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Ammonia and nitrite toxicity to false clownfish *Amphiprion ocellaris*

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Abstract False clownfish, *Amphiprion ocellaris*, is one of the most commercialized fish species in the world, highly produced to supply the aquarium market. The high stocking densities used to maximize fish production can increase ammonia and nitrite to toxic levels. In this study, *A. ocellaris* juveniles (1.20 ± 0.34 g) were exposed to six concentrations of ammonia ranged from 0.23 to 1.63 mg/L NH_3-N and eight concentrations of nitrite (26.3–202.2 mg/L NO_2^--N). The LC_{50}- 24, LC_{50}-48, LC_{50}-72 and LC_{50}-96 h were estimated to be 1.06, 0.83, 0.75 and 0.75 mg/L for NH_3-N and 188.3, 151.01, 124.1 and 108.8 mg/L for NO_2^--N. Analysis of gill lesions caused by sublethal concentrations of these nitrogenous compounds showed that both nitrogenous compounds induced tissue lesions such as hyperplasia of epithelium cells, hypertrophy of chloride cells and lamellar lifting to all concentrations tested. However, histopathological alterations were more conspicuous accordingly the increase of ammonia or nitrite in fish exposed to 0.57 mg/L NH_3-N or 100 mg/L NO_2^--N. Based on our results, we recommend to avoid concentrations higher than 0.57 mg/L of NH_3-N and 25 mg/L of NO_2-N in water.

Keywords Marine ornamental · Nitrogenous compounds · Histopathology · LC_{50}

Introduction

The false clownfish, *Amphiprion ocellaris*, was one of the main marine ornamental fish imported by USA from May 2004 to May 2005 (Rhyne et al. 2012). The genus *Amphiprion* was one of the first marine ornamental species successfully cultured (Hoff 1996). These

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fish have been raised commercially since 1950, and currently the reproduction and juvenile production have established protocols with high larval survival (Hoff 1996; Wittenrich 2007). Nowadays, with the popularity of marine aquariums, strongest restrictions to wild fish capture and the preference of some aquarists for captive bred fish, the demand for cultured fish individuals has enhanced. In order to supply the demand, clownfish are often raised in recirculating aquaculture systems (RAS), which allow high stocking densities and production maximization (Watson and Hill 2006).

The high fish densities, added to the high protein levels of fish diets, increase the content of nitrogenous compounds dissolved in the water (Ellis et al. 2002). Ammonia is the main nitrogenous compound excreted by fish, and it occurs in two forms: un-ionized (NH_3) and ionized (NH_4^+). The NH_3/NH_4^+ ratio depends on water pH, temperature and salinity (Thurston et al. 1981). Nitrite (NO_2^-) is the intermediate product of nitrification, which ends with the formation of nitrate (Rijn 1996). Ammonia and nitrite can reach toxic levels in fish within a few days, leading to physiological impairment and eventually death of the exposed animals (Randall and Tsui 2002; Kroupova et al. 2005).

The acute toxicity of ammonia and nitrite has been studied in several fish species using the traditional median lethal concentration test (LC_{50}) (Sampaio et al. 2002; Weirich and Riche 2006a, b; Rodrigues et al. 2007). Although nitrogenous compounds not always reach lethal concentrations, they may cause no visible tissue damage (Lease et al. 2003). In this regard, histological techniques are a good and usual procedure to evaluate and demonstrate tissue lesions caused by sublethal concentrations of aquatic pollution in fish (Bernet et al. 1999; Santos et al. 2014). Several studies have been carried out by combining both techniques (LC_{50} and histology) to determine safe levels of nitrogenous compounds to fish culture (Rodrigues et al. 2011; Wuertz et al. 2013; Rodrigues et al. 2014).

Therefore, the present study aims to expand the knowledge on culture of *A. ocellaris*, by determining the LC_{50} of ammonia and nitrite and evaluating gill lesions caused by sublethal concentrations of both nitrogen compounds.

