

Original article

Water pH and hardness alter ATPases and oxidative stress in the gills and kidney of pacu (*Piaractus mesopotamicus*)

Carlos Eduardo Copatti¹, Bernardo Baldisserotto², Carine de Freitas Souza²,
José María Monserrat³ and Luciano Garcia⁴

This study aimed to assess the effects of low and high water hardness in interaction with different water pH in pacu (*Piaractus mesopotamicus*). Pacu juveniles were subjected to low (50 mg CaCO₃ L⁻¹ – LWH) or high water hardness (120 mg CaCO₃ L⁻¹ – HWH) at water pH of 5.5 (acidic), 7.5 (circumneutral) or 9.0 (alkaline) for 15 days. Gills and kidneys were collected (days 1, 5 and 15). Gill Na⁺/K⁺-ATPase (NKA) and vacuolar-type H⁺-ATPase (V-ATPase) activities were higher in alkaline pH with HWH on day 1. Gill and kidney NKA and V-ATPase activities were higher in acidic pH with LWH on day 15. Gill NKA activity of pacus under alkaline pH with LWH was higher than those exposed to HWH. Reduced antioxidant capacity in the gills and kidney and enhanced thiobarbituric acid reactive substances (TBARS) levels were demonstrated in fish exposed to acidic or alkaline pH, mainly with LWH. HWH increased glutathione-S-transferase (GST) activity and reduced TBARS levels in the gills and kidney. On day 15, GST activity was increased at acidic pH with LWH. In conclusion, circumneutral pH presents less oxidative stress and fewer variations in ATPases and HWH reduced deleterious effects in fish exposed to acidic or alkaline pH.

Keywords: Acidic pH, Alkaline pH, GST, Na⁺/K⁺-ATPase, TBARS.

Este estudo objetivou analisar o efeito de baixa e alta dureza da água em interação com diferentes pH da água em pacu (*Piaractus mesopotamicus*). Juvenis de pacu foram submetidos a baixa (50 mg CaCO₃ L⁻¹ – BDA) ou alta dureza da água (120 mg CaCO₃ L⁻¹ – ADA) em pH da água de 5.5 ácido), 7.5 (circum-neutro) ou 9.0 (alcalino) por 15 dias. Foram coletados brânquias e rim (dias 1, 5 e 15). Atividade de Na⁺/K⁺-ATPase (NKA) e H⁺-ATPase do tipo vacuolar (V-ATPase) branquial foram maiores em pH alcalino com ADA no dia 1. Atividade de NKA e V-ATPase branquial e renal foram maiores em pH ácido com BDA no dia 15. Atividade de NKA branquial de pacus submetidos a pH alcalino com BDA foi maior que aqueles expostos para ADA. Em peixes expostos a pH ácido ou alcalino com BDA houve redução da capacidade antioxidante nas brânquias e rim e aumento dos níveis de “substâncias reativas ao ácido tiobarbitúrico” (TBARS). Em ADA aumentou a atividade da “glutathione-S-transferase” (GST) e reduziu níveis de TBARS nas brânquias e rim. No dia 15, a atividade da GST foi maior em pH ácido com BDA. Em conclusão, pH circum-neutro apresentou menor estresse oxidativo e poucas variações na atividade de ATPases e ADA reduziu efeitos deletérios em peixes expostos a pH ácido ou alcalino.

Palavras-chave: GST, Na⁺/K⁺-ATPase, pH ácido, pH alcalino, TBARS.

Introduction

The relationship between water pH and hardness influences fish development and welfare (Baldisserotto, 2011; Copatti *et al.*, 2011a,b). Water hardness and pH are interrelated water quality parameters and they present profound effects

on fish productivity and physiology and therefore deserve special attention (Copatti *et al.*, 2019). Hardness is important since the dissolved minerals (more specifically calcium and magnesium) are essential in the biological processes of aquatic animals, for example, bone and scale formation in fish (Guerreiro *et al.*, 2004; Canario, Flik, 2007). Calcium

¹Instituto de Biologia, Universidade Federal da Bahia (UFBA), Av. Adhemar de Barros, s/n, Salvador, BA, 40170-110, Brazil. (CEC) carloseduardocopatti@yahoo.com.br, <https://orcid.org/0000-0002-0114-0334> (corresponding author).

²Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria (UFSM), Prédio 21, Sala 5116, Av. Roraima, 1000, Camobi, 97105-900 Santa Maria, RS, Brazil. (BB) bbaldisserotto@hotmail.com, <https://orcid.org/0000-0002-8770-0100>; (CFS) carinedefs@yahoo.com.br, <https://orcid.org/0000-0001-9978-0454>.

³Instituto de Ciências Biológicas, Universidade Federal do Rio Grande (FURG), Av. Itália, km 8, 96203-000 Rio Grande, RS, Brazil. josemmonserrat@gmail.com, <https://orcid.org/0000-0002-9463-3049>.

⁴Laboratório de Aquacultura Continental, Instituto de Oceanografia, Universidade Federal do Rio Grande (FURG), 96203-900 Rio Grande, RS, Brazil. garcia_log@hotmail.com, <https://orcid.org/0000-0001-9133-7212>.

is crucial for osmoregulation and maintenance of heart, muscle and nerve function (Parra, Baldisserotto, 2007; Boyd *et al.*, 2016). Freshwater fish survival is greater at very acidic or alkaline water pH when fish are in high rather than in low water hardness (Baldisserotto, 2011). Calcium is important at stabilizing tight junctions in the gills and reduces their permeability, consequently contributing to osmoregulatory homeostasis and survival of fish by limiting diffusive branchial ion loss to the water by paracellular route (Baldisserotto, 2011; Chasiotis *et al.*, 2012). Apparently, one of the survival mechanisms of species living in waters of very low hardness is a high affinity for calcium, which would contribute to closing paracellular junctions in the gills (Gonzalez *et al.*, 1998).

In addition to the potential detrimental effects of low water hardness on fish osmoregulation, conditions leading to water acidification or alkalisation may result in the decline of fish populations due to changes in osmoregulatory homeostasis (Gonzalez *et al.*, 1998; Parra, Baldisserotto, 2007; Zahangir *et al.*, 2015). Osmoregulation in freshwater teleosts is essential to maintain cell volume and prevent loss of ions (Evans *et al.*, 2005). However, several biological or environmental processes may modify water pH and compromise cellular osmoregulation. For instance, photosynthesis and pond fertilisation increase water pH, while acidic rainfall decreases water pH (Boyd *et al.*, 2016). In addition, acidic waters can promote ionic imbalance due to high concentrations of H⁺, which causes the efflux of Na⁺ and K⁺ ions (Aride *et al.*, 2007). This results in a greater energetic expenditure, which compromises energy destined for growth to the osmoregulatory mechanisms (Bolner, Baldisserotto, 2007; Lemos *et al.*, 2018a). Exposure to very alkaline pH may inhibit Na⁺ and Cl⁻ transport by the gills, causing disturbances in ion influx (Yesaki, Iwama, 1992). In addition, the main problems in alkaline waters seem to be the inhibition of ammonia excretion and increase of CO₂ excretion in gills (Wood, 2001).

The transfer of ions is driven by highly specialized cells of the branchial epithelium called ionocytes (or chloride cells or mitochondrion-rich cells) (Evans *et al.*, 2005), which, if damaged, impair the transport of ions in fish (Christensen *et al.*, 2012). Numerous mitochondria in these cells are thought to supply the ATP for ion-transport proteins to drive the vectorial transport of ions as part of ion and acid base regulation (Marshall, 2002). Therefore, to maintain high ion levels in the blood relative to the environment, freshwater teleosts take up ions by active transport driven by ATP located in the apical and basolateral membranes of ionocytes and pavement cells (Wilson, Laurent, 2002).

