ACTA BIOLÓGICA COLOMBIANA
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Facultad de Ciencias
Departamento de Biología
Sede Bogotá

UNIVERSIDAD NACIONAL DE COLOMBIA

ARTÍCULO DE INVESTIGACIÓN / RESEARCH ARTICLE — CONSERVACIÓN

DIFFERENCES IN THE ACUTE AMMONIA TOXICITY BETWEEN SEXES OF GUPPY *Poecilia reticulata* (POECILIIDAE)

Diferencias en la toxicidad aguda del amoníaco entre sexos de Guppy *Poecilia reticulata* (Poeciliidae)

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Received: 19th October 2019. Returned for revision: 25th August 2021. Accepted: 1st December 2021.
Associated Editor: Alan Giraldo

Citation/ citar este artículo como: Forneck, S.C., Dutra, F.M., Maltauro, R.A, and Cunico, A.M. (2023). Differences in the acute ammonia toxicity between sexes of guppy *Poecilia reticulata* (Poeciliidae). *Acta Biol Colomb*., *28*(1), 57-63. https://doi.org/10.15446/abc. v28n1.91052

ABSTRACT

This study evaluated the sensitivity of male and female of guppies (*Poecilia reticulata)* to ammonia. LC_{50}-96h for males, females and combined sexes of *P. reticulata* was 37.33, 48.34, and 42.45 mg/L of total ammonia or 1.03, 1.34, and 1.17 mg/L of un-ionized ammonia. The mortality at the concentration of 40 mg/L of total ammonia differed between the sexes with higher mortality in males. These results have implications for production systems, since recommendations of ammonia toxicity reference values that do not consider the difference between the sexes, can lead to the mortality of males, and cause economic losses because males have higher commercial value in the ornamental market.

Keywords: lethal concentration, nitrogen compound, ornamental fish, sexual dimorphism, toxicology.

RESUMEN

Este estudio evaluó la sensibilidad de machos y hembras de guppies (*Poecilia reticulata)* al amoníaco. La $_{CL50}$-96h para machos, hembras y sexos combinados de *P. reticulata* fue de 37,33; 48,34 y 42,45 mg/L de amoníaco total o 1,03; 1,34 y 1,17 mg/L de amoníaco no ionizado. La mortalidad a la concentración de 40 mg/L de amoníaco total difirió entre los sexos con mayor mortalidad de los machos. Estos resultados tienen implicaciones para los sistemas de producción, una vez que las recomendaciones de valores de referencia de toxicidad del amoníaco que no consideren la diferencia entre sexos, pueden conducir a la mortalidad de los machos y provocar pérdidas económicas, debido a que los machos tienen mayor valor comercial en el mercado ornamental.

Palabras clave: compuesto de nitrógeno, concentración letal, dimorfismo sexual, peces ornamentales, toxicología.

INTRODUCTION

Ornamental fish trade is a consolidated market worldwide, accounted USD 351 million in global exports in 2019 (ITC, 2020). Brazil is one of the leading exporters of ornamental fishes of the world, exporting US$ 6.8 million in 2019 (ITC, 2020). Although part of the freshwater ornamental fish traded worldwide comes from capture, approximately 90 % are captive bred (Sommerville et al., 2016). The success of ornamental production depends on environmental quality, fish health and welfare (Florindo et al., 2017), because changes in water quality, stress, and high stocking densities lead to economic losses (Martins et al., 2002).

In Brazil, most of the ornamental fish farms perform the production in family and rudimentary system, using small production units, *e.g.* bottles, plastic boxes and aquaria (Vidal-Jr., 2006; Ribeiro and Fernandes, 2008). These systems facilitate the deterioration of water quality due to small water volume and low water exchange, in addition to high feeding frequency, fish excretion and high stocking densities (Ribeiro and Fernandes, 2008; Nandi et al., 2009; Eiras et al., 2019).

