Are Amazonian fish more sensitive to ammonia? Toxicity of ammonia to eleven native species

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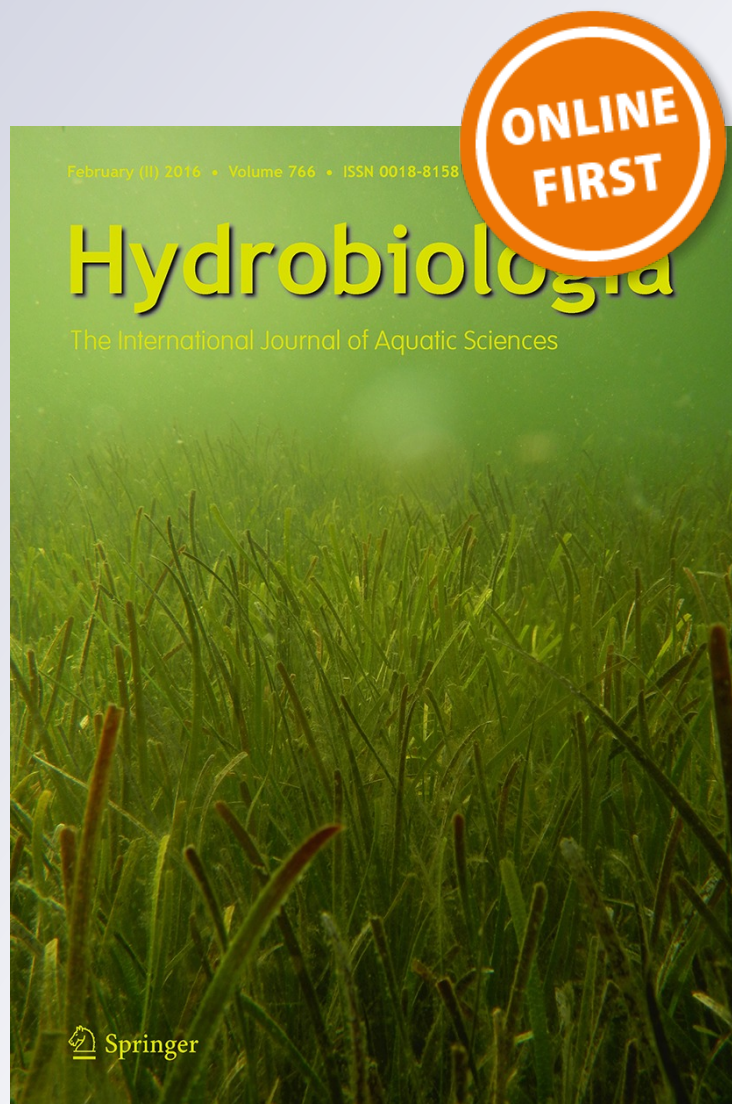**Luciana R. Souza-Bastos · Adalberto Luis Val · Chris M. Wood**

Abstract Little is known about the tolerance of Amazonian fish to ammonia. However, elevated ammonia of anthropogenic origin may now occur. As Amazonian fish evolved in waters which are generally acidic (i.e., low NH_3), we hypothesized that they would be more sensitive to ammonia than other freshwater fish. The acute (96-h) toxicity of NH_4Cl was tested in native ion-poor soft water (pH 7.0, ~ 28 °C) using semi-static tests with 11 species. Species sensitivity distributions (SSDs) for $LC50_{96\ h}$ and $LC10_{96\ h}$ and calculations of the hazardous concentrations to the most sensitive 5% (HC5 values) were tabulated. Values of $LC50_{96\ h}$/$LC10_{96\ h}$ (in mM total ammonia) ranged from 2.24/0.78 for *Paracheirodon axelrodi* (most sensitive) to 19.53/16.07 for *Corydoras schwartzi* (most tolerant). These results confirm our hypothesis that Amazonian fish are more sensitive to ammonia than other freshwater species. High levels of ammonia may be associated with hypoxia, especially during dry periods. Simultaneous hypoxia (15–20% saturation) exacerbated ammonia toxicity in the most sensitive species (*P. axelrodi*), but not in *Astronotus ocellatus* or *Corydoras schwartzi*, a facultative air-breather where prevention of air access doubled ammonia toxicity. The present data are useful in generating regulatory guidelines in Amazonian waters and indicate that further studies incorporating hypoxia and air access/denial are needed.

Keywords Air-breathing · Amazonian fish · Ammonia · HC5 · Hypoxia · LC50 · SSD

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Introduction

Ammonia exists in solution as both a gas (NH_3) and an ion (NH_4^+), which are in physico-chemical equilibrium with a pK of about 9–10, depending on solution chemistry and temperature. As the pH values typical of most natural waters and animal body fluids are well below the pK, NH_4^+ greatly predominates over NH_3 in most circumstances. Both are toxic, but the NH_3 form is generally considered to be more dangerous. In this paper, the term ammonia is used to refer to the total of $NH_3 + NH_4^+$, and these chemical symbols are used to refer to the two constituents separately. Ammonia is

almost universally found in the aquatic environment, derived from the metabolic processes of animals and plants, the decomposition of organic debris and organisms, the degradation of fertilizers, industrial emissions, sewage treatment plant effluents, and also volcanic activity (reviewed in Wilkie, 1997; Ip et al., 2001; Randall & Tsui, 2002; Wilkie, 2002; Camargo & Alonso, 2006). Keeping water ammonia concentrations at safe levels is very important to the health of aquatic organisms (Ip et al., 2001; Camargo & Alonso, 2006; Martinez et al., 2006).

