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Effects of iodized salt on the histopathology of the gills and liver in *Rhamdia quelen* and *Metynnis maculatus*

Thayzi de Oliveira Zeni[1,2], André Luiz Vicente[1], Gisela Geraldine Castilho-Westphal[1,2], Aline Horodesky[1,2], Roberto Montanhini Neto[1,3] & Antonio Ostrensky[1,2,4]

[1]Department of Animal Sciences, Integrated Group for Aquaculture and Environmental Studies, Curitiba, Paraná, Brazil

[2]Department of Biological Sciences, Federal University of Paraná, Curitiba, Paraná, Brazil

[3]Adisseo France S.A.S., Antony, France

[4]Department of Animal Sciences, Federal University of Paraná, Curitiba, Paraná, Brazil

Correspondence: T de Oliveira Zeni, Department of Animal Sciences, Integrated Group for Aquaculture and Environmental Studies, Rua dos Funcionários, 1540 Juvevê, Curitiba, Paraná, Brazil. E-mails: thayzi@yahoo.com.br; thayzi_zeni@yahoo.com.br

Abstract

Table salt has been used as a reference substance in toxicological studies on fish and as an antiparasitic agent in aquaculture. The objectives of this work were to evaluate the sensitivity of *Rhamdia quelen* and *Metynnis maculatus* to iodized salt and to assess the possible changes in the gills and liver resulting from subchronic exposure of fish to this compound. The iodized salt toxicity levels after 96 h of exposure were 11.4 and 10.8 g L^{-1} for *R. quelen* and *M. maculatus*. None of the observed changes in the livers of the studied fish could be unequivocally correlated with salt exposure. In turn, it could not be entirely ruled out that the changes found in the gills, including epithelial lifting, oedema formation and vascular congestion, were caused by exposure of fish to salt.

Keywords: reference substance, biomarker, sensitivity

Introduction

Biological markers or biomarkers, are defined as measurable biological tools, which vary proportionally to the extent of exposure of a given organism to one or more pollutants (Gagnon & Hodson 2012). Thus, a biomarker can function as a stress indicator at several levels of biological organization (Muñoz *et al.*, 2015). Biomarkers can be used to evidence changes in the below mentioned morphological parameters: biochemical, physiological (Melancon 1995), behavioural, cellular and energetic responses; and the body fluids of organisms exposed to xenobiotics (Livingstone 1993).

In turn, toxicity tests are used to detect and to assess the inherent capacity of a given toxic agent to produce deleterious effects in test organisms (Cruz *et al.*, 2008).

Among the main tools in toxicity testing, reference substances are used for comparisons between the results from toxicity tests, interlaboratory calibrations, the comparison of methods using different organisms, evaluations of reproducibility and validations of toxicity tests (Hunt & Anderson 1989; Resgalla & Laitano 2002). Reference substances further allow for a better understanding of the effects of xenobiotics on aquatic organisms (Jorge & Moreira 2005). These substances also help unveil differences in the sensitivity of different batches of one and the same test organism as a result of certain factors, such as acclimation, the occurrence of diseases, the density used and the stress level the organisms were subjected to prior to testing (Ong & Din 2001).

Thus, an ideal reference substance must be toxic at low concentrations, rapidly lethal, stable, non-selective, and detectable through standard analysis techniques, and it must yield consistent laboratory results (Ong & Din 2001). Reference substances should also be commonly available for easy acquisition, be recognizably used in scientific studies, cover different classes of substances (including pharmaceutical products, biocides or industrial

chemicals) and exhibit specificity regarding the toxic response (e.g. in the liver, gills, blood or nervous system (Hoffmann *et al.*, 2010). Because salt (NaCl) meets most of the aforementioned requirements, it is considered an adequate reference substance for toxicological studies (USEPA, 2002; ABNT, 2004).

Refined salt or table salt, is widely used in fresh water aquaculture, despite the iodine in its composition, which is an essential micromineral to all animal species (Miller & Ammerman 1995); however, refined salt can be toxic to fish at high concentrations (He *et al.*, 2014).

Among other purposes, salt is used during live fish transportation to reduce the osmotic difference between the external environment and the plasma of the fish, thus reducing stress and increasing animal resistance to diseases (Wurts & Durborow 1992; Garcia, Becker, Copatti, Baldisserotto & Radünz Neto 2007; Oyoo-Okoth, Cherop, Ngugi, Chepkirui-Boit, Manguya-Lusega, Ani-Sabwa & Charo-Karisa 2011; Tacchi, Lowrey, Musharrafieh, Crossey, Larragoite & Salinas 2015). Salt is also used in the treatment and prevention of diseases caused by fungi, bacteria and parasites (Anderson 1992; Wangen 2012).