In production systems, the factors that most affect aquatic organisms are nitrogen compounds (Camargo and Alonso, 2006; Damato and Barbieri, 2011). Among them, ammonia affects the health of these organisms, quickly reaching toxic concentrations, mainly due to decomposition of organic matters, food leftovers and fish excretion (Randall and Tsui, 2002; Campos et al., 2012). In natural environments, Brazilian legislation allows maximum total ammonia concentration of 3.7 mg/L (CONAMA, 2005), while in production systems its concentration can reach 3 mg/L (Boyd and Tucker, 1998). High ammonia concentrations in the environment may cause structural and functional damage to gills, liver and kidney, hindering the oxygen absorption, affecting metabolism, in addition to making it difficult the detoxification and excretion of ammonia by organism (Benli et al., 2008; Ip and Chew, 2010; Barbieri and Doi, 2012). The decreased function of these organs can lead to sublethal (*e.g.* growth inhibition, reproductive alterations, changes in immune and behavioral responses, and susceptibility for diseases) and lethal effects (Camargo and Alonso, 2006; Benli et al., 2008; Ip and Chew, 2010). Un-ionized ammonia is recognized as its most toxic form, due to small molecular size, which easily crosses the gill membranes of fish (Sung et al., 2012; Yang et al., 2015). Its proportion in water increases with increasing pH and temperature (Boyd and Tucker, 2012).

Toxicological studies have been conducted on fish species to better understand these effects, *e.g. Pimephales promelas* (Armstrong et al., 2012), *Premnas biaculeatus* (Rodrigues et al., 2014) and *Takifugu obscurus* (Cheng et al., 2015). However, there is a lack of information on the difference in sensitivity to this compound between males and females. It is known that the sensitivity of certain fish species to toxic agents may depend on the sex, *e.g.*, guppy *Poecilia reticulata* Peters 1959 to phenol (Colgan et al, 1982); *Cnesterodon decemmaculatus* (Jenyns 1842) to herbicides (Di Marzio et al., 1998) and *Jenynsia multidentate* (Jenyns 1842) (Ballesteros et al., 2007) and *Aphanius iberus* (Valenciennes 1846) (Varó et al., 2008) to pesticides.

The guppy *P. reticulata* is one of the ornamental freshwater fish most widespread worldwide, with great acceptance and popularity in aquarium trade, due to their several varieties and coloring patterns (Froese and Pauly, 2019), and its remarkable ability to adapt to confinement (Mukherjee et al., 2009). Due to its vibrant colors, males have great commercial value in the global export market (Mukherjee et al., 2009). The species has short generations, with males reaching sexual maturity at two months and females at three months (Riehl, 1991). Males and female can reach 2.8 and 6 cm total length, respectively (Froese and Pauly, 2019).

Therefore, based on the hypothesis that males of *P. reticulata* are more sensitive to ammonia than females, the aim of the study was to assess the effect of ammonia on different sexes of the guppy *P. reticulata*.

MATERIALS AND METHODS

The experiment was conducted at the Laboratory of Ecology, Fishery and Ichthyology (LEPI) at the Federal University of Paraná - UFPR, Palotina Sector, Brazil, during 96 h. *P. reticulata* individuals were provided by a local fish farmer. The animals were weighed, measured in their total length and divided into males (0.12 ± 0.03 g and 2.38 ± 0.15 cm; mean ± SD) and females (0.24 ± 0.11 g and 2.84 ± 0.39 cm). The experimental protocols of this study using animals were submitted to an ethical review process by the ethics committee on the use of animals (CEUA – UFPR – Palotina), under protocol n° 42/2015. The methodology used here in assessing the median lethal concentration (LC_{50}-96h) was based on the manual of the United States Environmental Protection Agency (EPA, 2002).

Experimental design

Bioassays used 240 males and 240 females of *P. reticulata* randomly distributed into 24 experimental units, with ten males and ten females, simultaneously stocked per unit. The experimental structure was composed of polypropylene units with a volume of 2 L (Static system), duly equipped with forced aeration system, and 12/12 hours photoperiod (light/dark). The animals were fasted 24 hours before the onset of the experiment and during the whole experimental period. The water used in the experimental units came from the public supply system.

The design was completely randomized, with six different total ammonia concentrations (control (no added total ammonia), 20, 40, 60, 80 and 100 mg/L ammonium chloride PA - Synth®; São Paulo; Brazil) and four replicates. The concentrations were determined by preliminary tests during 24 hours of exposure. The ammonia levels resulted from the addition of appropriate volumes of the stock solution of NH_4Cl (1000 mg/L NH_4Cl).

Mortality was determined by the total absence of motion or reaction to mechanical stimulation by means of a glass rod. The mortality assessment was performed every hour, for the first eight hours. After this period until the end of the 96 hours, the observations were carried out every six hours.