Na⁺K⁺-ATPase (NKA) and vacuolar-type H⁺-ATPase (V-ATPase) are considered as indexes of the ionoregulatory capacity status in gills and kidney of teleosts (Evans, 1993; Marshall, 2002; Perry *et al.*, 2003). NKA is an ionoregulatory enzyme expressed in animal cells to create an electrochemical gradient providing the driving force for ion transport in osmoregulatory organs, including fish gills

and kidney (Whittamore, 2012). V-ATPase is a multitasking enzyme which provides the driving force for NaCl uptake in freshwater teleosts and is essential to this the linkage indirectly with an epithelial Na⁺ channel through which Na⁺ can be driven down its electrochemical gradient into the cell (Marshall, 2002). Ionocytes participate in response regulation when fluids become alkaline (alkalosis), and it is possible that exposure to alkaline pH also stimulates the proliferation of ionocytes (Goss *et al.*, 1994). During respiratory acidosis, there is an increase in V-ATPase activity in kidney, which ensures that HCO₃⁻ levels accumulate in the body fluids to restore pH (Perry *et al.*, 2003). An increase in the rates of O₂ uptake led to even greater ion losses probably due to distortion and widening of the gill tight junctions (Gonzalez, McDonald, 1994). Thus, during osmoregulation alterations, macromolecules may experience damage from oxidative stress as a consequence of the changes in cell volume and membrane rearrangement (Rosas-Rodríguez, Valenzuela-Soto, 2010).

Oxidative stress, *i.e.* the imbalance between pro- and anti-oxidants, is characterised by environmental variables in fish in which antioxidant defence systems are ineffective at neutralising reactive oxygen species (ROS) formation, thus affecting cellular function via the oxidation of proteins, nucleic acids and lipids (Jones, 2006; Stoliar, Lushchak, 2012). Additionally, ROS can break down into free radicals that can perpetuate the destructive cycle of lipid peroxidation (LPO) chain reactions in cell membranes of various organ systems (Martínez-Álvarez *et al.*, 2005), which may affect osmoregulation. To prevent tissue damage in situations when there is the danger of inordinate accumulation of ROS, animals have developed a defence system to rapidly break down ROS (Birmie-Gauvin *et al.*, 2017). In this sense, fish tissues have antioxidant defence systems (*e.g.*, Glutathione-S-transferase – GST; Total antioxidant capacity against peroxy radicals – ACAP) to protect them from oxidative stress and to maintain the cellular redox balance (Do Carmo *et al.*, 2018).

The gills and kidney are complex organs that have a central role in fish physiology and contribute to maintain osmotic balance and acid-base regulation in freshwater teleosts (Wood *et al.*, 1999; Duffy *et al.*, 2011). As the ionic composition of freshwater fish blood is always different from that of the surrounding water, the transcellular transport of solutes across the gills and kidney contributes significantly to the overall maintenance of salt and water balance in fish (Evans *et al.*, 2005). The gills are the first target of acidic or alkaline water, since they are intimately in contact with the environment (Atli, Canli, 2011). The parenchyma of gills is a heterogeneous epithelium that is directly exposed to the surrounding aqueous environment. The gill epithelium presents a large surface area for the movement of biological material between blood and water, and is simultaneously involved in respiration, iono/osmoregulation, acid-base balance and nitrogen waste excretion (Evans *et al.*, 2005). The kidney is the primary organ for the elimination of water

and is particularly important for freshwater species due to the efficient ion reabsorption mechanisms to minimise ion loss (Atli, Canli, 2011). The kidney contributes to the maintenance of hydromineral balance by assisting in the elimination of excess water and the reabsorption of systemic salts (Duffy *et al.*, 2011), complementing the function of the gills. Obviously, the main function of the kidney in freshwater teleosts is to produce dilute urine, allowing for the excretion of the excess body water that has entered by osmosis through the gills (Bolner, Baldisserotto, 2007). Considering the relevance of these organs for osmoregulation and antioxidant capacity, the gills and kidney were the focal points of study.

Pacu (*Piaractus mesopotamicus* (Holmberg, 1887)) is a Neotropical Freshwater Characin native to the Paraná, Paraguay and Uruguay basins with herbivorous/omnivorous habits, and is widely cultured in fish farms in South America (Valladão *et al.*, 2016). A recent study found that high water hardness (HWH) has a protective effect on pacus exposed to acid or alkaline waters because HWH improved biochemical and physiological variables (Copatti *et al.*, 2019). However, the oxidative stress (thiobarbituric acid reactive substances (TBARS), glutathione-S-transferase (GST) and total antioxidant capacity against peroxy radicals (ACAP)) and ATPase responses (NKA and V-ATPase) to variations in water pH and hardness are still unknown for pacu.

Therefore, the present study was designed to investigate the effects of LWH and HWH in interaction with circumneutral, acidic and alkaline water pH in the pacu through the analysis of oxidative stress and ATPase activity in the gills and kidney. We hypothesised that an increase in water hardness may reduce ATPase activity and improve antioxidant capacity in pacu juveniles exposed to acidic or alkaline pH.

Material and Methods

Experimental conditions. Pacu juveniles (39.56 ± 2.64 g) were purchased from a fish farmer in Ajuricaba, Brazil and brought to the Continental Aquaculture Laboratory at the Universidade Federal do Rio Grande (FURG), Rio Grande, Brazil. Juveniles were maintained for ten days in 18 continuously aerated 250-L tanks in six recirculating aquaculture system (12 fish per tank, 216 fish in total), with biological and mechanical filters, water hardness of $50 \text{ mg CaCO}_3 \text{ L}^{-1}$ and water pH 7.5. The same system used for maintenance was used for the experimental procedures, except for water pH and hardness, which were maintained according to the specified treatments.

The design was organized into six treatments and three replicates for 15 days, under the following experimental conditions: low water hardness ($50 \text{ mg CaCO}_3 \text{ L}^{-1}$ – LWH) or high water hardness ($120 \text{ mg CaCO}_3 \text{ L}^{-1}$ – HWH) at water pH of 5.5 (acidic), 7.5 (circumneutral) or 9.0 (alkaline) according with Copatti *et al.* (2019). Experiments were run with catholyte pH automatically controlled (Tekna

TPR 603, USA) via feedback by using 50% NaOH or 98% H_2SO_4 (Copatti *et al.*, 2011a). In HWH, water hardness was increased by the addition of di-hydrated calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) according to Copatti *et al.* (2011a). We used this compound because its solubility is much higher than CaCO_3 , for example.

These water pH values were chosen because in silver catfish (*Rhamdia quelen*) exposed to water pH 5.5, 7.0 or 9.0 with LWH (30 or 60 $\text{mg CaCO}_3 \text{ L}^{-1}$) or HWH (120 or 180 $\text{mg CaCO}_3 \text{ L}^{-1}$) the survival was higher than 92.7% (Copatti *et al.*, 2011a). Previous study verified that at zero water hardness silver catfish mortality was superior to 62 and 40% at pH 7.0 and 8.0 (Copatti *et al.*, 2001b). This demonstrates that zero water hardness can be lethal to fish, and then it was not evaluated in our study.

The water physical-chemical variables were monitored daily for pH (5.5 ± 0.01 , 7.5 ± 0.01 or 9.0 ± 0.01) (pH meter Hanna HI 8424), temperature ($24.55 \pm 0.19^\circ\text{C}$), dissolved oxygen ($7.62 \pm 0.08 \text{ mg L}^{-1} \text{ O}_2$) (oximeter YSI 5908), nitrite ($0.10 \pm 0.02 \text{ mg L}^{-1} \text{ N-NO}_2$) (Boyd, Tucker, 1998), hardness (50 ± 0.01 or $120 \pm 0.10 \text{ mg CaCO}_3 \text{ L}^{-1}$), total ammonia ($0.80 \pm 0.23 \text{ mg L}^{-1} \text{ N-NH}_3$) (Eaton *et al.*, 2005) and unionised ammonia ($0.030 \pm 0.01 \text{ mg L}^{-1} \text{ N-NH}_3$) calculated from a conversion table for fresh water.

The fish were fed commercial food (Supra Acqua Line®, São Leopoldo, Brazil, 320 g kg^{-1} crude protein; 3,600 kcal digestible energy) twice a day (7:30 a.m. and 5:30 p.m.) until apparent satiety. The water that was lost due to evaporation or cleaning of the tanks to remove excess residues (faeces and feed) was replaced with fresh water under the respective treatment conditions. The methodology of this experiment was approved by the Ethical and Animal Welfare Committee of the Instituto de Biologia at the Universidade Federal da Bahia, Salvador, Brazil (number 29/2015). Vouchers were deposited in the ichthyological collection of the Museu Oceanográfico of FURG (catalog number 2929).

Sample collection. For the gills and kidney analyses, three fish from each tank ($n = 9$ per treatment) were randomly sampled on days 1, 5 and 15 to verify possible deleterious effects on acute, medium and longer exposure under the experimental conditions. Fish were immediately euthanised with a lethal concentration of hydrochloride benzocaine (50 ppm), because benzocaine did not cause oxidative stress (Stringhetta *et al.*, 2017), and samples of the gills and kidney were collected. Prior to tissue collection, animals underwent 24 h of fasting.