Materials and methods

Experimental fish

Fish were produced from natural spawning of clownfish broodstocks maintained in recirculating aquaculture systems at the Laboratory of Estuarine and Marine Fish Culture of the Universidade Federal do Rio Grande—FURG (Brazil), following standard protocols described by Wittenrich (2007). Prior to the experiments, juveniles were fed a commercial diet (55 % protein, 9 % lipids, 400–800 µm, Inve Aquaculture, NRD, USA) ad libitum three times a day. At the time of the experiments, the mean body weight of fishes was 1.20 ± 0.34 g.

Acute toxicity assays

Two independent experiments were conducted to determine the acute toxicity of ammonia and nitrite. Juvenile clownfish were exposed to six concentrations of ammonia: 0.23 ± 0.02, 0.57 ± 0.03, 0.97 ± 0.06, 1.05 ± 0.06, 1.22 ± 0.05 and 1.63 ± 0.14 mg/L NH_3-N obtained by adding ammonium chloride (Synth, Brazil) to water and eight concentrations of nitrite: 26.3 ± 2.6, 53.3 ± 3.9, 72.5 ± 2.8, 111.3 ± 4.6, 132.4 ± 5.6,

Table 1 Important factor and score value used to quantify histological lesions adapted from Bernet et al. (1999)

Important factor (*w*)	Score value (*a*): % of lamellae presenting alterations
1. Minimal pathological importance, the damage is reversible	0—Unchanged
	1—0.1 to 16.6
2. Moderate pathological importance, the lesions are reversible in some cases	2—16.7 to 33.3
	3—33.4 to 49.9
3. Marked pathological importance, usually irreversible and leads to loss of organ function	4—50.0 to 66.6
	5—66.7 to 83.3
	6—83.4 to 100

157.4 ± 2.8, 178.4 ± 3.4 and 202.2 ± 6.0 mg/L NO_2^--N with addition of sodium nitrite (Synth, Brazil) to water and a control, all in triplicates (Table 1).

Tests were carried out in 15-L fiberglass cylindrical tanks in static system. During all tests, tanks were held in a water bath for temperature control and were aerated by blowers connected to air stones. Photoperiod was maintained at 12/12 light/dark cycle. Ions such as chloride, calcium and magnesium were measured in the experimental water before the start of the trials. Chloride (15.41 ± 0.32 g/L) was determined according to the method proposed by Clesceri et al. (1989) and calcium (0.320 ± 0.011 g/L) and magnesium (1.004 ± 0.011 g/L) according to Basset et al. (1981). During the trial, water quality parameters were measured once a day. Salinity was measured with hand refractometer (Atago, S/Mill-E, Japan) and was maintained at 27 g/L. Dissolved oxygen concentration and temperature were measured with a digital oximeter (YSI 550A, USA). Dissolved oxygen concentration was always above 6 mg/L O_2, while temperature was held at 27 ± 0.1 °C. The pH was measured with a digital pH meter (Mettler Toledo, FiveEasy FE20, Switzerland) and remained at 8.15 ± 0.02. The alkalinity was measured by titrimetry (Rocha et al. 2010) and was 180 ± 5 mg/L $CaCO_3$. Total ammonia nitrogen (TA-N) and nitrite were measured according to Grasshoff et al. (1999) and Aminot and Chaussepied (1983). Un-ionized ammonia (NH_3) concentrations were calculated following TA-N, and temperature, salinity and pH values following the equations of Ostrensky et al. (1992), adapted from Whitfield (1974) and Bower and Bidwell (1978).

Fish starved 24 h before and throughout the trials to minimize the nitrogenous compounds excretion during the test. At the start of the experiment, five fish were distributed in each tank. Mortality and swimming behavior, such as erratic swimming, lethargic behavior and loss of equilibrium, were checked every 12 h. Immobile individuals, with no opercular movement and no response to mechanical stimuli, were considered dead and removed from the tanks. This work was approved by the Ethic Committee of Universidade Federal do Rio Grande—FURG (process number 23116.006275/2013-88).