In teleost fish, ammonia is the main waste product of nitrogen metabolism which is excreted, derived from the degradation of proteins and nucleic acids (reviewed in Wilkie, 1997; Ip et al., 2001; Wilkie, 2002; Chew et al., 2005; Randall & Ip, 2006). In general, ammonia is considered less costly to produce metabolically and easier to excrete than urea, though potentially more toxic. Approximately 80–90% of the ammonia produced is promptly excreted via the gills to the water under normal circumstances and thus does not become toxic for the organism itself (Wilkie, 1997; Ip et al., 2001; Randall & Tsui, 2002; Wilkie, 2002; Chew et al., 2005; Martinez et al., 2006; Randall & Ip, 2006).

However, the accumulation of ammonia in water can result in the buildup of ammonia in the blood and tissues of fish, resulting in lethal or sublethal toxicity (Tomasso et al., 1980; Wilkie, 1997; Ip et al., 2001; Randall & Tsui, 2002; Chew et al., 2005; Camargo & Alonso, 2006; Martinez et al., 2006; Randall & Ip, 2006; Wee et al., 2007; Miron et al., 2008; Dinesh et al., 2013). Fish exposed to increased ammonia concentrations in water have difficulty in eliminating this metabolite from the body, due to decrease in the partial pressure gradient (for NH_3) and/or electrochemical gradient (for NH_4^+) between the blood and the water (Wilkie, 1997; Ip et al., 2001; Wilkie, 2002; Chew et al., 2005; Camargo & Alonso, 2006; Piedras et al., 2006; Randall & Ip, 2006; Wee et al., 2007; Dinesh et al., 2013). These effects may be increased if there is a co-occurrence of other stress factors such as unfavorable pH, temperature, oxygen, etc. Stressed fish may suffer more drastic effects when exposed to ammonia (Wajsbrot et al., 1991; Ip et al., 2001; Randall & Tsui, 2002; Camargo & Alonso, 2006; Miron et al., 2008).

Toxic internal levels are harmful to the health, growth, and survival of fish (Wilkie, 1997; Ip et al.,

2001; Randall & Tsui, 2002; Chew et al., 2005; Martinez et al., 2006; Wee et al., 2007; Miron et al., 2008; Dinesh et al., 2013). The effects of this internal buildup of ammonia have been widely studied (Tomasso et al., 1980; Wilkie, 1997; Ip et al., 2001; Randall & Tsui, 2002; Wilkie, 2002; Chew et al., 2005; Camargo & Alonso, 2006; Martinez et al., 2006; Randall & Ip, 2006; Dinesh et al., 2013), but the number of species studied has been somewhat limited.

The Amazon River Basin covers the largest remaining tropical forest and has the highest diversity of freshwater fish species of any region of the world (Val & Almeida-Val, 1995; Lévêque et al., 2008; Langeani et al., 2009). As human settlement increases, ammonia discharges of anthropogenic origin are also increasing because of inputs of raw sewage (e.g., Perz, 2000; Couceiro et al., 2007). The natural geology of the region is characterized by soils which are generally very low in alkalinity, resulting in waters which are similarly low in alkalinity, and therefore in pH (Furch, 1984; Val & Almeida-Val, 1995; Cunha & Pascoaloto, 2009; Duncan & Fernandes, 2010; Bringel & Pascoaloto, 2012; Brito et al., 2014). Thus, Amazonian species evolved in environments where the concentration of the more toxic form of ammonia (NH_3) would routinely be very low. We hypothesized that in consequence, they would be more sensitive to ammonia than freshwater fish in other parts of the world. However, studies on the ability of Amazonian fish to tolerate high concentrations of environmental ammonia are rare (e.g., Cavero et al., 2004; Oliveira et al., 2008). Thus, the primary objective of this study was to measure the 96-h toxicity of ammonia to 11 species of Amazonian fish under standardized conditions. Tests were performed at pH 7.0 to allow direct comparison with the extensive dataset recently compiled by the U.S. EPA (2013). The Amazonian species were selected because of their commercial importance for fisheries, aquaculture, and the ornamental fish trade. Species sensitivity distribution plots (SSDs) for $LC50_{96 h}$ and $LC10_{96 h}$ values of potential use for regulatory purposes were generated based on the results for these 11 species.

Severe hypoxia is a common environmental stressor in the Amazon region, on both a seasonal and diurnal basis (Val & Almeida-Val, 1995). This is especially true during periods when water levels are low and ammonia concentrations may be elevated because fish are concentrated at high density in

"varzea" lakes. Therefore, three species differing widely in ammonia tolerance were retested in the presence of severe hypoxia (15–20% air saturation). Additionally, one species, which is a facultative air-breather, was retested with and without access to air to evaluate whether air-breathing is a potential mechanism to escape ammonia toxicity.

Materials and methods

Sources and maintenance of experimental animals

Eleven species of Amazonian fish, belonging to 4 different orders and 7 different families, were selected for use in this study because these animals are of high commercial and ornamental interest. Further information on these is shown in Table 1. These species were obtained from cultivation farms located near the city of Manaus (05°50′11.0″S; 61°16′54.3″W and 02°44′38.1″S; 59°28′38.6″W) and/or from the company Prestige Aquarium Ltda (Manaus, Brazil) between the months of August 2014 and January 2015.