Samples of the gills and kidney were stored in an ultra-freezer (-80°C) and then homogenised (1:5; w/v) in a Tris-HCl (100 mM, pH 7.75) buffer with EDTA (2 mM) and Mg^{2+} (5 mM) (Da Rocha *et al.*, 2009). Afterwards, the homogenate was centrifuged for 20 min ($10,000 \times g$, 4°C); the supernatant obtained was used for all analysis. The protein concentration was determined using the Coomassie blue staining method, where bovine serum albumin was used as standard protein, according to Bradford (1976).

Analysis. NKA and V-ATPase activities were measured simultaneously. NKA activity was analysed using the method described by Gibbs, Somero (1989) and V-ATPase activity was analysed according to Toni *et al.* (2014). The data are expressed as mmol ADP mg protein⁻¹ h⁻¹.

Glutathione-S-transferase (GST) activity was determined according to Habig, Jakoby (1981). The data are expressed as nmol CDNB-GSH conjugate min⁻¹ mg wet tissue⁻¹.

Total antioxidant capacity against peroxy radicals (ACAP) was determined through the detection of ROS in samples treated or not with a peroxy radical generator according to Amado *et al.* (2009). All samples were first diluted with homogenisation buffer to 2.0 mg protein mL⁻¹ and then exposed to peroxy radicals generated by the thermal (37°C) decomposition of 2,2'-azobis (2-methylpropionamide) dihydrochloride (ABAP, 4 mM). Peroxy radicals reacted with a fluorescent substrate (2',7'-dichlorofluorescein diacetate-H₂DCF-DA) and fluorometry (excitation 485 nm; emission 520 nm) were measured on a microplate reader (Victor 2, Perkin Elmer) with readings every 10 min for 70 min. The data were expressed as the relative area, where a higher relative area (the difference between the ROS area with and without ABAP relative to that without ABAP) means a lower antioxidant capacity.

The lipid peroxidation levels were measured according to Oakes, Van Der Kraak (2003) by thiobarbituric acid reactive substances (TBARS) content. The TBARS content was determined by the quantification of MDA (malondialdehyde) following the protocol described in detail by Maltez *et al.* (2018). The data are expressed as nmol TMP (tetramethoxypropane (ACROS Organics)) mg wet tissue⁻¹.

Statistical analysis. The results are expressed as the mean ± standard error of the mean (SEM). Levene's test demonstrated the homogeneity of data variances. Data were then compared using a two-way ANOVA (pH vs. hardness) followed by Tukey's post hoc test within the same sampling day. Differences were considered significant at P < 0.05.

Results

ATPase activity. No mortality occurred in any group. On day 1, gills NKA and V-ATPase activities in pacus exposed to alkaline pH with HWH were significantly higher than those subjected to other pH values at the same hardness (P < 0.05). On the same day (day 1), gill V-ATPase activity in pacus subjected to acidic pH with LWH was significantly higher than those exposed to HWH at the same pH (P < 0.05) (Tab. 1).

Tab. 1. Na⁺/K⁺-ATPase (NKA) and vacuolar-type H⁺-ATPase (V-ATPase) activities (mmol ADP mg protein⁻¹ h⁻¹) in the gills and kidney of pacu (*Piaractus mesopotamicus*) juveniles submitted to different pH and water hardness at different times. Note. LWH = low water hardness (50 mg CaCO₃ L⁻¹); HWH = high water hardness (120 mg CaCO₃ L⁻¹). Data are presented as the means ± SEM (n = 9 fish per treatment). Different uppercase letters indicate statistically significant differences between pH at the same hardness (P < 0.05). Different lowercase letters indicate statistically significant differences between hardness at the same pH (P < 0.05).

pH	Hardness	Gills NKA	Kidney NKA	Gills V-ATPase	Kidney V-ATPase
Day zero					
7.5	LWH	2.35 ± 0.39	0.33 ± 0.04	2.28 ± 0.46	0.33 ± 0.01
Day 1					
5.5	LWH	3.34 ± 0.95 ^{Aa}	0.32 ± 0.05	5.86 ± 1.16 ^{Aa}	0.31 ± 0.03
5.5	HWH	1.76 ± 0.70 ^{Ba}	0.45 ± 0.11	2.00 ± 0.62 ^{Bb}	0.42 ± 0.10
7.5	LWH	2.39 ± 0.52 ^{Aa}	0.25 ± 0.03	2.80 ± 0.58 ^{Aa}	0.26 ± 0.06
7.5	HWH	1.10 ± 0.48 ^{Ba}	0.25 ± 0.03	1.44 ± 0.53 ^{Ba}	0.28 ± 0.04
9.0	LWH	5.00 ± 0.78 ^{Aa}	0.38 ± 0.10	3.83 ± 0.70 ^{Aa}	0.39 ± 0.10
9.0	HWH	5.54 ± 0.81 ^{Aa}	0.25 ± 0.05	6.56 ± 1.21 ^{Aa}	0.27 ± 0.05
Day 5					
5.5	LWH	3.30 ± 1.34	0.25 ± 0.03	3.97 ± 1.15	0.19 ± 0.03 ^{Ba}
5.5	HWH	3.07 ± 0.69	0.24 ± 0.05	3.47 ± 1.07	0.25 ± 0.04 ^{Aa}
7.5	LWH	2.64 ± 1.05	0.24 ± 0.02	3.36 ± 1.07	0.19 ± 0.03 ^{Ba}
7.5	HWH	1.99 ± 0.67	0.25 ± 0.07	2.43 ± 0.47	0.23 ± 0.06 ^{Aa}
9.0	LWH	1.38 ± 0.71	0.31 ± 0.07	2.16 ± 0.64	0.41 ± 0.06 ^{Aa}
9.0	HWH	1.30 ± 0.57	0.29 ± 0.04	1.68 ± 0.72	0.20 ± 0.03 ^{Ab}
Day 15					
5.5	LWH	9.80 ± 1.10 ^{Aa}	0.79 ± 0.12 ^{Aa}	6.51 ± 1.49 ^{Aa}	0.68 ± 0.14 ^{Aa}
5.5	HWH	4.65 ± 1.05 ^{Ab}	0.31 ± 0.07 ^{Ab}	1.90 ± 0.99 ^{Ab}	0.30 ± 0.07 ^{Ab}
7.5	LWH	2.39 ± 1.33 ^{Ba}	0.42 ± 0.08 ^{Ba}	2.09 ± 0.52 ^{Ba}	0.40 ± 0.10 ^{Ab}
7.5	HWH	1.84 ± 0.56 ^{Aa}	0.21 ± 0.04 ^{Aa}	1.93 ± 0.62 ^{Aa}	0.21 ± 0.06 ^{Aa}
9.0	LWH	5.34 ± 0.28 ^{Ba}	0.37 ± 0.06 ^{Ba}	3.93 ± 1.23 ^{ABa}	0.27 ± 0.07 ^{Ba}
9.0	HWH	1.13 ± 0.21 ^{Ab}	0.41 ± 0.10 ^{Aa}	1.60 ± 0.53 ^{Aa}	0.24 ± 0.06 ^{Aa}

On day 5, kidney V-ATPase activity of fish subjected to alkaline pH with LWH was significantly higher than those exposed to the other treatments at the same water pH or hardness ($P < 0.05$) (Tab. 1).

On day 15, in general, pacus exposed to acidic pH with LWH presented gill and kidney NKA and V-ATPase activities significantly higher than other treatments at the same water pH or hardness ($P < 0.05$). On the same day (day 15), gills NKA activity of pacus exposed to alkaline pH with LWH was significantly higher than those subjected to alkaline pH with HWH ($P < 0.05$) (Tab. 1).

Oxidative stress. On days 1 and 5, gill GST activity in fish exposed to circumneutral pH with LWH was significantly higher than in those exposed to the other treatments at the same water pH or hardness ($P < 0.05$) (Fig. 1a). In addition, on day 1, gill GST activity in juveniles subjected to alkaline

pH with LWH was significantly lower than those subjected to HWH at the same pH ($P < 0.05$) (Fig. 1a). On day 5, gill GST activity in juveniles subjected to acidic pH with HWH was significantly higher than those subjected to LWH at the same pH ($P < 0.05$) (Fig. 1a). At the end of the exposure period (day 15), gill GST activity in pacus exposed to acidic pH was significantly higher than those exposed to circumneutral or alkaline water pH ($P < 0.05$) (Fig. 1a).