If used correctly, refined salt is safe for both fish and aquatic ecosystems (Altinok & Grizzle 2003; Garcia *et al.* 2007; Wangen 2012). However, excess salt can cause osmoregulatory dysfunctions in these animals (Barton & Zitzow 1995), leading to increased oxygen consumption (Maceina, Nordlie & Shireman 1980; Wang, Lui, Po & Fan 1997; Altinok & Grizzle 2003) and reduced food intake, factors that affect fish growth (Luz, Martínez-Álvarez, Pedro & Delgado 2008).

The objectives of the present work were to test the sensitivity of two very common fish species in Brazilian aquaculture, *Rhamdia quelen* (silver catfish) and *Metynnis maculatus* (spotted metynnis), to exposure to refined salt and evaluating responses using gill and liver histopathology as biomarkers of the exposure of fish to salt.

Materials and methods

Fry of *R. quelen* and *M. maculatus* were purchased from a commercial fish distributor in the Municipality of Curitiba, Paraná State, Brazil. Animals were packaged and transported in plastic bags (containing 1/3 water and 2/3 pure oxygen) to the Research Laboratory of Aquatic Organisms (Laboratório de Pesquisas com Organismos Aquáticos – LAPOA) of the Integrated Group of Aquaculture and Environmental Studies (Grupo Integrado de Aquicultura e Estudos Ambientais – GIA/UFPR), Curitiba, Paraná State, Brazil, where all experiments were conducted.

The fish were gradually acclimated to the temperature and pH of the receiving tanks. Animals were then subjected to a prophylactic saltwater (6 g L^{-1}) bath for 2 h. Subsequently, the fish were transferred to a polyethylene tank (1000 L) equipped with an internal biological filter and were kept under constant aeration (6.0 ± 1 mg L^{-1} dissolved oxygen) and controlled temperature (25 ± 1 °C) for either 60 days (for fish in the acute experiment) or 70 days (for fish in the subchronic experiment). Animals were fed *ad libitum* on a daily (Akaishi, Silva De Assis, Jakobi, Eirasstofella, ST-Jean, Courtenay, Lima, Wagener, Scofield & Oliveira-Ribeiro 2004) basis with commercial pellet food containing 35% crude protein.

Independent and sequential experiments were performed individually for each species.

Pilot experiment

To identify the concentrations to be used in the acute experiment, fish (density of 5 fish/aquarium) were transferred to glass aquaria (10 L) for 96 hours under aeration and controlled temperature, photoperiod 12L/12D, to nine concentrations ranging from 7 to 15 g L^{-1}. Fish exposed to concentrations of 8 and 9 g L^{-1} did not die, while in concentrations above 13 g L^{-1}, the mortality was 100%. Because of this, in the acute experiment, fish were exposed at five concentrations (9, 10, 11, 12 and 13 g L^{-1}).

Acute experiment

In the acute experiment, individuals (*R. quelen:* 7.45 ± 2.21 g and 8.9 ± 0.94 g; *M. maculatus:* 3.76 ± 1.95 g and 4.6 ± 0.73 cm) were transferred to glass aquaria (14 L). Fish (at a density of 7 fish/aquarium) were acclimated to the experimental conditions (controlled aeration and temperature and once-daily feeding) for 96 h (Agamy 2013). Salt was administered at five different concentrations (9, 10, 11, 12 and 13 g L^{-1}), whereas the control group received no salt. At the end of this period, salt was dissolved and added to the

aquaria at the respective experimental concentrations. The experiment consisted of the tested species to salt for 96 h. Analyses were performed every 24 h, and water quality parameters were recorded, exhibiting no significant differences. Temperature (kept at 23 ± 1 °C) and dissolved oxygen concentration (kept at 6.5 ± 1.0 mg L^{-1}) were measured using a portable oximeter (YSI 550A, USA). The pH value (median ranging between 6.94 and 7.31) was assessed with a benchtop pH meter (AZ86505, Brazil). Total ammonia nitrogen (TA-N = NH_3+NH_4^+) (median ranging between 0.43 and 0.68 mg L^{-1}) was assessed by spectrophotometry, according to the method described by Baptista, Baumgarten and Niencheski (1987). The concentration of gaseous ammonia (NH_3–N) (median of 0.02 for all treatments) was calculated according to the method described by Ostrensky, Marchiori and Poersch (1992).

At the end of the analyses, fish were fed, and 100% of the water volume of the aquaria was changed. Median lethal concentrations (LC_{50}) for 24, 48, 72 and 96 h were calculated using Probit Analysis Software (Russell, Robertson & Savin 1977).

Subchronic experiment

For subchronic exposure, three concentrations were used in the following percentages of LC50-96 h: 25, 55% and 80%. The highest concentration was chosen, since in the acute experiment, no animal died after exposure to concentration of 9 g L^{-1} for 96 h.