Histopathological analyses

At the end of the trials, three fish per tank were randomly sampled and euthanized in a benzocaine solution (300 ppm) for histopathological analyses. The samples were fixed in Bouin liquid, dehydrated in a graded series of ethanol and embedded in regular paraplast with an automatic processor (LUPETEC, PT 05, Brazil). Sections (5 μm width) were prepared with a microtome (LUPETEC, MRP03, Brazil) and stained with hematoxylin and

Table 2 Median lethal concentrations (95 % confidence intervals) of NH_3-N and NO_2^--N to juvenile false clownfish *Amphiprion ocellaris* at different exposure times

Time (h)	LC_{50} (mg/L)	
	NH_3-N	NO_2^--N
24	1.06^a (0.96–1.16)	188.3^a (185.3–191.3)
48	0.83^b (0.77–0.88)	151.0^b (141.7–160.9)
72	0.75^b (0.72–0.78)	124.1^c (116.4–132.2)
96	0.75^b (0.72–0.78)	108.8^c (100.1–118.2)

Different letters indicate significant differences after one-way ANOVA ($P < 0.05$) among treatments

eosin, and slides were analyzed using optical microscope (NIKON, Eclipse E200, Japan). Quantitative analyses of tissue lesions were based on at least 200 gill secondary lamellae of each fish. The organ lesion index was calculated as $I_{org} = \Sigma_{rp}\Sigma_{alt}$ $(a.w)$ (Bernet et al. 1999), where "Iorg" is the organ index, "rp" is the reaction pattern, "alt" is the alteration, "a" is a score value related to the alteration frequency, and "w" is the important factor related to the relevance of the alteration (Table 2).

Statistical analyses

Median lethal concentrations (LC_{50}) were calculated for 24, 48, 72 and 96 h using the Trimmed Spearman–Karber method (Hamilton et al. 1977). Comparisons between LC_{50} and histopathological index were analyzed using one-way ANOVA followed by Newman–Keuls post hoc test with a significant level of 5 %. Data of LC_{50} are presented as mean ± 95 % confidence interval and other data as mean ± standard error.

Results

Median lethal concentrations for 24, 48, 72 and 96 h and their confidence intervals are shown in Table 2. The LC_{50}-96 h for unionized ammonia and nitrite was 0.75 mg/L NH_3-N and 108.8 mg/L NO_2^--N, respectively. No mortality was observed at concentrations equal to or lower than 0.57 mg/L NH_3-N or 50 mg/L NO_2^--N.

Fish exposed to 0.23 or 0.57 mg/L NH_3-N did not show any behavioral changes. All fish died at concentrations equal or higher than 1.22 mg/L NH_3-N, and just one fish survived at 0.97 and 1.05 mg/L NH_3-N, with no behavioral impairment. Throughout the nitrite exposure trial, the swimming performance of fish exposed to 25 mg/L NO_2^--N was not affected. Nevertheless, fish exposed to concentrations equal to or higher than 50 mg/L NO_2^--N presented lethargic behavior, erratic swimming and hyperventilation. At the end of 96 h, all fish exposed to concentrations equal to or higher than 125 mg/L NO_2^--N were dead. All the mortalities are shown in Table 3.