On day 1, kidney GST activity in pacus subjected to acidic pH with LWH was significantly lower than those subjected to acidic pH with HWH ($P < 0.05$) (Fig. 1b). On day 5, kidney GST activity in pacus exposed to alkaline pH was significantly lower than those exposed to other water pH levels (except for circumneutral pH with HWH) ($P < 0.05$) (Fig. 1b). At the end of the exposure period (day 15), kidney GST activity in fish subjected to acidic pH with HWH was significantly higher than those subjected to circumneutral pH ($P < 0.05$) (Fig. 1b).

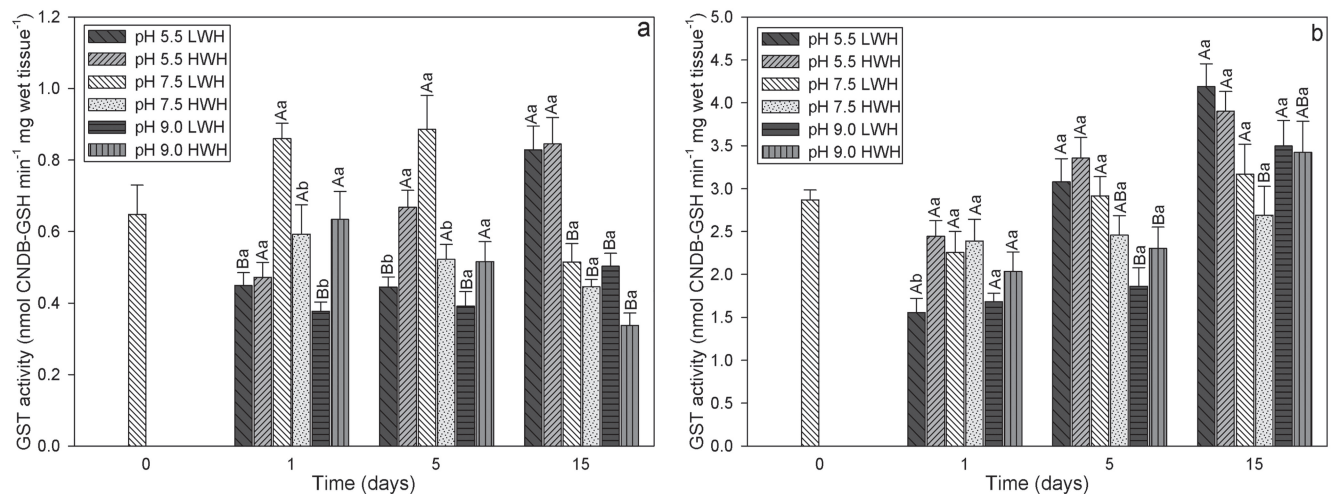


Fig. 1. Glutathione-S-transferase (GST) activity ($\text{nmol CNDB-GSH min}^{-1} \text{mg wet tissue}^{-1}$) in **a.** gills and **b.** kidney of pacu (*Piaractus mesopotamicus*) juveniles under different water hardness and pH at different times. LWH = low water hardness ($50 \text{ mg CaCO}_3 \text{ L}^{-1}$); HWH = high water hardness ($120 \text{ mg CaCO}_3 \text{ L}^{-1}$). Data are presented as the means \pm SEM ($n = 9$ fish treatment $^{-1}$). Different uppercase letters indicate statistically significant differences between pH at the same hardness ($P < 0.05$). Different lowercase letters indicate statistically differences between hardness at the same pH ($P < 0.05$).

Fish exposed to alkaline pH with HWH on day 1 showed significantly lower gill ACAP (higher relative area) compared to acidic pH with HWH ($P < 0.05$) (Fig. 2a). Fish exposed to acidic pH with LWH on day 5 showed significantly higher kidney ACAP (lower relative area) compared to those exposed to other water pH levels at the same hardness ($P < 0.05$) (Fig. 2b). No significant differences were registered among treatments for gill ACAP on days 5 and 15 or kidney ACAP on days 1 and 15 (Fig. 2 a and b).

On day 1, gill TBARS levels in pacus subjected to alkaline pH with LWH were significantly higher than in fish subjected to other treatments at the same water pH or hardness ($P < 0.05$) (Fig. 3a). On day 5, gill TBARS levels in pacu exposed to alkaline pH, with HWH, were significantly higher than in pacus exposed to other treatments at the same water pH or hardness ($P < 0.05$) (Fig. 3a). No significant

differences were observed among treatments for gill TBARS levels on day 15 (Fig. 3a).

Pacus exposed to alkaline pH with LWH on day 1 showed significantly higher kidney TBARS levels than those exposed to other water pH levels at the same hardness ($P < 0.05$) (Fig. 3b). On day 5, juveniles subjected to acidic or alkaline pH with LWH had significantly higher kidney TBARS levels than those subjected to circumneutral pH at the same hardness ($P < 0.05$) (Fig. 3b). In addition, at the same day (day 5), kidney TBARS levels in fish exposed to acidic pH with LWH were significantly higher than of those exposed to the same pH with HWH ($P < 0.05$) (Fig. 3b). On day 15, pacus exposed to alkaline pH with LWH showed significantly higher kidney TBARS levels than those exposed to circumneutral pH with LWH or alkaline pH with HWH ($P < 0.05$) (Fig. 3b).

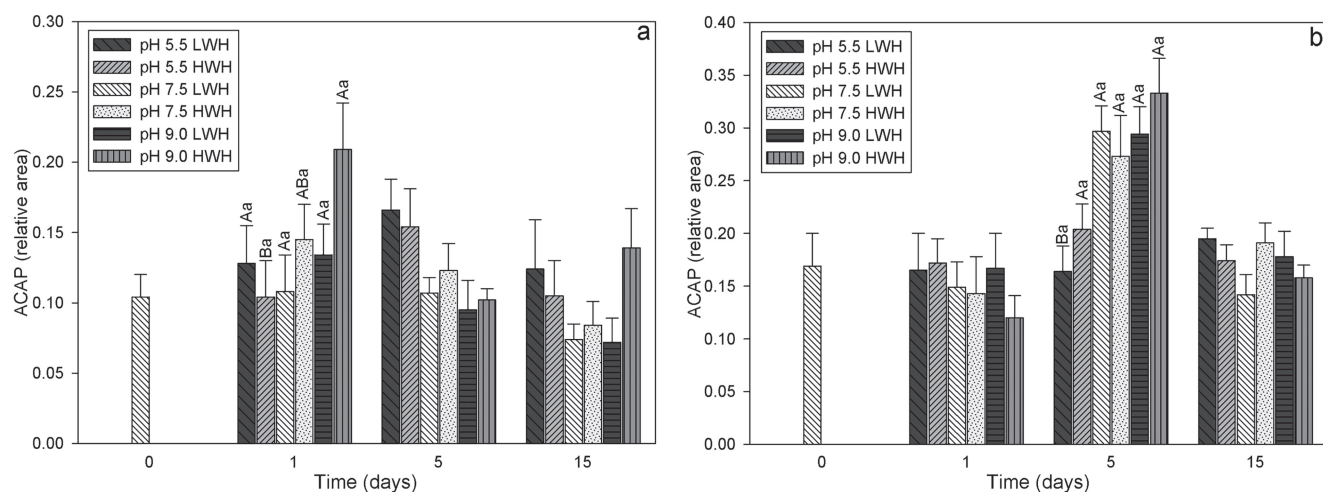


Fig. 2. Total antioxidant capacity against peroxy radicals (ACAP) (relative area) in **a.** gills and **b.** kidney of pacu (*Piaractus mesopotamicus*) juveniles under different water hardness and pH at different times. LWH = low water hardness ($50 \text{ mg CaCO}_3 \text{ L}^{-1}$); HWH = high water hardness ($120 \text{ mg CaCO}_3 \text{ L}^{-1}$). Data are presented as the means \pm SEM ($n = 9$ fish treatment $^{-1}$). Different uppercase letters indicate statistically differences between pH at the same hardness ($P < 0.05$).