Because of this, in the subchronic 40-day experiment, fish (*R. quelen:* 9.61 ± 1,45 g and 8,1 ± 1,03 cm, *M. maculatus:* 8.94 ± 2.15 g and 7,6 ± 0,88 cm) were subjected to three different concentrations of iodized salt in the water (3, 6 and 9 g L^{-1}), accompanied by a control group that received no salt, and all tests were performed in triplicate. For this experiment, 15 fish per tank, each containing 50 L of water, were used. In an additional tank, 15 fish were acclimated for 96 h and then immediately killed and processed for histopathology. The latter tank served as a reference for the comparison of changes, representing the condition prior to the beginning of the experiments. At day 30, fish from all treatments were transferred to freshwater (0 salinity) and remained there for 10 days.

At 20, 30 and 40 days after the beginning of the experiment, five individuals were removed from each aquarium, anesthetized with clove oil (35 μl L^{-1}), immobilized by severing the spinal cord and killed by means of exsanguination; the fish were then processed for subsequent analysis (Ethics committee for animal from the biological science section of the UFPR – 23075.064636-2015-04). Water quality parameters were recorded every 48 h, following the methods described above for the acute experiment. After animal feeding and water analysis, 30 % of the water volume in the tanks was changed. There was no difference in experimental conditions between the treatment and control groups. Temperature and dissolved oxygen concentration were kept at the same levels as in the acute experiment (25 ± 1 °C; 6.5 ± 1.0 mg L^{-1} respectively). The median pH value ranged from 6.98 to 7.34. Total ammonia ranged from 0.43 to 0.68 mg L^{-1} of TA–N, and the median gaseous ammonia was 0.02 mg L^{-1} of N–NH_3.

Fish were fed twice a day with commercial fish food (Hued, Oberhofer & Bistoni 2012). The feed was made available to the fish for 10 min and then removed to avoid decreasing water quality.

Histological analysis

After killing, fish were weighed (total weight measured with a Sartorius Analytic Scale, Germany) and measured (both total and standard lengths, with a calliper; Vonder, Brazil). Fish were then immersed in AFA (alcohol, formalin, acetic acid) fixative for 48 h (Mela, Guiloski, Doria, Randi, Ribeiro, Pereira, Maraschi, Prodocimo, Freire & Silva de Assis 2013), and a gill arch and a liver fragment were extracted from each specimen and were subjected to routine histological processing (Beçak & Paulete 1976). The test specimens were cut into 5-μm thick sections using a microtome and were stained with Harris' haematoxylin and eosin (HE) (Horobin & Brancoft 1998).

Permanent slides were analysed according to two impact indices. The organ index (I_{org}), which expresses the degree of damage caused by a given change to the analysed organ [according to the method proposed by Bernet, Schmidt, Meier, Burkhardt-Holm and Wahli (1999)].

$$I_{org} = \sum_{rp} \sum_{alt} (\alpha_{or\ rp\ alt} \times W_{or\ rp\ alt})$$

where org = organ, alt = alteration, rp = reaction pattern, α = lesion extension and W = pathological relevance of the alteration.

The total gill impact index (TGII) establishes the ratio between the sum of all changes and the total number of analysed gill lamellae per fish (Cardoso 2006).

$$\mathrm{GII} = (N_1/N_2)$$

where N_1 = number of altered lamellae, and N_2 = number of lamellae analysed.

The sum of the gill impact indices from all lamellae analysed was obtained.

$$\mathrm{TGII} = \sum_{i=1}^{5} \mathrm{GII}_n$$

where TGII = total gill impact index and GII_n = gill impact index of lamella n.

Slides were analysed using an Olympus BX41 phase contrast microscope (Japan) and were evaluated with Dino® Software (2012). Gills from all fish were analysed, whereas only the livers of three fish per aquarium for each concentration were analysed, totalling nine fish per treatment for each experimental period.

Statistical analysis

Data from histopathology and water quality parameters were subjected to the Shapiro–Wilk test for normality. The results obtained at the different concentrations and exposure times were compared using the Kruskal–Wallis test, followed by general linear models test. The overall results of each tested species were compared using the Mann–Whitney *U*-test. Specific tissue changes observed in the treatments applied compared to their controls were analysed by means of the proportions test. All statistical analyses were performed using Statistica Software, version 8.0®.

Results

Acute exposure

During the acute experiment (LC_{50}), fish exposed to control and low-salt concentrations (9 and 10 g L^{-1}) swam more actively than fish exposed to higher salt concentrations. At concentrations above 10 g L^{-1}, fish did not feed past day one of the experiment. No fish from the control treatment died during the experiment.

Rhamdia quelen was relatively more tolerant to salt than *M. maculatus*. The 96-LC_{50} calculated for *R. quelen* juveniles was 11.4 g L^{-1} (11.1–11.8), whereas that of *M. maculatus* was 10.8 g L^{-1} (10.4–11.1). The toxicity curve obtained for *R. quelen* exhibited barely any changes between 24 and 96 h. In contrast, the stabilization of salt toxicity for *M. maculatus* occurred after 72 h of exposure (Fig. 1).