The fish were transported in water saturated with O_2 to the Laboratory of Ecophysiology and Molecular Evolution (LEEM) at the Brazilian National Amazon Research Institute (INPA). In the laboratory, the animals were acclimated in groups of up to 50–300 fish for 5–7 days in 250–550 l tanks (depending on the size of the animals) under natural photoperiod with constant aeration. The same water, from an artesian well on the INPA campus, was used for acclimation and experimentation. This has physical and chemical characteristics typical of the very soft, ion-poor waters of the Amazon region (Cunha & Pascoaloto, 2009; Duncan & Fernandes, 2010; Bringel & Pascoaloto, 2012; De Boeck et al., 2013). This water was stored as a common pool of approximately 1000 l for all experimentation, with the pH adjusted to 7.0 by addition of NaOH (0.1 M), and its chemistry was measured (see Table 2). Dissolved oxygen, pH, temperature, and conductivity of INPA's water were measured by different meters, respectively (Yellow Springs Instruments—YSI®, digital model 55, model 60 and model 85, Yellow Springs, OH, USA). Ionic concentrations (Na^+, K^+, Ca^{2+}, and Mg^{2+}) were determined by atomic absorption spectrophotometry in flame mode (AAnalyst 800, Perkin Elmer®, Singapore). Cl^- concentration was determined by the colorimetric method of Zall et al., (1956), with absorbance read at 480 nm. Dissolved organic carbon (DOC) was read on a total carbon analyzer (Apollo 9000 combustion TOC analyzer, Teledine Tekmar) using an injection volume of 5 ml. For this, water samples were pre-filtered (Hydrophilic PVDF Millipore Millex—HV 0.45 μm). The standard curve was prepared with dilute solution of potassium hydrogen phthalate (KHP). Alkalinity and hardness were determined according to protocols described by Adad (1982).

The animals were fed every morning to satiation with commercial feed (Nutripiscis-Presence®, 36%

Table 1 Orders, families, species, common names (from http://www.fishbase.org) and biometric data of the fish used in this study

Order	Family	Species	Common name	Weight (g)	Length (cm)
Characiformes	Bryconidae	*Brycon amazonicus*	Matrinchã	0.92 ± 0.05	4.59 ± 0.08
	Characidae	*Hemigrammus rhodostomus*	Rodóstomo	0.43 ± 0.01	3.65 ± 0.04
		Hyphessobrycon socolofi	Rosa céu	0.54 ± 0.009	3.69 ± 0.03
		Paracheirodon axelrodi	Cardinal tetra	0.13 ± 0.005	2.43 ± 0.03
		Paracheirodon simulans	Neon tetra	0.06 ± 0.003	1.73 ± 0.03
	Gasteropelecidae	*Carnegiella strigata*	Borboleta	0.35 ± 0.02	3.19 ± 0.05
	Serrasalmidae	*Colossoma macropomum*	Tambaqui	3.40 ± 0.16	6.08 ± 0.11
Osteoglossiformes	Arapaimidae	*Arapaima gigas*	Pirarucu	13.73 ± 0.28	13.78 ± 0.08
Perciformes	Cichlidae	*Apistogramma agassizii*	Agassízi	0.5 ± 0.01	2.84 ± 0.04
		Astronotus ocellatus	Oscar	9.16 ± 0.16	8.48 ± 0.04
Siluriformes	Callichthyidae	*Corydoras schwartzi*	Coridora	1.94 ± 0.03	4.68 ± 0.02

Table 2 Chemical composition of the INPA well water used for acclimation and experiments

Chemical parameters	Concentration
Ions (µmol/l)	
Na^+	37
Cl^-	24
K^+	8
Ca^{2+}	8
Mg^{2+}	3
pH	7.03
O_2 (mg/l)	7.13
DOC (mgC/l)	1.09
Alkalinity (mgCaCO$_3$/l)	2.0
Hardness (mgCaCO$_3$/l)	1.1
Conductivity (µS/cm)	17.92
Temperature (°C)	28.3

DOC dissolved organic carbon

protein; SP Rações, São Paulo, Brazil). After 2–3 h, the tanks were cleaned and 80% of the water was replaced. Water ammonia concentrations during acclimation never exceeded 0.07 mM. There were no mortalities during the acclimation period. The food supply was suspended 48 h before the start of the experiments.

Experimental design

Lethal concentration (LC) tests

Each species was subjected to semi-static tests (80% replacement every 24 h), to determine the lethal concentrations of ammonia over 96 h. The assays were performed in tanks with volumes ranging between 1 and 15 l (depending on species), but always maintaining the ratio of 100 ml of water for every 1 g of fish, based on our previous experience with some of these same species (Wood et al., 2007, 2009, 2014; Robertson et al., 2015; C. M. Wood, unpubl. results). Ten individuals of each species were placed in each of eight tanks, corresponding to eight experimental conditions which were control (only freshwater, without ammonia) and nominally 2.5, 5, 7.5, 10, 15, 20, and 40 mM of total ammonia ($NH_3 + NH_4^+$). Specifically for *Paracheirodon axelrodi*, in addition to the experimental concentrations already mentioned,

three more were tested: 1, 1.5, and 2 mM of total ammonia. Furthermore, for *Corydoras schwartzi*, which is a facultatively air-breathing species (Graham, 1999; Nelson, 2014), the experiments were performed twice, with and without placing a net in the water column approximately 3 cm below the water surface, which prevented the animals from reaching the surface and thereby stopped them from air-breathing.

To prepare the concentrations of each of the experimental conditions, a stock solution of ammonium chloride (NH_4Cl, 1 M) was prepared and diluted in each of the tanks to obtain the required concentrations. Throughout the experimental period aquaria were closed, at ambient temperature (26–30 °C), natural photoperiod (approximately 12 h light: 12 h dark), and constant aeration, which maintained O_2 levels at greater than 80% air saturation. The pH of the water was kept at pH 6.9–7.1, adjusted by the addition of HCl (0.1 M) or NaOH (0.1 M), as required.