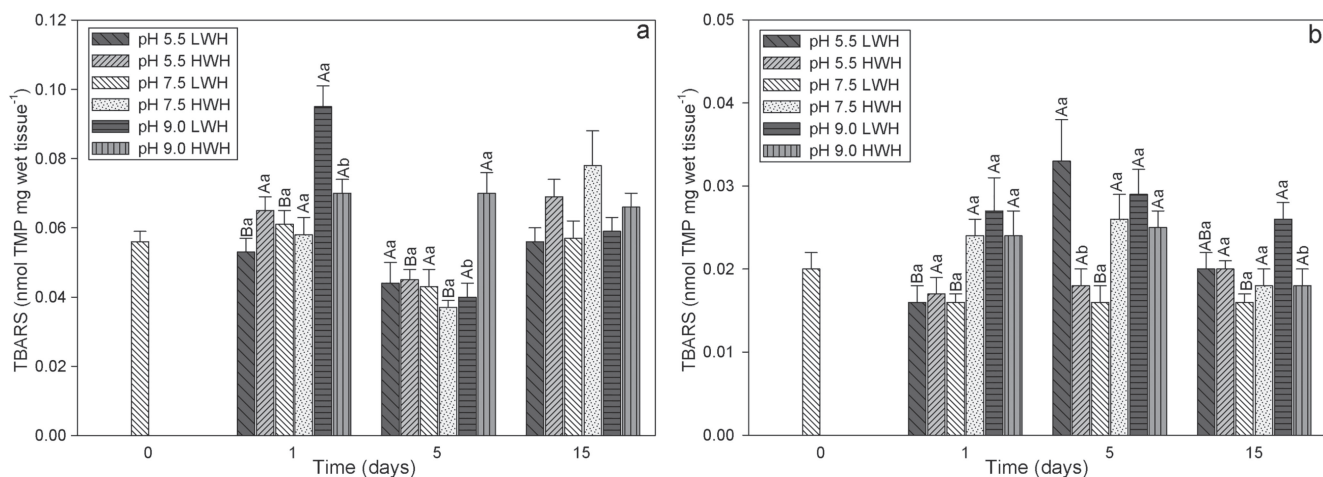


Fig. 3. Thiobarbituric acid reactive substances (TBARS) content ($\text{nmol TMP mg wet tissue}^{-1}$) in **a.** gills and **b.** kidney of pacu (*Piaractus mesopotamicus*) juveniles under different water hardness and pH at different times. LWH = low water hardness ($50 \text{ mg CaCO}_3 \text{ L}^{-1}$); HWH = high water hardness ($120 \text{ mg CaCO}_3 \text{ L}^{-1}$). Data are presented as the means \pm SEM ($n = 9$ fish treatment $^{-1}$). Different uppercase letters indicate statistically differences between pH at the same hardness ($P < 0.05$). Different lowercase letters indicate statistically differences between hardness at the same pH ($P < 0.05$).

Discussion

In the present study, pacus apparently showed tolerance to changes in water pH, because no fish exposed to the experimental pH (5.5, 7.5 or 9.0) died throughout the experiment. In addition, in the current study, both acidic and alkaline pH were harmful to juveniles, mainly with LWH, as shown by deleterious effects in relation to ATPase and antioxidant levels in fish kept under these conditions. Copatti *et al.* (2011a) stated the protective effect of HWH occurs because the high ion loss is reduced by increasing the water hardness in acidic or alkaline pH, which could cause an improvement in the osmoregulation and antioxidant

capacity of the fish in these water pH values. In this sense, our initial hypothesis was that an increase in water hardness would reduce ATPase activity and improve antioxidant capacity in pacu juveniles exposed to acidic or alkaline pH. This hypothesis was confirmed, because the ATPase levels and oxidative stress variables in pacus exposed to acidic (mainly) or alkaline pH under HWH were reduced or showed fewer changes compared to fish subjected to circumneutral pH. The effect of HWH on increased fish survival in acidic and alkaline pH has been reported in previous studies because their ion loss was reduced with HWH (Townsend, Baldisserotto, 2001; Parra, Baldisserotto, 2007; Copatti *et al.*, 2011a).

An increase in NKA and V-ATPase activities in the gills and kidney may be attributed to the specialised structure located in these tissues in fish. So, this increase in ATPase activities could then lead to physiological and ionoregulatory alterations (Copatti *et al.*, 2015). In the kidney of teleosts, ATPase provides energy for ion transport, because solute and water absorption usually occurs via the retention of Na⁺, driven by ATPases in the basolateral membrane (Evans, 1993); an increase in ATPases in the kidney can be the result of its pivotal role in both detoxification and osmoregulation (Atli, Canli, 2011). This is consistent with the increase in NKA and V-ATPase activities in the gills and kidney of pacu juveniles observed in the current study at different times during acidic or alkaline pH exposure. This has been verified mainly in LWH in comparison to HWH, because the greater ion loss would induce higher NKA and V-ATPase activities in an attempt to regulate ion levels. Thus, higher ATPase activities would also contribute to reduce ion loss. In addition, this mechanism of ion transport could be better elucidated through morphological analysis. Therefore, it is suggested that in future studies, morphological studies should be carried out to better understand the ion transport mechanisms with the ATPases activity.

The gills and kidney of fish tend to have normal functioning affected under exposition to very acidic or alkaline waters (Wood *et al.*, 1999; Bolner, Baldisserotto, 2007; Parra, Baldisserotto, 2007). Wilkie *et al.* (1999) showed that alkaline water causes transient decreases in the capacity of the ion transport system by directly acting on the gill's respective Cl⁻ and Na⁺ transport sites. It seems probable, therefore, that exposure to alkaline pH with LWH stimulated the proliferation of ionocytes or increased their activity in the gills and kidney in pacu, preventing the impaired active transport of ions through the epithelium of these organs.

The primary effects of acid water exposure on fish is increased rates of passive Na⁺ losses (Kwong *et al.*, 2014). Na⁺ uptake in freshwater fish is primarily coupled to H⁺ secretion through the actions of Na⁺/H⁺ exchanger (not evaluated in the current study) and V-ATPase (Evans *et al.*, 2005), so a reduction in water pH would reduce the gradient to drive Na⁺ influx. The increased activity of NKA and V-ATPase in gills and kidney could increase acid excretion, contributing to the necessary enhancement of HCO₃⁻ reabsorption (Perry *et al.*, 2006). In addition, in acidic waters, there is a reduction in NKA and V-ATPase activities in the gills and kidney, probably due to the generation of ionocyte lesions, which could increase ion loss (Wood, 2001; Evans, 2011).

The increased ion losses during acid exposure are thought to be largely associated with the disruption of paracellular tight junctions (Kwong, Perry, 2013), which apparently is caused by calcium displacement from these junctions (Kwong *et al.*, 2014). So, it would be expected that the increase in water hardness would minimize changes in osmoregulation on pacus exposed to acid waters, as demonstrated by the reduction in NKA and V-ATPase activities. Thus, decreased

NKA and V-ATPase activities could also occur due to the minor disturbance in ion regulation and the positive effects of HWH. In this context, HWH could reduce diffusive ion losses in silver catfish (Townsend, Baldisserotto, 2001).

The alterations of NKA activity, along with the increasing level of ROS causes functional disorders in osmoregulation as well as tissue damage (Atli, Canli, 2007). In the current study, we found that, in pacus, LPO and antioxidant defences may be increased or inhibited under acidic or alkaline pH depending on water hardness and the duration of exposure, and gills and kidney showed variability in antioxidant competence. The kidney demonstrated greater antioxidant capacity compared to the gills, since GST activity was higher in this tissue, although ACAP was slightly higher in the gills (lower relative area). TBARS levels, in turn, were higher in the gills, indicating more LPO end products compared to the kidney. Peroxidative damage to gills may result from oxidative deterioration of polyunsaturated fatty acids, thereby impacting the osmoregulatory functions of the gills (Evans, 1987) and, consequently, increasing the production of ROS and thereby increasing lipid damage.