Subchronic exposure

In the gills of fish subchronically exposed to salt, 11 types of tissue lesions and infestation with the parasitic ciliate *Ichthyophtirius multifiliis* were recorded. For *R. quelen*, fish exposed to salt exhibited higher prevalence rates of vascular congestion, epithelial lifting and oedema formation than fish from the control group. These results were not confirmed in *M. maculatus*, which only exhibited significant differences between treatment and control with respect to lamellar fusion and the presence of parasites. In both cases, control fish exhibited higher prevalence rates than those exposed to salt (Table 1 and Fig. 2).

Regarding the impact indices, TGII were higher in *R. quelen* specimens exposed to 9 g L^{-1} of salt. The lowest indices were measured 20 days after the beginning of the experiment, and the highest after 40 days, i.e. 10 days after transferring the

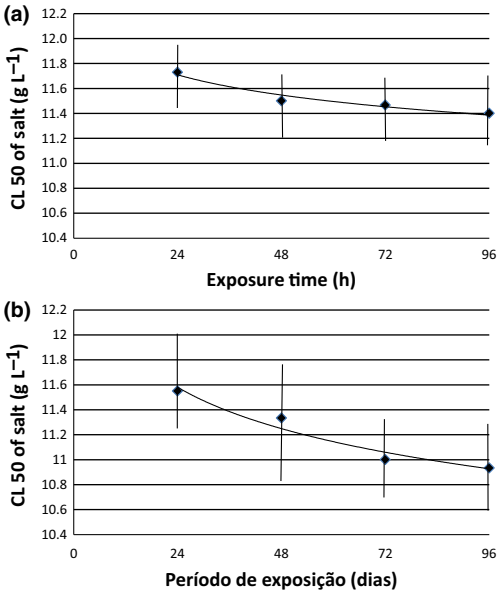

Figure 1 LC50 curves of iodized salt, with the respective confidence intervals, for (a) *Rhamdia quelen* and (b) *Metynnis maculatus*.

Table 1 Frequencies (in %) of *Rhamdia quelen* (silver catfish) and *Metynnis maculatus* (spotted metynnis) specimens with changes in the gills of control and iodized salt (NaCl)-exposed groups

	Frequency (%)			
	Rhamdia quelen		*Metynnis maculatus*	
Changes	Treatments *n* = 135	Control *n* = 60	Treatments *n* = 135	Control *n* = 60
Pigment accumulation	0.74	0.00	0.00	2.00
Aneurism	0.00	0.00	0.74	2.00
Vascular congestion	25.19[a]	10.00[b]	6.67	8.00
Epithelial desquamation	0.74	2.00	0.00	0.00
Epithelial lifting	64.44[a]	26.00[b]	73.33	62.00
Oedema	37.04[a]	10.00[b]	66.67	54.00
Lamellae under regeneration	4.44	0.00	0.00	0.00
Lamellar fusion	4.44	2.00	3.70[b]	14.00[a]
Haemorrhage	0.00	0.00	0.74	0.00
Epithelial hyperplasia	29.63	18.00	30.37	40.00
Epithelial hypertrophy	3.70	2.00	2.22	8.00
Parasites	6.67	8.00	0.74[b]	8.00[a]

Letters indicate statistically significant differences ($p < 0.05$) between treatments and control, according to the proportions test.

fish to water without significant salt concentrations. Furthermore, there was a constant pattern 30 days after the beginning of the experiment. Specifically, all indices of all concentrations, including the control, were higher in *M. maculatus* than in *R. quelen* (Table 2).

When grouping indices for analyses, i.e. considering the set of changes quantified at different salt concentrations and exposure times, *M. maculatus* exhibited higher gill histopathological indices than *R. quelen*.

Furthermore, exposure time had a significant influence on the histopathological indices in both species. In contrast, the tested salt concentrations had no significant effects ($p > 0.05$), which was also the case with respect to the interaction between exposure time and the tested salt concentrations.

Liver histopathology

In the liver analysis, seven tissue changes were observed, of which none exhibited differences between treatments and control in *M. maculatus*. However, in *R. quelen*, oedema and pigment accumulation were prevalent in the control compared to salt-exposed fish, a relationship that was inverted with respect to the occurrence of vascular congestion (Table 3 and Fig. 3).

After calculating the indices of tissue changes, *M. maculatus* specimens exhibited increased THII and I_{org} indices over the experimental period when exposed to a concentration of 3 g L^{-1} of salt (Table 4).

In the comparison of the indices between both species, differences were only observed for the exposure to 3 g L^{-1} of salt at 40 days after the beginning of the experiment. Of note, *M. maculatus* exhibited higher values than *R. quelen* regarding all two indices. However, after grouping all salt-exposure treatments and exposure times, there were no statistically significant differences between the two tested species with respect to the indices of liver changes.

Furthermore, no isolated effects of the tested salt concentrations or exposure time on the final results obtained for each species could be observed. However, the interactions between both factors exhibited significant effects ($p < 0.05$) on THII and I_{org} in *R. quelen*.