Analysis of water quality

Water quality variables were evaluated independently for each treatment to maintain adequate levels for the biology of the species and ensure the absence of effect from other variables. Dissolved oxygen (Alfakit®T-160 oximeter), temperature (Incoterms® digital thermometer) and pH (Tekna®T-100 pH meter) were measured daily. The total ammonia and nitrite at the beginning and the end of the experiment were determined according to American Public Health Association (APHA, 2005) and measured in a spectrophotometer (BEL photonics 2000 UV). Nitrate concentrations were not evaluated due to its low toxicity to aquatic organisms (Tomasso, 1994). The security level of the median lethal concentration of ammonia is determined as recommended by Sprague (1971). The un-ionized ammonia fraction was calculated according to Emerson et al. (1975):

$$N-NH_3 = \frac{\left[N-NH_3 + N-NH_4^+\right]}{1+10^{(pka-pH)}}$$

Where: $N\text{-}NH_3$= un-ionized ammonia; $N\text{-}NH_3$ + $N\text{-}NH_4^+$= total ammonia; pKa = - log Ka, calculated as pKa = 0,09018 + 2729,92 / *T; T*= temperature in Kelvin.

Statistical analysis

One-Way-ANOVA tests were performed to verify differences in water quality variables among treatments. The median lethal concentration for 24, 48, 72 and 96 h, and their respective confidence intervals (95 %) were calculated using the Trimmed Spearman-Kerber method for combined sexes, and males and females separately. The method was chosen because it is not subject to the deficiencies of the commonly used Probit and Logit methods (Hamilton et al., 1977). The estimate does not consider the results of treatments that did not register mortality and those whose mortality reached 100 %. Subsequently, the chi-squared test was used to compare the mortality between the sexes (Dolan et al., 2007). For this analysis, we used the mortality at the concentration closest to the calculated LC_{50}-96h for both sexes, *i.e.* 40 mg/L of total ammonia. The analysis tested statistical significance for 5 % alpha and were performed in the software R (R Core Team, 2019).

RESULTS

The physical and chemical variables related to the water quality did not differ among treatments (p> 0,05), except for the test variable (total ammonia), indicating no influence of other variables on mortality rates in the different treatments (Table 1).

Males and females combined had mortality of 1 ± 3,5 % (mean ± SD) at the control treatment after 96 h. At the concentrations of 20 and 40 mg/L the average mortalities were 11 ± 8 % e 33 ± 15 % after 96 hours, respectively. The concentration of 60 mg/L caused mortality of 86 ± 6 % after 96 hours, with 79 % average mortality in 24 hours. Concentrations of 80 and 100 mg/L led to 100 % mortality in 24 hours. The lethal concentration for 50 % of the fish (males and females combined) at 96 h was 42.45 mg/L of total ammonia and 1.17 mg/L of un-ionized ammonia (Table 2, Fig. 1a). The mortality at the concentration of 40 mg/L differed between the sexes (χ^2 = 4.62; DF = 1; p = 0.032), with higher mortality of males.

Males at the control treatment had mortality of 3 ± 5 % after 96 hours, with mortality of only one individual in one of the replicates. At concentrations of 20 and 40 mg/L of total ammonia the average mortality was of 20 ± 14 % and 45 ± 19 % after 96 hours, respectively. Concentrations of 60, 80 and 100 mg/L led to 100 % mortality in 24 hours. The

Table 1. **Water quality variables (mean ± SD) during the acute toxicity test to determine the LC50-96h for *Poecilia reticulata*.**

Variables	Total ammonia concentration						Secure values for the species
	0 mg/L	20 mg/L	40 mg/L	60 mg/L	80 mg/L	100 mg/L	
Dissolved oxygen (mg/L)	6.97 ± 0.21	7.03 ± 0.38	6.98 ± 0.23	7.09 ± 0.30	7.39 ± 0.36	7.42 ± 0.41	> 3[a]
pH	7.74 ± 0.04	7.63 ± 0.10	7.65 ± 0.02	7.60 ± 0.04	7.58 ± 0.05	7.54 ± 0.04	7 – 8[b]
Temperature (°C)	27.13 ± 0.12	26.95 ± 0.10	27.02 ± 0.16	27.08 ± 0.23	26.33 ± 0.22	26.15 ± 0.13	18 – 28[b]
Total ammonia (mg/L)	0.01 ± 0.02	21.34 ± 1.07	39.84± 2.69	62.33 ± 0.92	80.22 ± 0.94	100.16 ± 0.47	–
Un-ionized ammonia (mg/L)	0.001 ± 0.001	0.592 ± 0.135	1.126 ± 0.119	1.585 ± 0.157	1.882 ± 0.236	2.117 ± 0.207	< 2[c*]
Nitrite (mg/L)	0.055 ± 0.019	0.216 ± 0.222	0.416 ± 0.225	0.174 ± 0.211	0.081 ± 0.072	0.059 ± 0.076	3[d]

Recommended values for the species according to: a) Weber and Kramer, 1983; b) Riehl, 1991; c) Latap et al., 2015; d) Doleželová et al., 2011 (estimated safe level value). * Secure values for fish in general.