Enhanced ROS formation during an environmental stress situation causes the activation of redox-sensitive transcription factors (Nrf2, for example) and ROS-mediated covalent modification of antioxidant proteins leading to enhanced antioxidant defences (Hermes-Lima *et al.*, 2015). In addition, previous evidence indicates that acidic or alkaline pH can trigger a pro-oxidant scenario in different fish organs (Mai *et al.*, 2010; Maqsood, Benjakul, 2011). The higher LPO (TBARS) and antioxidant defences (GST and ACAP) in the gills and kidney of pacu juveniles exposed to acidic or alkaline pH, mainly with LWH, observed in our study was expected and demonstrates that the function of these organs was affected. Under these conditions, fish were not able to neutralise ROS in these tissues. The capacity to neutralise ROS was greater in fish subjected to LWH at circumneutral pH. Despite the negative effects of acid and alkaline waters, our results demonstrate that oxidative stress variables are early indicators of acidic or alkaline pH-induced toxic effects and suggest that HWH presented positive effects, reducing LPO and increasing GST activity and ACAP (lower relative area). In this way, Copatti *et al.* (2019) verified that HWH improved biochemical and physiological responses in pacu under acidic or alkaline pH.

GST activity is involved in detoxification, and elevated GST activity demonstrates that an attempt to neutralise elevated levels of ROS has occurred (Monteiro *et al.*, 2009) and serves to re-establish the balance between pro-oxidants and antioxidants to alleviate ROS-induced oxidative damage (Wilhelm Filho *et al.*, 2005). In the present study, HWH increased GST activity in kidney on day 1 and gills on day 5 in pacus subjected to acidic and/or alkaline pH. Interestingly, in circumneutral pH, an elevation of water hardness reduced GST activity in the gills on these same days (1 and 5), which suggests that, in this tissue, HWH had no positive effect when pacus were exposed to circumneutral pH.

The ACAP assessment sheds light on the overall antioxidant status of the fish against peroxyl radicals (Maltez *et al.*, 2018), which was compromised in the kidney of pacus through alkaline pH exposure on day 5. In addition, a possible explanation for lower gills ACAP (higher relative area) in alkaline pH with HWH on day 1 was due to a compensatory effect, because an increase in GST activity and a reduction in TBARS levels were observed under these conditions.

The TBARS assay quantifies the end products of LPO, which is a very sensitive marker of oxidative damage in the gills and kidney. TBARS are also thought to be involved in triggering the up-regulation of antioxidant enzymes (Lushchak, Bagnyukova, 2006). Acidic or alkaline pH with LWH cause oxidative stress in pacu, because the elevated TBARS concentration observed in the gills and kidney indicates high levels of LPO. Concomitantly, the results of antioxidant enzymes and ATPases in the gills and kidney can be also interpreted as an indication of a more oxidative environment in cells, leading to protein oxidation in acidic or alkaline waters with LWH. In addition, after the increase in TBARS in the gills on day 1 in fish exposed to alkaline pH with LWH, the gills showed lower TBARS levels on day 5. This result may indicate a decrease in polyunsaturated fatty acid levels due to early oxidative degradation (Sun *et al.*, 2014). Finally, HWH reduced LPO in fish kidney subjected to acidic (day 5) or alkaline pH (day 15), according to the TBARS results.

Another important aspect that we observed in the present study was that alkaline pH presented lower antioxidant capacity and/or higher LPO than acidic pH. Similarly, in tambaqui (*Colossoma macropomum*), Nile tilapia (*Oreochromis niloticus*) and Amazon catfish (*Pseudoplatystoma reticulatum* x *Leiarius marmoratus*), alkaline pH present more deleterious effects than acidic pH (Aride *et al.*, 2007; Lemos *et al.*, 2018a,b). In addition, high pH levels (> 9.0) can harm fish by denaturing cellular membranes (Zahangir *et al.*, 2015). Nevertheless, synergism between exposure to acidic water and ROS can cause DNA damage (Phillips, Ward, 2001) as a result of increased cations (Jolly *et al.*, 2004). If cations are responsible for the changes in pH, they can react with biological molecules, and/or act as signalling molecules which will play a role in mediating extensive DNA damage (Leonard *et al.*, 2004). Our results suggest that the occurrence of LPO in acidic pH with LWH might be initiated by ROS.

In this sense, HWH could decrease cell permeability and potentially ameliorate the effects of high pH on passive ion loss (Yesaki, Iwama, 1992; Wilkie, Wood, 1996). For instance, calcium ions appear to be important in mediating white shrimp responses to acidic or alkaline pH stress, decreasing oxidative stress (Wang *et al.*, 2009). Saglam *et al.* (2014) showed that HWH has a protective role in Nile tilapia exposed to metals (Cd, Cu), and HWH was also found to trigger antioxidant enzyme responses. In the current study, we observed increased antioxidant defence capacity in pacus under acidic or alkaline pH with HWH,

which shows that enzymatic defence mechanisms are used for ROS scavenging. In addition, to our knowledge, this is the first study that evaluated oxidative stress in fish under different water pH at LWH and HWH.

The oxidative stress produced in tissues (*e.g.*, gills and kidney) are maintained within the physiological levels when the antioxidant defences are in a normal physiological state (Wilhelm Filho, 1996). Our results demonstrated that, even in acidic (mainly) or alkaline waters, oxidative stress in pacu gills and kidney is less intense after a longer exposure period (15 days), because, in acidic pH, fish were able to increase their antioxidant defences and, in alkaline pH (except to kidney TBARS at LWH), oxidative stress variables were stable independently of hardness on day 15. Maltez *et al.* (2018) suggests that, in some cases, a recovery period (10 days or more) is necessary to complete restoration of homeostasis after the end of the stressful conditions. Thus, we believed that pacu requires 15 days or more to acclimate to waters with acidic or alkaline pH and to restore the activity of osmoregulatory and oxidative stress enzymes in gills and kidney.

In conclusion, circumneutral pH is indicated to raise pacu juveniles because at this pH there is less oxidative stress and fewer variations in ATPase activity. In addition, an increase in NKA and V-ATPase activities in the gills and kidney in acidic or alkaline water with LWH could indicate important osmoregulatory changes for the adjustment of pacu juveniles in these water pH; HWH exposure reduced ATPase activity in both organs. In an integrated view with our observation that acidic or alkaline pH induced oxidative stress and antioxidant responses primarily under HWH, we can conclude that pacu exposed to acidic or alkaline pH are less affected by the deleterious effects with water hardness of 120 mg CaCO₃ L⁻¹.

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References

- Amado LL, Garcia ML, Ramos PB, Freitas RF, Zafalon B, Ferreira JL, Yunes JS, Monserrat JM. A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: Application to evaluate microcystins toxicity. *Sci Total Environ.* 2009; 407(6):2115–23. <https://doi.org/10.1016/j.scitotenv.2008.11.038>
- Aride PHR, Roubach R, Val AL. Tolerance response of tambaqui *Colossoma macropomum* (Cuvier) to water pH. *Aquac Res.* 2007; 38(6):588–94. <https://doi.org/10.1111/j.1365-2109.2007.01693.x>