Discussion

One of the main applications of salt in aquaculture is its use as an antiparasitic agent (Tonguthai 1997; Miron, Silva, Golombieski & Baldisserotto 2003). The higher frequency of parasites (*I. multifiliis*) in *M. maculatus* specimens of the control group compared to salt-exposed fish supports the role of salt against fish ectoparasites. However, this result was not observed in *R. quelen*. Specifically, there was no difference between the treatments

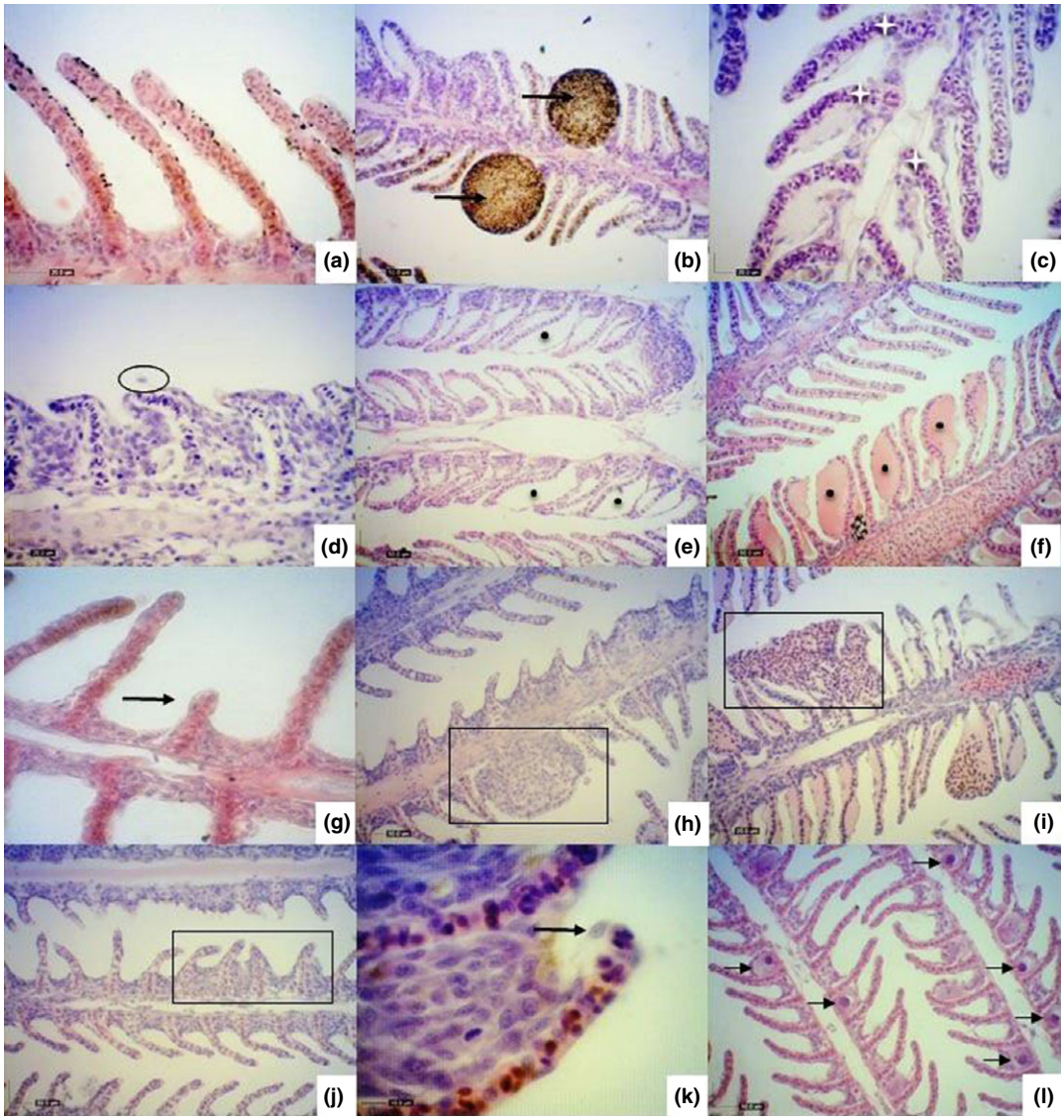

Figure 2 Photomicrograph of gills of *Rhamdia quelen* and *Metynnis maculatus* exposed to iodized salt and stained with HE (haematoxylin and eosin). (a) Pigment accumulation; (b) aneurysm (arrow) ; (c) vascular congestion (star); (d) epitelial desquamation (circle); (e) epithelial lifting (black ball); (f) oedema (black ball); (g) lamellae under regeneration (arrow) ; (h) lamellar fusion (rectangle); (i) haemorrhage (rectangle); (j) epithelial hyperplasia (rectangle); (k) epithelial hypertrophy (arrow); (l) parasites (arrow).

and the control with respect to the prevalence of the above parasites, which were detected on some *M. maculatus* specimens. The results support the hypothesis that at least with respect to *I. multifiliis*, the antiparasitic efficiency of salt is not guaranteed, as had already been observed during routine activities in our laboratory.