The mortality of the animals was monitored every hour during the first 12 h of experiment. On subsequent days, mortality was checked three times daily, and at the time of 80% water replacement. Water samples were collected before and after the partial exchange and used to determine the real concentrations of total ammonia in each experimental condition. In addition to mortality, dissolved oxygen (Yellow Springs Instruments—YSI®, digital model55, Yellow Springs, OH, USA), pH and temperature (Yellow Springs Instruments—YSI®, digital model60), and total ammonia were monitored three times daily, with 6-h intervals between the individual measurements (Online resource 1). Measurements of the total ammonia concentrations ($NH_3 + NH_4^+$) were performed according to the colorimetric method of Verdouw et al. (1978). The unionized ammonia fraction (NH_3) was calculated using the formula previously described by Ip et al. (2001), which uses the dissociation coefficient (Keq) determined by Emerson et al. (1975).

Lethal concentration (LC) tests in combination with hypoxia

In the second series, three of the species were retested for ammonia tolerance in the simultaneous presence of hypoxia (15–20% air saturation). The species were chosen based on results of the first series of tests: *C.*

schwartzi, which is a facultative air-breather, was chosen as the most tolerant species, *Astronotus ocellatus* was chosen because it was the second most tolerant species but lacking in air-breathing capability, and *P. axelrodi* was chosen as the least tolerant species. The experimental protocol followed the same pattern as the above-described experiment. Acute hypoxia was induced by continuous injection of N_2 and air mixtures into the water, so that the O_2 levels stayed between 15 and 20% saturation. The mortality and abiotic parameters such as dissolved oxygen, pH, temperature, and concentration of total ammonia were determined as previously described and listed in Online resource 2. Specifically for *Corydoras schwartzi,* this experiment was performed with the use of a net in the water column, as previously described.

All experiments in this study were approved by the Ethics Committee on Animal Experiments of INPA under the registration number 047/2012.

Statistical analyses

Toxicity data are presented as means with 95% confidence limits (CL), whereas all other values are presented as mean ± SEM. The LC50$_{96 h}$ values (concentration which is lethal to 50% of the population in 96 h) and the LC10$_{96 h}$ values (concentration which is lethal to 10% of the population in 96 h), along with their 95% confidence intervals, were calculated by Probit analysis (Finney, 1978). Measured rather than nominal concentrations were used in all calculations. In those few cases where mortality occurred in the controls (see "Results" section), the experimental data were corrected for control mortality as described by Sprague (1969). Correlation analyses were performed to assess various potential interactions (software SigmaStat 3.5®). The species sensitivity distributions (SSDs) and hazardous concentration based on toxicity to the most sensitive 5% of the species (HC5), along with their respective 95% confidence intervals, were calculated based on the LC50 or LC10 total ammonia results, using the Burriloz 2.0 program (CSIRO v. I.O.14; Perth, Australia). Where comparisons with data from other studies were made, the pH normalization calculator supplied by the U.S. EPA (2013) as

part of its most recent ammonia criterion document was used to normalize the latter data to the test pH (7.0) of the present study. The theoretical rationale behind this calculation is described in U.S. EPA (1999).

Results

Lethal concentration tests

As expected, mortality increased with the concentration of ammonia exposure in all species, and was generally preceded by hyperactivity, changes in the pattern of swimming, and overturn, especially in the highest concentrations tested (10–40 mM).

For most species, the highest rates of mortality occurred within the first 12 or 24 h of experiment, especially in test concentrations between 15 and 40 mM (see Online resource1).

Only *Carnegiella strigata*, *Hemigrammus rodhostomus*, and *Paracheirodon simulans* exhibited mortality in the control condition, which did not exceed 30%, and experimental data were appropriately corrected (see "Methods" section).

The results of toxicity tests for total ammonia (LC50$_{96 h}$) and LC10$_{96 h}$ are shown in Table 3 grouped according to phylogeny. Key toxicant parameters during the tests are tabulated in Online resource 2. In Fig. 1A and B, the LC50$_{96 h}$ values are ordered in terms of rank sensitivity from most sensitive (bottom) to most tolerant (top). LC50$_{96 h}$ values (and their respective 95% confidence intervals) ranged from 2.24 mM (2.11–2.35) as total ammonia in the most sensitive species (*P. axelrodi*) to 19.53 mM (17.61–21.67) in the most tolerant species (*C. schwartzi*). Corresponding values in terms of NH_3 toxicity were 0.019 (0.018–0.021) mM to 0.146 (0.131–0.161) mM. Note that the species sensitivity order would change slightly (Fig. 1B vs. A) when the data are presented as NH_3 concentrations because of minor variations in pH and temperature among the various trials.

Across species, correlation analysis revealed no significant effect of body mass on LC values ($P = 0.718$; $r = 0.123$; data not shown). There were no obvious phylogenetic patterns in the data.

Table 3 Total ammonia ($NH_3 + NH_4^+$) and unionized ammonia (NH_3) concentrations lethal to 50% ($LC50_{96h}$) and 10% ($LC10_{96h}$) of eleven species of Amazonian fish in 96-h tests (based on measured values) together with their respective 95% CL (in brackets)