Histopathological analyses of the gills of fish exposed to different ammonia and nitrite concentrations revealed alterations, and the frequency of abnormalities was more severe which accordingly increased the ammonia or nitrite concentrations (Figs. 1, 2). Fish exposed to ammonia and nitrite presented lamellar lifting (Fig. 2b), hyperplasia and hypertrophy of chloride cells and hyperplasia of the epithelial cells (Fig. 2c). Fish exposed to 0.57 mg/L NH_3-N or 100 mg/L NO_2^--N also presented lamellar fusion of the secondary

Table 3 Accumulated mortality in each treatment of juvenile false clownfish *Amphiprion ocellaris* acutely exposed to NH_3-N and NO_2^--N during 96 h

Time (h)	NH_3-N (mg/L)								
	0	0.23	0.57	0.97	1.05	1.22	1.63		
24	0	0	0	4	8	9	15		
48	0	0	0	10	12	15			
72	0	0	0	14	14				
96	0	0	0	14	14				
	NO_2^--N (mg/L)								
	0	26.3	53.3	72.5	111.3	132.4	157.4	178.4	202.2
24	0	0	0	0	0	1	0	1	15
48	0	0	0	0	0	6	6	12	
72	0	0	0	0	2	10	15	15	
96	0	0	0	1	6	15			

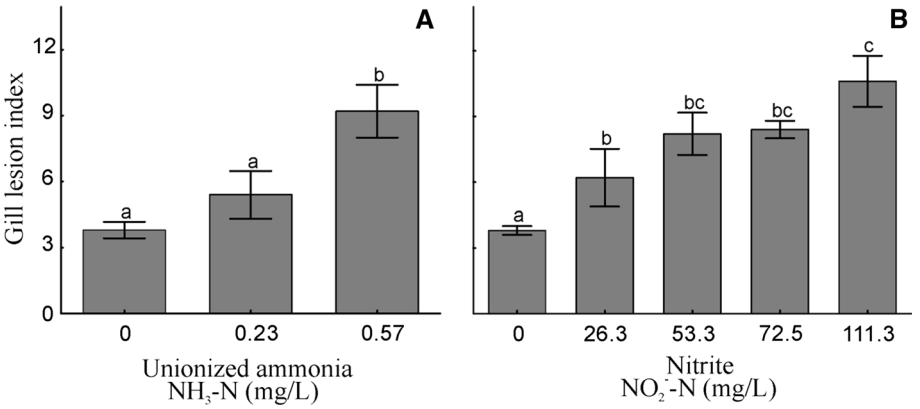


Fig. 1 Gill lesion index of juvenile false clownfish *Amphiprion ocellaris*: lesion index (mean ± standard error) after 96 h of exposure to different NH_3-N (**a**) or NO_2^--N (**b**) concentrations. *Different letters* represent significant differences after one-way ANOVA followed by Newman–Keuls ($P < 0.05$)

lamella (Fig. 2d). Significant differences ($P < 0.05$) in the Bernet's lesion index were found between the control and 0.57 mg/L NH_3-N, and the control and 25 or more mg/L NO_2^--N (Fig. 1a, b).

Discussion

Comparison of tolerance to any toxic among species is difficult, because toxicity depends on the ontogeny of the species, their nutritional status and water characteristics during the trials (Gomulka et al. 2014; Costa et al. 2008). The results of the present study showed that the tolerance of juvenile *A. ocellaris* to ammonia LC_{50} (LC_{50}-96 h 0.75 mg/L NH_3-N) is very close to the ammonia toxicity tolerated by the black sea bass *Centropristis striata*, the most sensitive marine fish species to this nitrogen compound (LC_{50}-96 h of 0.54 mg/L of

