to control levels after 96 h in clean water (Fig. 2b). Conversely,

the animals exposed to the metals did not maintain a standard pattern of recovery. The fish exposed to Al-Mn maintained low T levels after 24 and 96 h in clean water ( $P = 0.025$ ; Fig. 2b). However, when exposed to isolated Mn, despite the lower T levels after 24 h in

clean water ( $P = 0.013$ ), these values increased after 96 h compared with the CTR-n group ( $P = 0.013$ ). Fish exposed to Al recovered after 24 h in clean water; however, after 96 h in clean water, the T values were higher than in the CTR-n group ( $P = 0.013$ ; Fig. 2b).

#### Plasma concentration of $17\beta$ estradiol

The fish in the CTR-n, pH-ac, Al and Al-Mn groups during the acute experiment showed a pattern toward decreased  $E_2$  levels after 96 h (Fig. 3a) that were certainly due to experimental conditions. However, the fish exposed to Mn showed an increase in  $E_2$  concentration after 24 h compared with the CTR-n group ( $P = 0.014$ ). Notably, during the recovery period, all groups showed similar values compared with those of the CTR-n group (Fig. 3b). Hence, 24 h in clean water was sufficient for the animals to recover from the effects of the Mn (Fig. 3b;  $P = 0.452$ ).

#### Plasma concentration of cortisol

A period of 24 h was not sufficient to trigger any alterations in C levels (Fig. 4a); however, after 96 h of acidic pH, the C levels increased compared with the CTR-n group ( $P = 0.024$ ; Fig. 4a), and 24 h in clean water was sufficient to reestablish C levels ( $P = 0.138$ ). However, after 96 h in clean water, all experimental groups failed to reach higher CTR-n levels and showed a decrease in the C concentration ( $P < 0.001$ ; Fig. 4b).

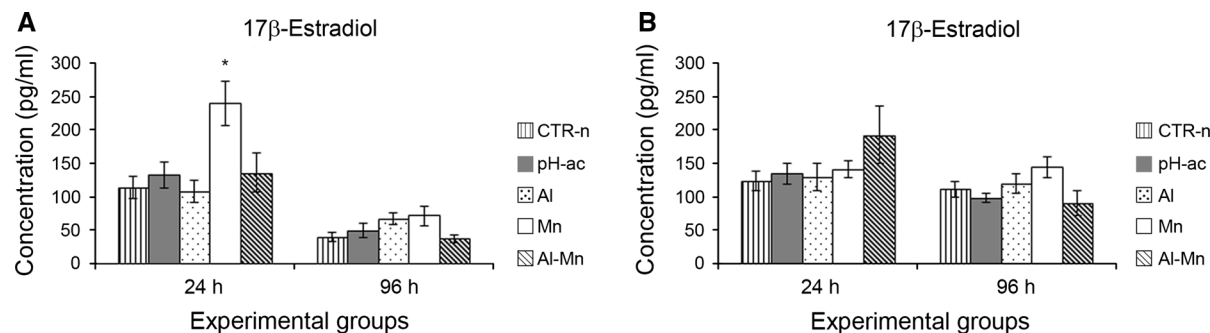
## Discussion

In the present work, we suggest that Al and Mn in acidic water act as EDs in mature male *A. altiparanae*;

however, keeping the animals for 96 h in metal-free water was usually sufficient to restore the concentration levels from the CTR-n group. Acidic pH, a necessary condition for the bioavailability of the studied metals, also triggered changes in the plasma concentrations of 11-KT and T, i.e., primarily acting in the androgen steroidogenic pathway. Hence, the acidification of water alone induced changes in the reproductive physiology of the studied fish.

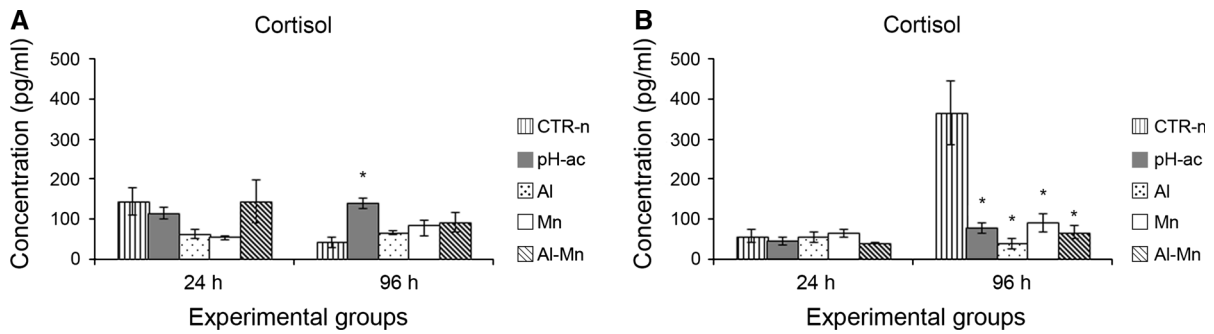
The effects of acidification on organisms and their ability to inhibit fish reproductive activity have been well known since the twentieth century (Sangalang et al. 1990). Monette and McCormick (2008) reported physiological effects and mortality in *Salmo salar* following episodic acidification and found that smolts are more sensitive than parrs to short-term exposure to Al and acidic pH. Water acidification enables the mobilization of inorganic aluminum, and its toxicity to fish varies with the Al concentration, pH, temperature,  $Ca^{2+}$  concentration, ionic strength, and dissolved organic material in addition to the duration of the exposure (Gensemer and Playle 1999).