Order	Family	Specie	Common name	Lethal concentrations ($LC_{50-96\ h}$) mM		Lethal concentrations ($LC_{10-96\ h}$) mM	
				Total ammonia ($NH_3 + NH_4^+$)	Unionized ammonia (NH_3)	Total ammonia ($NH_3 + NH_4^+$)	Unionized ammonia (NH_3)
Characiformes	Bryconidae	*Brycon amazonicus*	Matrinchã	6.00 (4.71–7.65)	0.049 (0.038–0.062)	2.92 (1.87–4.56)	0.024 (0.015–0.037)
	Characidae	*Hemigrammus rhodostomus*	Rodóstomo	6.00 (5.09–7.06)	0.039 (0.032–0.045)	4.18 (3.11–5.61)	0.026 (0.020–0.035)
		Hyphessobrycon socolofi	Rosa céu	4.63 (3.64–5.87)	0.036 (0.028–0.046)	3.83 (1.92–7.64)	0.030 (0.015–0.060)
		Paracheirodon axelrodi	Cardinal tetra	2.24 (2.11–2.35)	0.019 (0.018–0.021)	0.78 (n.d–1.75)	0.001 (n.d–0.0015)
		Paracheirodon simulans	Neon tetra	8.52 (6.79–10.68)	0.067 (0.053–0.083)	4.04 (2.68–6.08)	0.031 (0.021–0.047)
	Gasteropelecidae	*Carnegiella strigata*	Borboleta	3.16 (2.19–4.54)	0.022 (0.015–0.031)	1.44 (0.71–2.91)	0.010 (0.005–0.020)
	Serrasalmidae	*Colossoma macropomum*	Tambaqui	7.84 (6.79–9.13)	0.064 (0.055–0.075)	5.46 (4.36–7.00)	0.045 (0.036–0.057)
Osteoglossiformes	Arapaimidae	*Arapaima gigas*	Pirarucu	4.88 (3.71–6.41)	0.041 (0.031–0.054)	2.28 (1.37–3.80)	0.019 (0.011–0.031)
Perciformes	Cichlidae	*Apistogramma agassizii*	Agassízi	7.87 (6.07–10.20)	0.050 (0.039–0.065)	3.22 (1.96–5.27)	0.021 (0.013–0.034)
		Astronotus ocellatus	Oscar	12.51 (10.93–14.31)	0.103 (0.090–0.118)	9.18 (7.53–11.21)	0.076 (0.062–0.092)
Siluriformes	Callichthyidae	*Corydoras schwartzi* (without net)	Coridora	19.53 (17.61–21.67)	0.146 (0.131–0.161)	16.07 (14.24–18.12)	0.120 (0.106–0.135)
		Corydoras schwartzi (with net)	Coridora	10.23 (8.45–12.37)	0.068 (0.056–0.082)	5.69 (4.12–7.84)	0.038 (0.027–0.052)

Common names from http://www.fishbase.org

The values in parenthesis correspond to 95% confidence interval, and "n.d" indicates that the value could not be determined
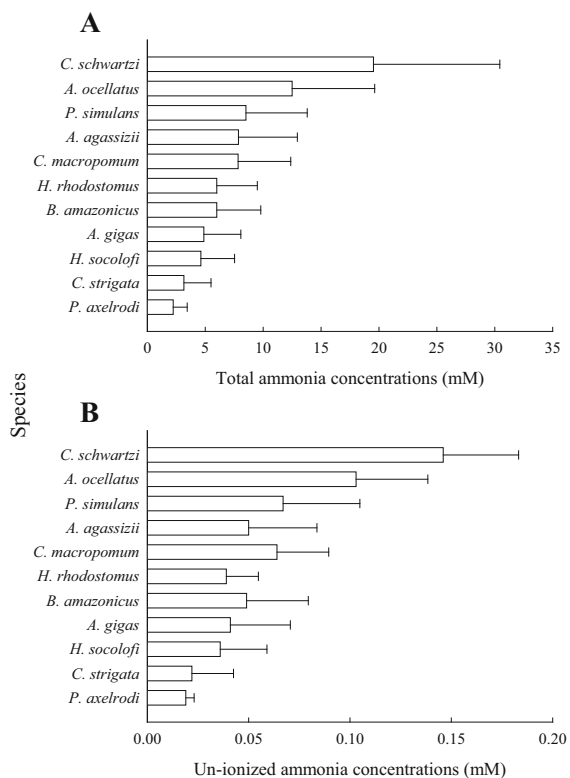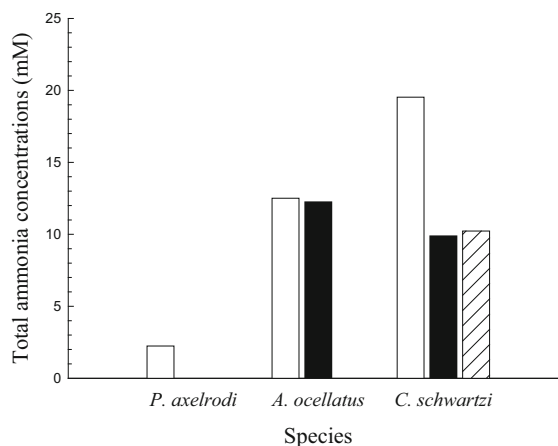
Fig. 1 Acute toxicity of ammonia over 96 h, based on measured values, expressed as LC50$_{96\ h}$ in terms of **A** total (NH$_3$ + NH$_4^+$) concentration, and **B** unionized ammonia (NH$_3$) concentration (both mM) determined for eleven species of Amazon fish. The data are displayed in the order of the most tolerant (*top*) to the least tolerant species (*bottom*) in terms of total ammonia toxicity, and the error bars represent 95% CL

Lethal concentration tests in combination with hypoxia

Simultaneous hypoxia (15–20% saturation) had no effect on the LC50$_{96\ h}$ value in *Astronotus ocellatus* (Fig. 2), which is an obligatory water-breather and the second most tolerant species under normoxia (Fig. 1A). However, the most sensitive species, *P. axelrodi*, did not tolerate the combination of the two factors, with 100% mortality by 24 h in all tested ammonia concentrations (Fig. 2). Notably in the control treatment (hypoxia alone, no ammonia), hypoxia caused 50% mortality. When the most tolerant species, *C. schwartzi*, a facultative air-breather, was denied access to air during the ammonia exposures, the LC50$_{96\ h}$ value dropped by 50% (Fig. 2)—i.e., toxicity was exacerbated. The simultaneous presence of