- Atli G, Canli M. Essential metal (Cu, Zn) exposures alter the activity of ATPases in gill, kidney and muscle of tilapia *Oreochromis niloticus*. *Ecotoxicol.* 2011; 20(8):1861–69. <https://doi.org/10.1007/s10646-011-0724-z>
- Baldisserotto B. Water pH and hardness affect growth of freshwater teleosts. *R Bras Zootec.* 2011; 40(suppl. 1):138–44.
- Birnie-Gauvin K, Costantini D, Cooke SJ, Willmore WG. A comparative and evolutionary approach to oxidative stress in fish: a review. *Fish Fish.* 2017; 18(5):928–42. <https://doi.org/10.1111/faf.12215>
- Bolner KCS, Baldisserotto B. Water pH and urinary excretion in silver catfish *Rhamdia quelen*. *J Fish Biol.* 2007; 70(1):50–64. <https://doi.org/10.1111/j.1095-8649.2006.01253.x>
- Boyd CE, Tucker CS. Pond aquaculture water quality management. Norwell: Kluwer Academic Publishers; 1998.
- Boyd CE, Tucker CS, Somridhivej B. Alkalinity and hardness: critical but elusive concepts in aquaculture. *J World Aquacult Soc.* 2016; 47(1):6–41. <https://doi.org/10.1111/jwas.12241>
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72(1–2):248–54. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)
- Canario A, Flik G. Endocrinology of calcium homeostasis. *Gen Comp Endocrinol* 2007; 152 (2–3):242.
- Chasiotis H, Kolosov D, Bui P, Kelly SP. Tight junctions, tight junction proteins and paracellular permeability across the gill epithelium of fishes: a review. *Respir Physiol Neurobiol* 2012; 184(3):269–81. <https://doi.org/10.1016/j.resp.2012.05.020>
- Christensen AK, Hiroi J, Schultz ET, McCormick SD. Branchial ionocyte organization and ion-transport protein expression in juvenile alewives acclimated to freshwater or seawater. *J Exp Biol.* 2012; 215(4):642–52. <https://doi.org/10.1242/jeb.063057>
- Copatti CE, Baldisserotto B, Souza CF, Garcia LO. Protective effect of high hardness in pacu juveniles (*Piaractus mesopotamicus*) under acidic or alkaline pH: Biochemical and haematological variables. *Aquaculture.* 2019; 502(1):250–57. <https://doi.org/10.1016/j.aquaculture.2018.12.028>
- Copatti CE, Bolner KCS, Rosso FL, Loro VL, Baldisserotto B. Tolerance of piava juveniles to different ammonia concentrations. *Semina: Ciênc Agrár.* 2015; 36(6):3991–4002. <http://dx.doi.org/10.5433/1679-0359.2015v36n6p3991>
- Copatti CE, Garcia LO, Cunha MA, Baldisserotto B, Kochhann D. Interaction of water hardness and pH on growth of silver catfish, *Rhamdia quelen*, juveniles. *J World Aquacult Soc.* 2011a; 42(4):580–85. <https://doi.org/10.1111/j.1749-7345.2011.00501.x>
- Copatti CE, Garcia LO, Kochhann D, Cunha MA, Becker AG, Baldisserotto B. Low water hardness and pH affect growth and survival of silver catfish juveniles. *Ciênc Rur.* 2011b; 41(8):1482–87. <http://dx.doi.org/10.1590/S0103-84782011005000101>
- Da Rocha AM, De Freitas DP, Burns M, Vieira JP, De La Torre FR, Monserrat JM. Seasonal and organ variations in antioxidant capacity, detoxifying competence and oxidative damage in freshwater and estuarine fishes from Southern Brazil. *Comp Biochem Physiol C.* 2009; 150(4):512–20. <https://doi.org/10.1016/j.cbpc.2009.07.012>
- Do Carmo TLL, Azevedo VC, De Siqueira PR, Galvão TD, Dos Santos FA, Dos Reis Martinez CB, Appoloni CR, Fernandes MN. Reactive oxygen species and other biochemical and morphological biomarkers in the gills and kidneys of the Neotropical freshwater fish, *Prochilodus lineatus*, exposed to titanium dioxide (TiO₂) nanoparticles. *Environ Sci Pollut Res.* 2018; 25(23):22963–76. <https://doi.org/10.1007/s11356-018-2393-4>
- Duffy NM, Bui P, Bagherie-Lachidan M, Kelly SP. Epithelial remodeling and claudin mRNA abundance in the gill and kidney of puffer fish (*Tetraodon biocellatus*) acclimated to altered environmental ion levels. *J Comp Physiol B.* 2011; 181(2):219–38. <https://doi.org/10.1007/s00360-010-0517-3>
- Eaton AD, Clesceri LS, Rice EW, Greenberg AE. Standard methods for the examination of water and wastewater. 21st ed. Washington: APHA; 2005.
- Evans DH. The fish gill: site of action and model for toxic effects of environmental pollutants. *Environ Health Perspect.* 1987; 71(1):54–58. <https://doi.org/10.1289/ehp.877147>
- Evans DH. Osmotic and ionic regulation. In: Evans DH, editor. The physiology of fish. Boca Raton: CRC Press; 1993. p.315–41.
- Evans DH. Freshwater fish gill ion transport: August Krogh to morpholinos and microprobes. *Acta Physiol.* 2011; 202(3):349–59. <https://doi.org/10.1111/j.1748-1716.2010.02186.x>
- Evans DH, Piermarini PM, Choe KP. The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol Rev.* 2005; 85(1):97–177. <https://doi.org/10.1152/physrev.00050.2003>
- Gibbs A, Somero GN. Pressure adaptation of Na⁺/K⁺-ATPase in gills of marine teleosts. *J Exp Biol.* 1989; 143(1):475–92.
- Gonzalez RJ, McDonald DG. The relationship between oxygen uptake and ion loss in fish from diverse habitats. *J Exp Biol.* 1994; 190(1):95–108.
- Gonzalez RJ, Wood CM, Wilson RW, Patrick ML, Bergman HL, Narahara A, Val AL. Effects of water pH and calcium concentration on ion balance in fish of the rio Negro, Amazon. *Physiol Zool.* 1998; 71(1):15–22.
- Goss GG, Wood CM, Laurent P, Perry SF. Morphological responses of the rainbow trout (*Oncorhynchus mykiss*) gill to hyperoxia, base (NaHCO₃) and acid (HCl) infusions. *Fish Physiol Biochem.* 1994; 12(6):465–77. <https://doi.org/10.1007/BF00004449>
- Guerreiro PM, Fuentes J, Flik G, Rotllant J, Power DM, Canario AVM. Water calcium concentration modifies whole-body calcium uptake in sea bream larvae during short-term adaptation to altered salinities. *J Exp Biol* 2004; 207(4): 645–53. <https://doi.org/10.1242/jeb.00765>
- Habig WH, Jakoby WB. Assays for differentiation of glutathione S-transferases. *Methods Enzymol.* 1981; 77(1):398–405. [https://doi.org/10.1016/S0076-6879\(81\)77053-8](https://doi.org/10.1016/S0076-6879(81)77053-8)
- Hermes-Lima M, Moreira DC, Rivera-Ingraham GA, Giraud-Billoud M, Genaro-Mattos TC, Campos ÉG. Preparation for oxidative stress under hypoxia and metabolic depression: Revisiting the proposal two decades later. *Free Radical Biol Med.* 2015; 89(1):1122–43. <https://doi.org/10.1016/j.freeradbiomed.2015.07.156>