Regarding the effects of Al, in general, an increase in androgen levels (T and 11KT) was observed. Hontela and Lacroix (2006) noted that the activity of the steroidogenic enzyme can be either stimulated or inhibited by metals. Additionally, all tissues and cell types can be targeted by an ED (Lawrence et al. 2003). EDs interfere with steroid biosynthesis by targeting the enzymes that metabolize steroid hormones to biologically active products in target cells, for example steroid acute regulatory protein (StAR), which is considered the limiting enzyme in steroidogenesis (Hampl et al. 2016). T and 11-KT are synthesized in the same pathway; thus, there may be an increase in the activity of enzymes involved in this pathway and a



**Fig. 3** Plasma concentration of  $17\beta$ -estradiol in male *Astyanax altiparanae* after acute exposure (a 24 and 96 h) and recovery (b 24 and 96 h) ( $n = 12$ /tank). \*Significant difference compared with the control (CTR-n) group within the same experimental period





**Fig. 4** Plasma concentration of cortisol in male *Astyanax altiparanae* after acute exposure (a 24 and 96 h) and recovery (b 24 and 96 h) ( $n = 12/\text{tank}$ ). \*Significant difference compared with the control (CTR-n) group within the same experimental period

higher production of these two hormones. High hormone production can be explained by a change in an animal's reproductive strategy (Lubzens et al. 2010) that can help in adapting to stressful environmental conditions caused not only by metals but also by acidic water. This hormone production accelerates the final maturation of gametes, a process that demands high concentrations of 11-KT, T, and  $17\alpha, 20\beta$ -dihydroxy-4-pregnen-3-one ( $17\alpha 20\beta$  DHP). This reproductive strategy ensures fish reproductive success, as observed in female *O. niloticus*, which showed higher progestogen levels when exposed to acidic pH (Correia et al. 2010). The hormone 11-KT induces spermatogenesis from spermatogonial proliferation to the spermiogenesis phase and enables the meiosis process until spermiation is initiated (Schulz et al. 2010).

In teleosts, T is less effective than 11-KT for spermatogenesis but is also modulated by the hypothalamus–pituitary axis for the activation of the testes (Weltzien et al. 2002). Zelennikov et al. (1999) noted an increase in the serum T levels in juvenile Russian sturgeon exposed to acidic pH and attributed that increase to the maintenance of homeostasis under stressful environmental conditions. The increase in androgen levels in *A. altiparanae*, which are triggered by Al and acidic pH, suggests a special need to adjust to the effects of metal exposure and a need for androgen hormones to spermiate. This is in light of the testes analyses (data not presented), which confirmed that all animals were sexually mature.

According to Lee et al. (2006), Al and low concentrations of Mn can also induce the production of LH, FSH, and T in male rats and trigger their maturation in the prepubertal phase. In *A. altiparanae*,

Mn did not alter androgen levels during the acute experiment; however,  $E_2$  levels increased after 24 h of exposure to Mn but quickly returned to the control levels during the recovery phase.  $E_2$  plays an important role in the early stages of spermatogenesis and cell renewal in teleosts (Knapp and Carlisle 2011), and Mn may interfere with aromatase enzyme conversion.

Unlike that observed for androgens and estrogens, the hormone C did not change upon exposure to metals, either isolated or combined. However, after 96 h of exposure to acidic pH, the C levels decreased compared with the CTR-n group. However, the general C level profile of the experimental fish differed from that of the control fish. This result confirms the literature; in a similar stress situation, corticosteroid pathways are activated, thereby increasing the production of C for rapid responses, such as the maintenance of homeostasis (Hontela and Lacroix 2006). The plasma concentration of C in fish during acute exposure to metals remained the same as in the fish in the CTR-n group. However, there were variations in the CTR-n group in both experimental periods, which can be assigned to factors such as time in the aquaria; the fish in this group were always in metal-free water. Somehow, the supposed stress caused by exposure to metals was not sufficient to induce a rapid response to a corticosteroid increase. Alternatively, the response may have occurred prior to the collection period after 24 h of metal exposure. An acidic pH was the only situation that triggered a C increase after 96 h of exposure. However, the transference to metal-free water was sufficient to reduce the C levels in 24 h.