Fig. 2 The effects, in three Amazonian fish, of continuous hypoxia (15–20% air saturation, *black bars*) versus continuous normoxia (>80% air saturation, *white bars*) on the acute toxicity of ammonia over 96 h expressed as LC50$_{96\ h}$, based on measured values, in terms of total ammonia (NH$_3$ + NH$_4^+$) concentration. The third bar (striped) presented for *Corydoras schwartzi* corresponds to the normoxic condition, but with the fish denied access to air

hypoxia under these conditions (i.e., no access to air) had no additional effect on the LC50$_{96\ h}$ in *C. schwartzi* (Fig. 2). Key toxicant parameters during these tests are tabulated in Online resource 3.

Discussion

Are Amazonian fish more sensitive to ammonia? Species sensitivity distributions (SSDs), hazardous concentration for 5% of species (HC5), and environmental guidelines

To our knowledge, this is the first detailed survey of acute ammonia toxicity to teleost fish of the Amazon basin. It is of interest to see how the current data are compared with those of fish from other regions, and with current environmental criteria. Recently, the U.S. EPA (2013) has surveyed acute ammonia toxicity data from a large number of studies on freshwater fish and aquatic invertebrates, and from these summaries has plotted a species sensitivity distribution (SSD) based on genus mean acute values for 69 genera. Fortuitously, the U.S. EPA (2013) expressed the data in terms of total ammonia, normalized all values to pH 7.0, and concluded that at least for fish, there was no

need for temperature normalization. Thus, the present data can be directly compared with the very extensive U.S. EPA (2013) dataset.

Figure 3A shows an SSD plot for the current LC50$_{96h}$ acute toxicity dataset. Application of the Burriloz program analysis to estimate the hazardous concentration based on toxicity to the most sensitive 5% of the species (HC5; see "Statistical analyses" section) yields a value of 2.50 (1.60–4.20) mM. A similar analysis of the U.S. EPA (2013) dataset yielded a very similar value (2.39 mM), called the
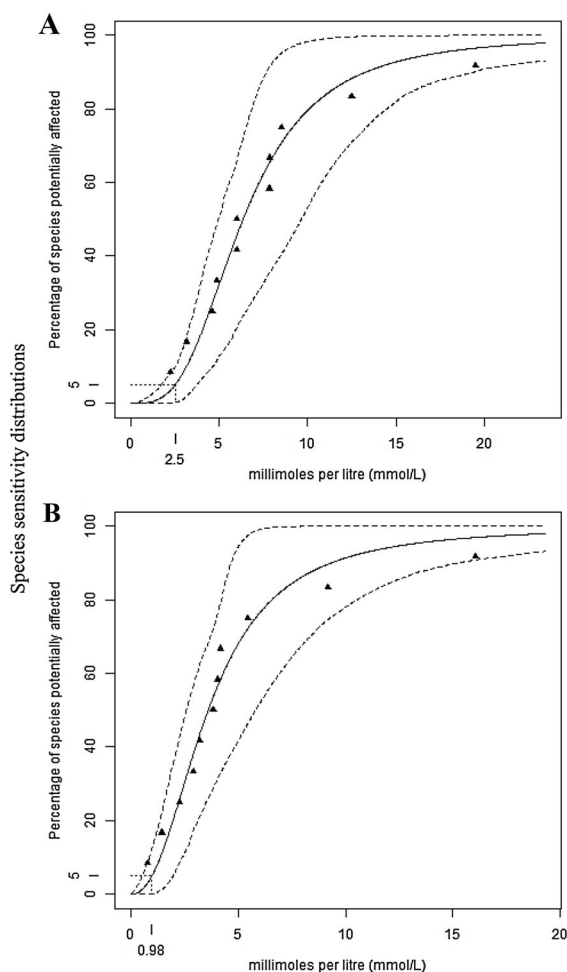
Fig. 3 Species sensitivity distributions (SSDs; *solid line*) and confidence intervals (*dotted lines*) obtained for **A** the LC50$_{96 h}$ values of the total (NH$_3$ + NH$_4^+$) ammonia (mmol/l), and **B** the LC10$_{96h}$ values of the total (NH$_3$ + NH$_4^+$) ammonia (mmol/l), for eleven species of Amazon fish (represented by the *symbols*). The values are represented in order of most tolerant (*upper right*) to least tolerant (*lower left*) species. The quadrants represent the HC$_5$ values (2.5 mmol/l for LC50$_{96 h}$, 0.98 mmol/l for LC10$_{96 h}$) for the species used in this study

Final Acute Value (FAV). However, this does not mean that Amazonian fish necessarily have similar sensitivity to those in the U.S. EPA (2013) database, because the latter included a large number of invertebrate genera. Indeed the 8 most sensitive genera, including all those which helped determine the HC5 or FAV, were freshwater molluscs, which were not evaluated in the present study.

To make a more cogent comparison, we added the current Amazonian fish LC50$_{96 h}$ data (11 species, 10 genera) to an SSD based only on the 25 fish genera in the U.S. EPA database, to create a blended SSD of 35 genera. Notably, none of the genera were common to the two datasets. The Amazonian fish occupied ranks 1 (*Carnegiella strigata*), 5 (*Hyphessobrycon socolofi*), 6 (*Arapaima gigas*), 11 (*Hemigrammus rhodostomus*), 12 (*Brycon amazonicus*), 13 (*Paracheirodon sp.*), 18 (*Colossoma macropomum*), 20 (*Apistogramma agassizii*), 29 (*Astronotus ocellatus*), and 34 (*Corydora schwartzi*) of the 35 positions in the blended SSD. Indeed if *Paracheirodon axelrodi* had been ranked by itself rather than averaged with *Paracheirodon simulans*, it would have held position 1 (most sensitive), just behind *Carnegiella strigata* as 2.