The tolerance of fish to a given compound can vary depending on the age/size of the specimens used Marchioro and Baldisserotto (1999). Nevertheless, the 96-h LC_{50} values calculated in this study (11.4 g L^{-1} for *R. quelen* and 10.8 g L^{-1} for *M. maculatus*) were very similar to those obtained in other studies available in the literature (Marchioro 1997; Marchioro & Baldisserotto 1999; Bringolf, Kwak, Cope & Larimore 2005; Zuanon, Salaro, Veras, Tavares & Chaves 2009).

Among the tissue changes observed in gills, this study, only four, namely vascular congestion, epithelial lifting, oedema formation and lamellar

Table 2 Indices of gill changes proposed by Cardoso (2006) (TGII) and Bernet *et al.* (1999) (I_{org}) ($p < 0.05$) applied over the course of *Rhamdia quelen* and *Metynnis maculatus* exposure to iodized salt (NaCl)

			Species			
Treatment	Conc. (g L^{-1})	Exposure time (days)	*R. quelen* TGII	*M. maculatus*	*R. quelen* I_{org}	*M. maculatus*
Control	0	4	0.87 (0.32–1.41)	1.00 (0.48–1.5)	2.97 (0–5.95)	3.51 (0.09–5.95)
	0	20	1.58 (0.78–2.27)	0.58 (0.32–1.04)	6.40 (2.52–20.99)	1.37 (0.00–5.00)
	0	30	0.48^{B} (0.34–0.53)	1.58^{A} (0.80–2.27)	1.47^{B} (0.50–2.97)	7.45^{A} (2.99–14.93)
	0	40	1.72 (1.11–2.71)	1.43 (0.86–2.12)	10.41 (5.50–14.96)	6.93 (2.06–10.99)
Exposure	3	20	0.33 (0.09–0.93)	0.76^{b} (0.20–1.20)	0.51 (0–3.50)	2.35 (0.00–5.00)
	3	30	0.64^{B} (0.40–1.93)	2.09^{aA} (1.11–4.00)	0.98^{B} (0–8.00)	10.93^{A} (3.57–23.56)
	3	40	0.65 (0.10–1.38)	1.39^{ab} (0.49–2.53)	2.47 (0–7.03)	4.90 (2.01–17.01)
	6	20	0.96 (0.78–1.15)	1.08 (0.23–2.67)	3.48 (2.97–4.99)	3.34 (0.00–14.59)
	6	30	0.73^{B} (0.18–1.08)	1.61^{A} (0.84–2.92)	2.00^{B} (0–4.00)	6.45^{A} (3.47–16.91)
	6	40	0.48 (0.38–1.61)	0.70 (0.53–1.73)	0.96 (0–8.00)	1.95 (0.00–8.97)
	9	20	0.38^{bB} (0.22–0.54)	2.10^{A} (0.88–2.47)	0.48 (0–2.00)^{B}	11.47 (2.98–14.00)^{A}
	9	30	0.88^{abB} (0.48–1.01)	1.80^{A} (1.09–3.01)	1.97^{B} (1.01–5.01)	10.00^{A} (4.00–16.50)
	9	40	1.15^{a} (0.57–1.47)	1.52 (0.87–2.05)	3.46 (1.01–7.00)	6.00 (0.00–11.00)
Exposure		Total	0.66^{B} (0.32–1.29)	1.41^{A} (0.70–2.52)	1.90^{B} (0.00–5.99)	5.92^{A} (2.08–13.93)
Concentration			0.462	0.811	0.126	0.866
Exposure time			0.043	0.003	0.063	0.003
Conc. × exposure time			0.220	0.723	0.235	0.835

Uppercase letters in the rows represent differences between species according to the Mann–Whitney *U*-test ($p < 0.05$); lowercase letters in the columns represent differences within each tested concentration in the course of the experimental period, according to the Kruskal–Wallis test ($p < 0.05$). Statistical significance of the relationship between concentration and exposure time was assessed by means of General Linear Models (GLM) test. Conc. – Salt concentration (g L^{-1}) to which fish were exposed.