- Jolly AJ, Wild CP, Hardie LJ. Acid and bile salts induce DNA damage in human oesophageal cell lines. *Mutagenesis*. 2004; 19(4):319–24. <https://doi.org/10.1093/mutage/geh035>
- Jones DP. Redefining oxidative stress. *Antioxid. Redox Signal*. 2006; 8(9-10):1865–79. <https://doi.org/10.1089/ars.2006.8.1865>
- Kwong RWM, Kumai Y, Perry SF. The physiology of fish at low pH: The zebrafish as a model system. *J Exp Biol*. 2014; 217(5):651–62. <https://doi.org/10.1242/jeb.091603>
- Kwong RWM, Perry SF. Cortisol regulates epithelial permeability and sodium losses in zebrafish exposed to acidic water. *J Endocrinol*. 2013; 217(3):253–64. <https://doi.org/10.1530/JOE-12-0574>
- Lemos CHP, Ribeiro CVM, De Oliveira CPB, Couto RD, Copatti CE. Effects of interaction between pH and stocking density on the growth, haematological and biochemical responses of Nile tilapia juveniles. *Aquaculture*. 2018a; 495(1):62–67. <https://doi.org/10.1016/j.aquaculture.2018.05.037>
- Lemos CHP, Chung S, Ribeiro CVM, Copatti CE. Growth and biochemical variables in Amazon catfish (*Pseudoplatystoma reticulatum* ♀ x *Leiarius marmoratus* ♂) under different water pH. *An Acad Bras Ciênc*. 2018b; 90(4):3573–81. <http://dx.doi.org/10.1590/0001-3765201820180241>
- Leonard SS, Harris GK, Shi XL. Metal-induced oxidative stress and signal transduction. *Free Radic Biol Med*. 2004; 37(12):1921–42. <https://doi.org/10.1016/j.freeradbiomed.2004.09.010>
- Lushchak VI, Bagnyukova TV. Effects of different environmental oxygen levels on free radical processes in fish. *Comp Biochem Physiol*. 2006; 144(3):283–89. <https://doi.org/10.1016/j.cbpb.2006.02.014>
- Mai WJ, Yan JL, Wang L, Zheng Y, Xin Y, Wang WN. Acute acidic exposure induces p53-mediated oxidative stress and DNA damage in tilapia (*Oreochromis niloticus*) blood cells. *Aquat Toxicol*. 2010; 100(3):271–81. <https://doi.org/10.1016/j.aquatox.2010.07.025>
- Maltez LC, Barbas LAL, Nitz LF, Pellegrin L, Okamoto MH, Sampaio LA, Monserrat JM, Garcia LO. Oxidative stress and antioxidant responses in juvenile Brazilian flounder *Paralichthys orbignyanus* exposed to sublethal levels of nitrite. *Fish Physiol Biochem*. 2018; 44(5):1349–62. <https://doi.org/10.1007/s10695-018-0526-9>
- Marshall WS. Na⁺, Cl⁻, Ca²⁺ and Zn²⁺ transport by fish gills: retrospective review and prospective synthesis. *J Exp Zool*. 2002; 93(3):264–83. <https://doi.org/10.1002/jez.10127>
- Maqsood S, Benjakul S. Comparative studies on molecular changes and pro-oxidative activity of haemoglobin from different fish species as influenced by pH. *Food Chem*. 2011; 124(3):875–83. <https://doi.org/10.1016/j.foodchem.2010.07.011>
- Martínez-Álvarez RM, Morales AE, Sanz A. Antioxidant defenses in fish: Biotic and abiotic factors. *Rev Fish Biol Fisher*. 2005; 15(1):75–88. <https://doi.org/10.1007/s11160-005-7846-4>
- Monteiro DA, Rantin FT, Kalinin AL. The effects of selenium on oxidative stress biomarkers in the freshwater characid fish matrinxã, *Brycon cephalus* (Günther, 1869) exposed to organophosphate insecticide Folisuper 600 BR® (methyl parathion). *Comp Biochem Physiol C*. 2009; 149(1):40–49. <https://doi.org/10.1016/j.cbpc.2008.06.012>
- Oakes KD, Van Der Kraak GJ. Utility of the TBARS assay in detecting oxidative stress in white sucker (*Catostomus commersoni*) populations exposed to pulp mill effluent. *Aquat Toxicol*. 2003; 63(4):447–63. [https://doi.org/10.1016/S0166-445X\(02\)00204-7](https://doi.org/10.1016/S0166-445X(02)00204-7)
- Parra JEG, Baldisserotto B. Effect of water pH and hardness on survival and growth of freshwater teleosts. In: Baldisserotto B, Mancera JM, Kapoor BG, editors. *Fish Osmoregulation*. Enfield: Science Publishers; 2007. p.135–50.
- Perry SF, Furimsky M, Bayaa M, Georgalis T, Shahsavariani A, Nickerson JG, Moon TW. Integrated responses of Na⁺/HCO₃⁻ cotransporters and V-type H⁺-ATPases in the fish gill and kidney during respiratory acidosis. *Biochim Biophys Acta*. 2003; 1618(2):175–84. <https://doi.org/10.1016/j.bbamem.2003.09.015>
- Perry SF, Gilmour KM. Acid-base balance and CO₂ excretion in fish: Unanswered questions and emerging models. *Respir Physiol Neurobiol*. 2006; 154(1–2):199–215. <https://doi.org/10.1016/j.resp.2006.04.010>
- Phillips RM, Ward TH. Influence of extracellular pH on the cytotoxicity and DNA damage of a series of indolequinone compounds. *Anticancer Res*. 2001; 21(3B):1795–801.
- Rosas-Rodríguez JA, Valenzuela-Soto EM. Enzymes involved in osmolyte synthesis: How does oxidative stress affect osmoregulation in renal cells? *Life Sciences*. 2010; 87(17–18):515–20. <https://doi.org/10.1016/j.lfs.2010.08.003>
- Saglam D, Atli G, Dogan Z, Baysoy E, Gurler C, Eroglu A, Canli M. Response of the antioxidant system of freshwater fish (*Oreochromis niloticus*) exposed to metals (Cd, Cu) in differing hardness. *Turk J Fish Aquat Sci*. 2014; 14(1):43–52. https://doi.org/10.4194/1303-2712-v14_1_06
- Stoliar OB, Lushchak VI. Environmental pollution and oxidative stress in fish. In: Lushchak VI, editor. *Oxidative stress-environmental induction and dietary antioxidants* InTech [Electronic version]; 2012. <https://doi.org/10.5772/38094>
- Stringhetta GR, Barbas LAL, Maltez LC, Sampaio LA, Monserrat JM, Garcia LO. Oxidative stress responses of juvenile tambaqui *Colossoma macropomum* after short-term anesthesia with benzocaine and MS-222. *An Acad Bras Ciênc*. 2017; 89(3):2209–18. <https://doi.org/10.1590/0001-3765201720160823>
- Sun H, Wang W, Li J, Yang Z. Growth, oxidative stress responses, and gene transcription of juvenile bighead carp (*Hypophthalmichthys nobilis*) under chronic-term exposure of ammonia. *Environ Toxicol Chem*. 2014; 33(8):1726–31. <https://doi.org/10.1002/etc.2613>
- Toni C, Becker AG, Simões LN, Pinheiro CG, De Lima Silva L, Heinzmann BM, Caron BO, Baldisserotto B. Fish anesthesia: Effects of the essential oils of *Hesperozygis ringens* and *Lippia alba* on the biochemistry and physiology of silver catfish (*Rhamdia quelen*). *Fish Physiol Biochem*. 2014; 40(3):701–14. <https://doi.org/10.1007/s10695-013-9877-4>
- Townsend CR, Baldisserotto B. Survival of silver catfish fingerlings exposed to acute changes of water pH and hardness. *Aquacult. Int*. 2001; 9(5):413–19. <https://doi.org/10.1023/A:1020592226860>

- Valladão GMR, Gallani SU, Pilarski F. South American fish for continental aquaculture. *Rev Aquacult.* 2016; 10(2):351–69. <https://doi.org/10.1111/raq.12164>
- Wang WN, Zhou J, Wang P, Tian TT, Zheng Y, Liu Y, Mai WJ, Wang AL. Oxidative stress, DNA damage and antioxidant enzyme gene expression in the Pacific white shrimp, *Litopenaeus vannamei* when exposed to acute pH stress. *Comp Biochem Physiol C.* 2009; 150(4):428–35. <https://doi.org/10.1016/j.cbpc.2009.06.010>
- Wilhelm Filho DW. Fish antioxidant defenses – a comparative approach. *Braz J Med Biol Res.* 1996; 29(12):1735–42.
- Wilhelm Filho D, Torres MA, Zaniboni-Filho E, Pedrosa RC. Effect of different oxygen tensions on weight gain, feed conversion, and antioxidant status in piapara, *Leporinus elongatus* (Valenciennes, 1847). *Aquaculture.* 2005; 244(1–4):349–57. <https://doi.org/10.1016/j.aquaculture.2004.11.024>
- Wilkie MP, Wood CM. The adaptations of fish to extremely alkaline environments. *Comp Biochem Physiol B.* 1996; 113(4):665–73. [https://doi.org/10.1016/0305-0491\(95\)02092-6](https://doi.org/10.1016/0305-0491(95)02092-6)
- Wilkie MP, Laurent P, Wood CM. The physiological basis for altered Na⁺ and Cl⁻ movements across the gills of rainbow trout (*Oncorhynchus mykiss*) in alkaline (pH = 9.5) water. *Physiol Biochem Zool.* 1999; 72(3):360–68. <https://doi.org/10.1086/316670>
- Wilson JM, Laurent P. Fish Gill morphology: inside out. *J Exp Zool.* 2002; 293(3):192–213. <https://doi.org/10.1002/jez.10124>
- Whittamore JM. Osmoregulation and epithelial water transport: lessons from the intestine of marine teleost fish. *J Comp Physiol B.* 2012; 182(1):1–39. <https://doi.org/10.1007/s00360-011-0601-3>
- Wood CM. Toxic responses of the gill. In: Schlenk D, Benson WH, editors. *Target organ toxicity in marine and freshwater teleosts.* London: Taylor & Francis; 2001. p.1–89.
- Wood CM, Milligan CL, Walsh PJ. Renal responses of trout to chronic respiratory and metabolic acidoses and metabolic alkalosis. *Am J Physiol.* 1999; 277(2):R482–92. <https://doi.org/10.1152/ajpregu.1999.277.2.R482>
- Yesaki TY, Iwama GK. Survival, acid-base regulation, ion regulation, and ammonia excretion in rainbow trout in highly alkaline hard water. *Physiol Zool.* 1992; 65(4):763–87. <https://doi.org/10.1086/physzool.65.4.30158538>
- Zahangir MM, Haque F, Mostakim GM, Islam MS. Secondary stress responses of zebrafish to different pH: Evaluation in a seasonal manner. *Aquacult Rep.* 2015; 2(1):91–96. <https://doi.org/10.1016/j.aqrep.2015.08.008>



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