Clearly, the Amazonian fish are on average more sensitive to acute ammonia toxicity than the wide range of species from other habitats surveyed by the U.S. EPA (2013). These data therefore confirm our initial hypothesis of greater ammonia sensitivity in Amazonian species (see "Introduction" section), perhaps reflecting their evolutionary history in native waters that are acidic (i.e., low in NH$_3$). Fresh waters elsewhere are often alkaline, which might over the long term result in greater ammonia tolerance. On the other hand, the present finding could be considered surprising, because high concentrations of ammonia are expected to occur naturally in the region during dry periods, when fish become concentrated in water bodies where degradation rates of organic matter are high.

The U.S. EPA divides the FAV by a safety factor of 2 to yield the acute Ambient Water Quality Criterion (AWQC) which is 1.20 mM as total ammonia at pH 7.0 (U.S. EPA, 2013). Applying the same procedure to the HC5 of Fig. 3A would yield an almost identical value of 1.25 mM as total ammonia at pH 7.0, based on Amazonian fish alone. This raises an interesting question for future study: are Amazonian invertebrates, especially molluscs, more sensitive than

Amazonian fish to ammonia? If so, this would necessitate a lower acute AWQC.

Many jurisdictions have AWQC designed to protect only against chronic rather than acute toxicity. Endpoints for chronic ammonia toxicity were not measured in the present study, but LC10, appropriately factored, is sometimes used as a surrogate in environmental regulations when other information is lacking. The SSD for $LC10_{96\ h}$ shown in Fig. 3B yields an HC5 of 0.98 (0.49–2.21) mM, and if divided by 2, this would yield 0.49 mM as total ammonia at pH 7.0. The U.S. EPA (2013) chronic AWQC, based directly on chronic toxicity measurements, is 0.14 mM as total ammonia at pH 7.0, while the corresponding Canadian chronic AWQC is 0.09 mM (CCME, 2010). The current Brazilian national chronic AWQC is 0.21 mM as total ammonia at pH 7.0 (Resolução CONAMA 357, 2005), so all three chronic AWQC would appear to be protective for Amazonian fish.

Comparison with other relevant data

Many fish species of the Amazon have high commercial value, both as ornamental and food fish, and ammonia toxicity can be an important constraint, impairing their health and survival during management (Ip et al., 2001; Martinez et al., 2006; Oliveira et al., 2008; Ip & Chew, 2010; Damato & Barbieri, 2011; Zhang et al., 2012; Dinesh et al., 2013; Barbieri & Bondioli, 2015). Thus, information on ammonia tolerance is valuable for any economic activity dependent on these factors. In general ammonia toxicity is well documented for a wide range of fish species (reviewed in Wilkie, 1997; Ip et al., 2001; Randall & Tsui, 2002; Wilkie, 2002; Camargo & Alonso, 2006; Randall & Ip, 2006; Ip & Chew, 2010; Chew & Ip, 2014). However, studies of ammonia tolerance in neotropical freshwater fish are few (e.g., Martinez et al., 2006; Piedras et al., 2006; Miron et al., 2008; Barbieri & Bondioli, 2015) and, more specifically, very rare for Amazonian fish (Cavero et al., 2004; Oliveira et al., 2008).

Paracheirodon axelrodi, which proved to be the least tolerant of the 11 species in the present investigation, is one of only two which has been studied previously. The present $LC50_{96\ h}$ of 2.24 mM is in good agreement with the value of 2.78 mM reported earlier by Oliveira et al. (2008), confirming the high

sensitivity of this species. Interestingly, in the present study *P. axelrodi* was 3.8-fold more sensitive than the congeneric *P. simulans* ($LC50_{96\ h} = 8.52$ mM). Some studies suggest that the tolerance of the different species of fish to ammonia is related to the size of the animals (e.g., Martinez et al., 2006; Piedras et al., 2006; Oliveira et al., 2008; Zhang et al., 2012). However, *P. axelrodi* was in fact twice as large as *P. simulans* (Table 1), so this is not the explanation. Indeed, overall, there was no relationship between body mass and $LC50_{96\ h}$ across the 11 species. *P. axelrodi* appears to be extremely sensitive to other abiotic parameters (for example, temperature change—Oliveira et al., 2008) and hypoxia (see below). Thus, it is an inherently fragile species. Given the high value of *P. axelrodi* in the ornamental fish trade, special care should be employed in its handling to avoid toxicity due to ammonia buildup, hypoxia, and temperature change.

Arapaima gigas is the only other of these species which has been studied previously for ammonia toxicity. Cavero et al. (2004) reported that *A. gigas*, under temperature and pH conditions very similar to those of the present study, tolerated 1.07 mM total ammonia for 33 days without any apparent harm. As 33 days constitute a chronic exposure, comparison with the present acute $LC10_{96\ h}$ of 2.28 mM (Table 3) is appropriate, and in good agreement. *A. gigas* is an extremely important species in aquaculture, so it is beneficial that it tolerates moderate levels of ammonia exposure. Very small fish (13.7 g) were tested in the present study (Table 1). As it grows larger, this species becomes less and less dependent on gill function for gas exchange, and more dependent on air-breathing (e.g., Graham, 1997; Brauner et al., 2004), so it is likely that ammonia tolerance would increase at later life stages (see "Interactions of ammonia toxicity with air-breathing" section).