Table 3 Percentages of *Rhamdia quelen* and *Metynnis maculatus* specimens exhibiting liver changes in the control and iodized salt (NaCl)-exposed groups

	% affected fish			
	R. quelen		*M. maculatus*	
Changes	Exposed to salt *n* = 81	Control *n* = 24	Exposed to salt *n* = 81	Control *n* = 24
Pigment accumulation	31.58^{b}	66.67^{a}	20.63	29.41
Vascular congestion	47.37^{a}	11.11^{b}	36.51	23.53
Macrovesicular fatty degeneration	3.51	0.00	4.76	5.88
Hyaline degeneration	0.00	0.00	4.76	5.88
Hydropic degeneration	3.51	0.00	0.00	5.88
Oedema	3.51^{b}	22.22^{a}	25.40	11.76
Haemorrhage	1.75	0.00	1.59	0.00

Letters indicate statistically significant differences ($p < 0.05$) between treatments and control, according to the proportions test.

fusion, exhibited differences in the frequency of occurrence between treatments and controls. However, none of these changes exhibited the same pattern when comparing between the analysed species. Of note, lamellar fusion was more frequently observed in the control group of *M. maculatus*. Hence, only the remaining three tissue changes cannot be ruled out *a priori* as being a result of exposure to salt.

Epithelial lifting, oedema formation, hyperplasia and lamellar fusion are considered non-specific changes that can be caused by an array of stressor agents, such as ammonium (Cengiz & Unlu 2006) or different pesticides (Albinati, Moreira, Albinati, Carvalho, Lira, Santos & Vidal 2009; Kumar, Prasad, Srivastava, Tripathi & Srivastav 2010; Al-Ghanbousi, Ba-Omar & Victor 2012), and can even be observed in animals collected directly in

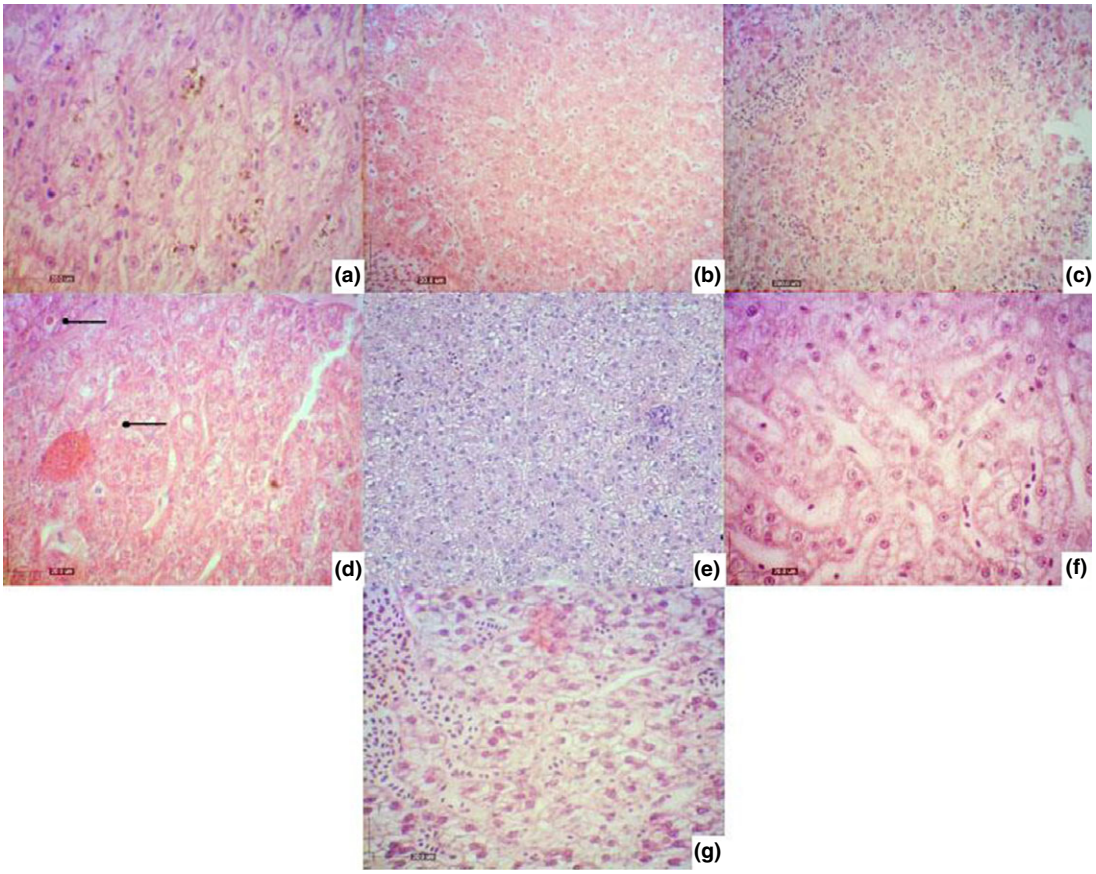

Figure 3 Photomicrograph of liver of *Rhamdia quelen* and *Metynnis maculatus* exposed to iodized salt and stained with HE (haematoxylin and eosin). (a) Pigment accumulation; (b) vascular congestion; (c) macrovesicular fatty degeneration; (d) hyaline degeneration; (e) hydropic degeneration; (f) oedema and (g) haemorrhage.

the environment, with no signs of exposure to these pollutants (Camargo & Martinez 2007).