Table 2. LC_{50} calculated, their 95 % confidence intervals and safe level of total ammonia and un-ionized ammonia to 24, 48, 72 and 96 h for pooled males and females, and separately males and females of *Poecilia reticulata*.

Gender	Hours	LC_{50} (mg/L of total ammonia)	Confidence interval (95 %)	Safe level (mg/L of total ammonia)	LC_{50} (mg/L of un-ionized ammonia)	Safe level (mg/L of un-ionized ammonia)
Pooled males and females	24	46.91	44.02 – 50.00	4.69	1.30	0.13
	48	45.03	41.83 – 48.48	4.50	1.25	0.13
	72	43.75	40.37 – 47.42	4.38	1.21	0.12
	96	42.45	39.02 – 46.19	4.25	1.17	0.12
Males	24	42.26	38.62 – 46.24	4.23	1.17	0.12
	48	41.28	36.36 – 46.88	4.13	1.14	0.11
	72	38.97	33.81 – 44.92	3.90	1.08	0.11
	96	37.33	31.96 – 43.60	3.73	1.03	0.10
Females	24	52.27	48.14 – 56.76	5.23	1.45	0.15
	48	49.81	45.81 – 54.15	4.98	1.38	0.14
	72	49.49	45.27 – 54.10	4.95	1.37	0.14
	96	48.34	44.13 – 52.95	4.83	1.34	0.13

lethal concentration for 50 % of males after 96 hours (LC_{50}-96h) was 37.33 mg/L of total ammonia and 1.03 mg/L of un-ionized ammonia (Table 2; Fig. 1b).

For female of *P. reticulata* there was no mortality in the control treatment. At concentrations of 20 and 40 mg/L the average mortality after 96 hours was of 2 ± 1 % e 20 ± 12 %, respectively. The concentration of 60 mg/L caused mortality of 73 ± 13 % after 96 hours, and within the first 24 hours the average mortality of females reached 57 %. There has been 100 % mortality on the treatments with concentrations of 80 and 100 mg/L of total ammonia in 24 hours. The lethal concentration for 50 % of the females after 96 hours was 48.34 mg/L of total ammonia and 1.34 mg/L of un-ionized ammonia (Table 2; Fig. 1c).

The safe levels ranged from 3.73 to 5.23 mg/L of total ammonia, and 0.10 to 0.15 mg/L of un-ionized ammonia (Table 2).

DISCUSSION

The LC_{50}-96 h value found in the present study for combined males and females of *P. reticulata* (1.17 mg/L of un-ionized ammonia) corroborates a previous study that found LC_{50}-96 h of 1.24 mg/L of un-ionized ammonia (Rubin and Elmaraghy, 1977). However, Rubin and Elmaraghy (1977) evaluated the sensitivity to ammonia for immature juveniles of *P. reticulata*, while our study used adult individuals. In addition, the authors tested the toxicity without considering the difference between sexes. Toxicity of chemicals compounds to aquatic organisms may vary depending on the species, size, age and sex (Dunnette, 1992; Allen et al., 2004; Pandey et al., 2005; Nwani et al., 2010). In this way, our results showed that females of *P. reticulata* are more resistant to ammonia than males. This difference in ammonia tolerance has important implication for ornamental aquaculture, since males of *P. reticulata* have higher commercial value than females due to vibrant colors (Mukherjee et al., 2009), one of the major factors that determine the price of ornamental fish (Mukherjee et al., 2015). In addition, the recommendation of a toxicity reference value that does not consider this difference may cause production losses, since the toxicity value for males is relatively lower than that observed for the combined sexes.

A difference in the sensitivity depending on sex to *P. reticulata* was observed to other substances like glyphosate herbicide (Antunes, 2013), in which females were more resistant. The greater sensitivity of males to ammonia may be related to animal size (Mayer and Ellersieck, 1986) given that Poeciliidae have a pronounced sexual dimorphism, with females larger than males. Smaller fish are more sensitive to toxic agents than larger fish because they receive higher dosages of toxic agent per weight unit (Piedras et al., 2006).