Ozaki et al. (2006) found that low doses of C can induce the mitosis of spermatogonia through the increased production of 11-KT in Japanese eels,

whereas high doses of C inhibit this process. The authors relate this result to the stimulatory ability of C and catecholamines in the mobilization process for energy purposes. Therefore, the unaltered maintenance of the C concentration in *A. altiparanae* suggests that metal exposure has not necessarily changed energy recruitment. In contrast, females of the same species altered the metabolism of lipids and proteins (Vieira et al. 2013) and reduced the C plasma concentration (Correia et al. 2012) after exposure to Al and Mn. Hence, the sensitivity of females to metals requires more pronounced metabolic adjustments. It is important to highlight that the fish exposed to the metals and acidic pH failed to trigger a sharp increase in C levels when transferred to the metal-free water, as did the fish in the CTR-n group. Thus, adverse conditions (metals or acidic pH) inhibited the hypothalamus–pituitary–interrenal response (Wingfield and Sapolsky 2003), as observed in *Oreochromis niloticus* (Correia et al. 2010).

Ninety-six hours in metal-free water was sufficient for the recovery of steroid plasma levels in most situations, as the hormone concentration showed similar values to those in the CTR-n group. This response may be attributed to several factors related to synthesis pathways and hormone excretion. Processes, such as sulfation and glucuronidation, may also be activated, making hormones more soluble and therefore enabling their elimination by the excretory system (Norman and Litwack 1997). The recovery response was rapid in *A. altiparanae*. In experiments with the Atlantic salmon, Nilsen et al. (2013) reported a 2-week period for the complete recovery of body functions after exposure to acidic pH and Al.

The teleost group has a wide variety of strategies and survival tactics. They show great plasticity by adjusting to environmental conditions. The Characiformes stand out due to their great adaptive phenotypic divergence. Among them, *Astyanax* is one of the most common and diverse characid genera with hundreds of species (Orsi et al. 2004). Due to the high plasticity of that genus, *Astyanax* can be expected to have various mechanisms to cope with the environment in which they live. For example, species of the *Astyanax* genus can alter their biological functions when they are in a polluted environment, as observed in *A. altiparanae*.

Environmental pollution by chemicals, known as EDs, has stimulated or blocked biological processes and interfered with sensitive hormone pathways that

regulate reproductive functions (Miura et al. 1999). Laskey and Phelps (1991) analyzed the possible mechanisms by which metals may act and suggested different routes for the action of cations, such as cadmium, nickel, cobalt, and zinc, in the inhibition or stimulation of T synthesis. The inhibition may occur in the early stages of P450scc activity (side-chain cleavage) in which the enzyme breaks down cholesterol side chains, converting this precursor to pregnenolone. According to those authors, the sites of stimulation of steroid production triggered by those cations are located in the subsequent action of P450scc and progesterone synthesis steps. Hence, cations can act in different inhibitory/stimulatory pathways in the plasma membrane, adenylate cyclase, or cholesterol from mitochondrial interactions with calcium ions, membrane proteins (P450scc), or lipids (Mgbonyebi et al. 1994). The current data show that metals can be considered EDs, and there are several ways by which they can act. An acidic pH may cause these effects alone or may also potentiate effects on plasma concentrations. The reason for a stimulation or inhibition of steroid hormone production remains unclear. However, given the high plasticity of fish, as suggested for *A. altiparanae* and most *Astyanax* species, sublethal metal concentrations can affect the endocrine system but not damage the entire body (Kime and Nash 1999). Given that physiological systems are intrinsically related, changes in the endocrine system can trigger altered responses in the reproductive tract, and there is a multitude of factors that influence those effects; thus, tools are needed to elucidate the mechanisms of action of EDs.

In conclusion, Al, Mn, as well as water acidity, can act as EDs in mature male *A. altiparanae*. Those EDs stimulate androgen synthesis, which triggers changes in the physiological system. Additionally, the maintenance of mature male *A. altiparanae* under experimental conditions also changes the concentration of plasma androgens regardless of the association with metals or pH variations. Our results suggest that the adoption of alternative reproductive tactics in *A. altiparanae* in an impacted natural environment may inhibit its reproductive strategy.

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