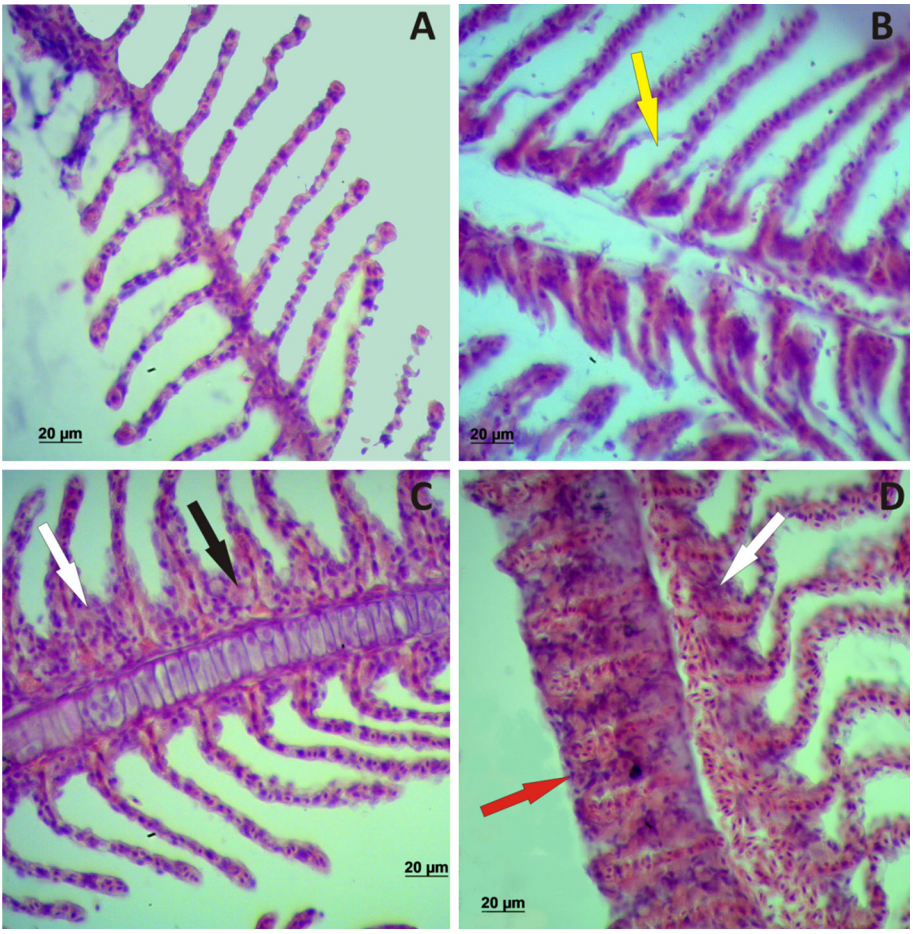


Fig. 2 Gill of juvenile false clownfish *Amphiprion ocellaris*: **a** control group; **b**, **c** fish exposed to 0.24 mg/L NH_3-N showing lamellar lifting (*yellow arrow*) hyperplasia of first lamellae (*white arrow*) and hypertrophy of chloride cells (*black arrow*); **d** fish exposed to 0.57 mg/L NH_3-N showing lamellar fusion (*red arrow*), hyperplasia of first lamellae (*white arrow*). H–E (×400). (Color figure online)

NH_3-N; Weirich and Riche 2006b). The false clownfish is also more sensitive than the maroon clownfish, *P. biaculeatus* (LC_{50}-96 h 0.89 mg/L NH_3-N) (Rodrigues et al. 2014), and some other table fish commercially produced, such as the Florida pompano, *Trachinotus carolinus* (LC_{50}-96 h 1.01 mg/L NH_3-N), the cobia, *Rachycentron canadum* (LC_{50}-96 h 1.13 mg/L NH_3-N) (Weirich and Riche 2006a; Rodrigues et al. 2007), and the turbot, *Scophthalmus maximus* (LC_{50}-96 h 2.6 mg/L NH_3-N). Therefore, special attention should be given to this compound, since the concentration of ammonia is the first, among nitrogenous compounds, to rise in aquariums or production systems, such as RAS with limited water exchange.