The different response after ammonia exposure can also be explained by the difference in body lipid content, since the lipids accumulated in the body protect the organism against the toxic effects of lipophilic chemicals (Geyer et al., 1994), such as ammonia. Lipophilic chemicals accumulate in adipose tissue, decreasing the fraction of the compound that can reach the organs (Geyer et al., 1994; Ballesteros et al., 2007). Thus, Poeciliidae females are more resistant to lipophilic chemicals than males (Geyer et al., 1994; Ballesteros et al., 2007), since females store fat aiming to allocate that energy for reproduction (Meffe and Snelson, 1993; Heulett et al., 1995).

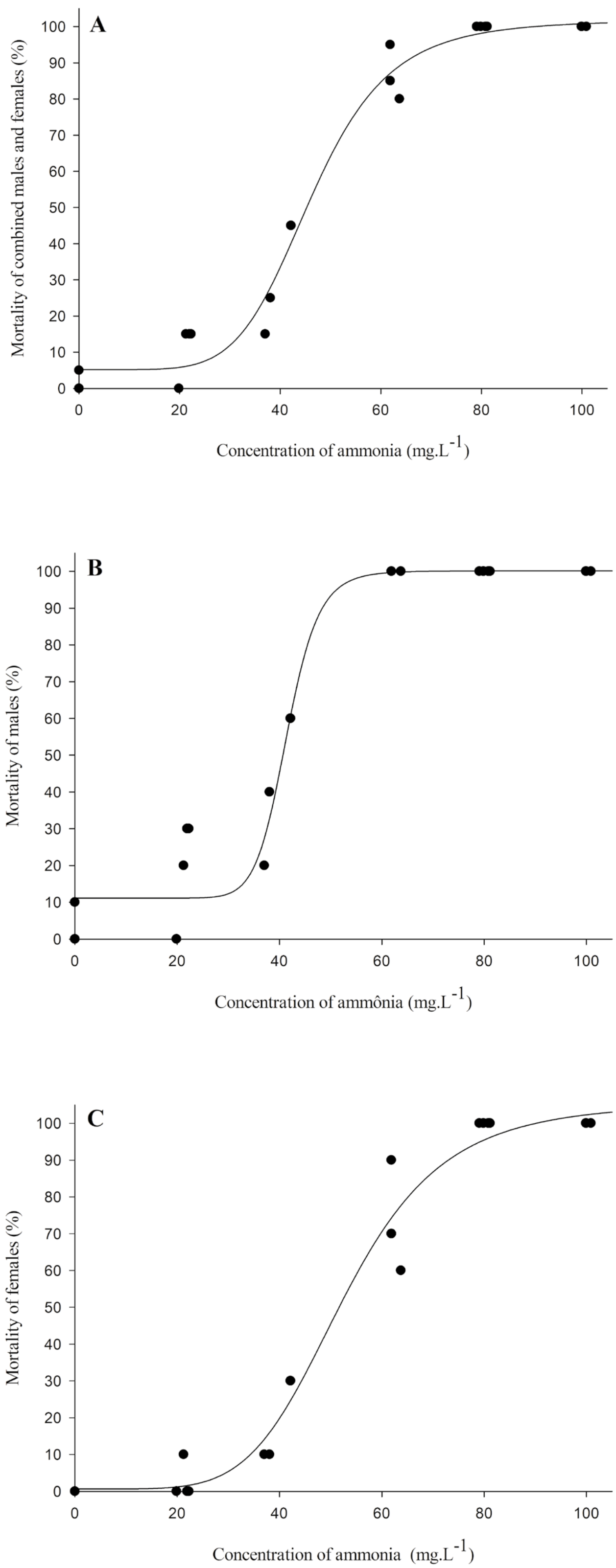

Figure 1. Mortality curves for *Poecilia reticulata* after 96 hours of exposure to ammonia. a) Combined males and females; b) males; c) females.

CONCLUSION

From the productive point of view, the greater sensitivity of males to ammonium exposure compared to that registered by females can cause economic losses, since males have a higher market value. Thus, recommendations for the productive management of *P. reticulata* must take into account the safety level of non-ionized ammonia for males, *i.e.* 3.73 mg/L of total ammonia or 0.10 mg/L of non-ionized ammonia, in order to achieve the best productive performance.

ACKNOWLEDGMENTS

The authors thank Dr. Eduardo Luiz Cupertino Ballester for providing the necessary structure and materials to carry out the experimental work. S. C. Forneck thanks the Coordination for the Improvement of Higher Education Personnel (Capes) for the scholarship (Funding Code 001). F. M. Dutra thanks the National Council for Scientific and Technological Development (CNPq) for the scholarship (Process no. 155338/2018-8).

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