The present data may also be compared with those previously reported for the few species of neotropical freshwater fish of economic and commercial interest that have been evaluated; data have been normalized to pH 7.0 at reported as total ammonia. These include *Piaractus mesopotamicus* ($LC50_{24\ h}$ 4.18 mM, Martinez et al., 2006), *Prochilodus lineatus* ($LC50_{24\ h}$ 3.64 mM, Martinez et al., 2006), and *Astyanax altiparanae* ($LC50_{24\ h}$ 3.26 mM, Martinez et al., 2006). The Amazonian species presented generally similar levels of tolerance. But, specifically when

comparing the present data for *Hyphessobrycon socolofi* (LC50$_{96 h}$ 4.63 mM) with a species of the same genus, *Hyphessobrycon callistus* (LC50$_{96 h}$ 9.50 mM, Damato & Barbieri, 2011), *H. socolofi* was shown to be approximately 50% less tolerant. However, the tolerance of different species of fishes to ammonia may be related to their normal living environments, especially pH and oxygen levels and water chemistry, which have direct influence on the toxicity of ammonia to fish. Thus, further studies are needed with Amazonian species exposed to the different and unique characteristics of the region to classify them in relation to tolerance ammonia. The present dataset is too small to determine whether phylogeny plays any role in ammonia tolerance of various Amazonian species, but again this is an important area for future investigation.

Interactions of ammonia toxicity
with air-breathing

In general, air-breathing fish have been reported to be more resistant to ammonia (Cavero et al., 2004; Randall & Ip, 2006; Wee et al., 2007; Ip & Chew, 2010; Chew & Ip, 2014; Lefevre et al., 2014). This conclusion is supported by our finding that the catfish *Corydoras schwartzi*, which is a facultative air-breather, was the most ammonia-tolerant of the 11 Amazonian species studied. This species swallows air periodically from the water surface, and uses its well-vascularized gut as an accessory breathing organ (Graham, 1999; Nelson, 2014). Notably, when air-breathing was prevented, ammonia toxicity to *C. schwartzi* approximately doubled—i.e., the LC50$_{96 h}$ value dropped by 50% (Fig. 2). Presumably, by air-breathing, this species can reduce the ventilation and perfusion of its gills, thereby reducing ammonia uptake from the environment. Some air-breathing fish feature changes in gill structure or even degenerate gills (Ip & Chew, 2010; Chew & Ip, 2014), and many air-breathing fish species have increased air-ventilation rates when in waters with high levels of ammonia (Randall & Ip, 2006), as well as in hypoxic waters (Brauner et al., 1995; Randall & Ip, 2006; Nelson, 2014), and acidic and hydrogen sulfide-rich waters (Brauner et al., 1995). Facultative air-breathing is a very common adaptive characteristic in many Amazonian fish (Val & Almeida-Val, 1995), especially for those living in areas with poor water quality, and it is at

least partially responsible for the high tolerance of these animals in these conditions, as demonstrated for *Hoplosternum littorale* (Brauner et al., 1995). In future studies, it will be important to test whether air-breathing is a widely used strategy to avoid ammonia toxicity.

Interactions of ammonia toxicity with hypoxia

Some studies have claimed that a reduction in environmental oxygen levels increases the toxicity of ammonia to aquatic animals (Wajsbrot et al., 1991; Ip et al., 2001; Lefevre et al., 2014). Given the prevalence of severe hypoxia in the Amazon basin (see Introduction), it was of interest to assess possible interactions between the two stressors in the present investigation. Clearly, hypoxia greatly exacerbated ammonia toxicity in the most sensitive species, *P. axelrodi* (Fig. 2), but here, hypoxia itself was part of the cause of mortality: 50% of the fish died in response to hypoxia alone (15–20% saturation). Branchial ion flux rates and endogenous ammonia production rates are exceptionally high in this species (Wood et al., 2014; Robertson et al., 2015), so this may contribute to their sensitivity. On the other hand, the same level of hypoxia had absolutely no effect on acute ammonia toxicity in *Astronotus ocellatus* (Fig. 2). *A. ocellatus* lacks air-breathing ability but is well known to be exceptionally tolerant of hypoxia (Muusze et al., 1998; Almeida-Val et al., 2000), and indeed suppresses branchial ion and ammonia fluxes during hypoxia (Wood et al., 2007, 2009). Similarly, hypoxia did not alter ammonia toxicity in *Corydoras schwartzi* when prevented from air-breathing (Fig. 2). *Corydoras schwartzi* and *Astronotus ocellatus* were, respectively, the most tolerant and second most tolerant of the 11 species we studied (Fig. 1). These results lend support to the proposal of Walsh et al. (2007) that there is a correlation between ammonia and hypoxia tolerance in fish, based on common mechanisms involving glutaminergic transmission in the brain.

Concluding remarks

With a few exceptions, Amazonian teleosts appear to be generally less tolerant of aquatic ammonia than other fish. We have attributed this to their long history in water of low alkalinity and therefore low pH. Additionally, for some Amazonian species, facultative

air-breathing may offer an escape from ammonia loading. In both cases, these factors would reduce the evolutionary pressures selecting for ammonia tolerance. Nevertheless, we cannot eliminate the possibility that it is a general characteristic of tropical fish. Indeed Kwok et al. (2007) concluded that at least for unionized ammonia (NH_3), tropical species appear to be more sensitive than temperate species. Regardless of the explanation, the present data suggest that increasing anthropogenic discharges of ammonia (see "Introduction" section) will be a significant threat to Amazonian teleosts

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