Despite the sensitivity to ammonia, juvenile false clownfish presented a moderate resistance to nitrite (LC_{50}-96 h 108.8 mg/L of NO_2^--N at salinity 27 with 15.41 g/L of Cl^-) when compared to other fish species. When compared to the fish species most

resistant to nitrite, *Chanos chanos* (LC_{50}-96 h 675 mg/L of NO_2^--N at salinity 16 with 8.8 g/L of Cl^-) (Almendras 1987), the false clownfish revealed itself to be six times more sensitive to this nitrogen compound. On the other hand, it can be considered more resistant than most of the marine species, such as *Paralichthys orbignyanus* (LC_{50}-96 h 30.6 mg/L of NO_2^--N at salinity 30), *T. carolinus* (LC_{50}-96 h 34.2 mg/L of NO_2^--N at salinity 25 with 13.5 g/L of Cl^-) (Bianchini et al. 1996; Weirich and Riche 2006a) and *Sciaenops ocellatus* (LC_{50}-96 h 85 mg/L of NO_2^--N at salinity 36) (Wise and Tomasso 1989).

The gill is very susceptible to toxics due to its direct and constant contact with the water (Bernet et al. 1999). Gill alterations due to ammonia or nitrite toxicity observed in the present study were also reported for maroon clownfish exposed to 0.75 mg/L of NH_3-N (Rodrigues et al. 2014) and for various other fish species exposed to ammonia such as Nile tilapia *Oreochromis niloticus* (Benli et al. 2008) and silver catfish *Rhamdia quelen* (Miron et al. 2008). The frequency of the lesions rose in accordance with the increasing levels of ammonia or nitrite, similar to the results presented by Lease et al. (2003); Rodrigues et al. (2014) and Wuertz et al. (2013).

Hyperplasia and hypertrophy of chloride cells, hyperplasia of the epithelial cells and lamellar fusion are probably reactive histopathologies of the epithelium to toxicant exposure. The hyperplasia and hypertrophy of chloride cells may occur to increase active excretion of these toxicants, whereas the other lesions may happen to reduce the surface area of gill in direct contact with the toxicant (Mallat 1985). Nevertheless, these histopathologies can compromise fish respiration and ionic regulation, impair excretion of nitrogenous compounds (Kroupova et al. 2005) and increase the toxicity of ammonia and nitrite. Branquial histopathologies are also especially important on nitrite toxicity, because the main nitrite toxicity occurs when nitrite oxidizes hemoglobin to methemoglobin, causing hypoxia (Kroupova et al. 2005).

A possible way to protect juvenile clownfish *A. ocellaris* from the toxic effect of ammonia is maintaining a slightly low pH to reduce the NH_3/NH_4^+ ratio, as NH_3 is more toxic due to its capacity to diffuse through gill membrane, while NH_4^+ needs an active transporter (Ip and Chew 2010). Likewise, higher salinities may also help to avoid nitrogen compound toxicity, as demonstrated for juvenile mullet *Mugil liza* (Sampaio et al. 2002) and for juvenile cobia *R. canadum* (Barbieri and Doi 2012). Due to drinking of sea water, in marine fish the main nitrite uptake to blood occurs in the intestine, competing with Cl^- in the $Na^+/K^+/2Cl^-$ and Na^+/Cl^- cotransporters (Grossel and Jensen 1999). Also, nitrite can reach fish blood by diffusion through the gill epithelium. However, due to high Cl^- concentration, the active transport of ions in the gill excretes Cl^- and Na^+ and in addition actively excretes the NO_2^- instead the Cl^- (Jensen 2003).

In conclusion, juvenile *A. ocellaris* are more sensitive to ammonia than to nitrite, but both toxics can cause mortalities, presenting LC_{50}-96 h = 0.75 mg/L to NH_3-N and 108.8 mg/L to NO_2^--N. Gill histology proved to be efficient to evaluate the effects of sublethal concentrations of ammonia and nitrite in these fish. Based on our results and according to the recommendation of Meade (1985), maximum safe levels of nitrogenous compounds must be based on gill histopathology in fish. This way we recommend to avoid concentrations higher than 0.57 mg/L of un-ionized ammonia and 25 mg/L of nitrite in water on clownfish production.

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