Vascular congestion was observed in all treatments and in both species. However, it was more prevalent in salt-exposed *R. quelen* specimens than in the respective control fish. The changes were observed in all treatments and in both species. However, in *R. quelen*, epithelial lifting, oedema formation and vascular congestion were more frequently observed in salt-exposed fish than in the control group.

If the evaluated histopathological indices were exclusively influenced by salt exposure, significantly increased indices should be expected after exposing fish to this agent. However, this was not the case. Additionally, the indices of tissue changes were not significantly related to the salt concentrations to which the fish were exposed but only to the exposure time.

Moreover, the results obtained in this study differed between the analysed species, indicating a species-specific relationship between gills and salt tolerance.

Among the tissue changes observed in the liver, none was more prevalent in treatments than in the control group in *M. maculatus.* In contrast, in *R. quelen*, three changes exhibited differences between treatments and control: vascular congestion, pigment accumulation and oedema formation. Of these changes, the latter two were more prevalent in the control than in the treated groups.

As with the gills, vascular congestion in the liver occurs due to the obstruction of blood flow, causing blood stasis. Congestion can be caused by either physical obstruction of small or large vessels or a failure in normal blood flow (Thomsom 1983). This change was observed with higher prevalence in salt-exposed *R. quelen* specimens than in the respective control, similarly to the results observed in the gills. A hypothesis could

Table 4 Indices of liver changes proposed by Cardoso (2006) (THII) and Bernet *et al.* (1999) (I_{org}) ($p < 0.05$) applied over the course of *Rhamdia quelen* and *Metynnis maculatus* exposure to iodized salt (NaCl)

Treatment	Conc. (g L^{-1})	Exposure time (days)	*R. quelen* THII	*M. maculatus*	*R. quelen* I_{org}	*M. maculatus*
Control	0	4	0.11 (0.00–0.11)	0	0	0
	0	20	0.06 (0.05–0.8)	0.01 (0.00 -0.05)	0.02 (0.00–0.16)	0
	0	30	5.00 (0.01–5.00)	3.87 (0.00–5.00)	28.83 (0.00–30.00)	25.86 (0.00–30.00)
	0	40	0	2.00 (1.14- 3.49)	0	11.95 (6.05–21.02)
Exposure	3	20	0.06 (0.04–0.07)	0.01^{b} (0.00–0.06)	0.26 (0.00–0.29)	0^{b}
	3	30	1.13 (0.00–1.58)	0.99^{ab} (0.06–1.07)	5.99 (0.00–30.00)	0.00^{ab} (0.00–2.02)
	3	40	0.01^{B} (0.00–2.75)	5.00^{aA} (4.29–5.00)	0.02^{B} (0.00–0.29)	11.95^{aA} (6.05–21.02)
	6	20	0.65 (0.20–4.11)	2.22 (0.00 -5.00)	0.09 (0.00–24.00)	0.00 (0.00–30.00)
	6	30	0.01 (0.00–1.58)	4.09 (0.00–5.00)	0.09 (0.00–7.51)	24.39 (0.00–30.00)
	6	40	0.01 (0.00–2.75)	0.04 (0.00–0.32)	0.09 (0–11.95)	0.00 (0.00–0.95)
	9	20	4.05 (2.17–5.04)	0.02 (0.00–5.00)	22.94 (18.04–30.00)	0.00 (0.00–30.00)
	9	30	2.55 (1.44–3.78)	2.50 (0.00–5.00)	12.94 (6.12–22.02)	15.00 (0.00–30.00)
	9	40	3.79 (1.21–5.00)	5.00 (2.53–5.00)	20.95 (6.04–30.00)	30.00 (15.00- 30.00)
Exposure		Total	0.96 (0.00–5.00)	0.27 (0.00–4.05)	0.01 (0.00–30.00)	0.01 (0.00–22.02)
Concentration			0.141	0.621	0.160	0.525
Exposure time			0.313	0.110	0.314	0.100
Conc. × exposure time			0.394	0.019	0.370	0.040

Uppercase letters in rows indicate differences between species, according to the Mann–Whitney *U*-test ($p < 0.05$), and lowercase letters in columns indicate differences within each tested concentration over the experimental period, according to the Kruskal–Wallis test ($p < 0.05$). Statistical significance of the relationship between concentrations and exposure times was assessed by means of general linear models (GLM) test. Conc. – Salt concentration (g L^{-1}) to which fish were exposed.

explain the occurrence of vascular congestion in this study: a possible fluid–electrolyte imbalance, leading to changes in membrane permeability and consequently to cell disruption, the formation of emboli and thrombi, and finally the obstruction of blood vessels, generating congestion. The emboli and thrombi formation were not observed in the histopathological sessions analysed.

Thus, among the observed changes, only vascular congestion observed in *R. quelen* specimens could not be ruled out *a priori* as having been caused by the exposure of fish to salt. Moreover, it must be noted that neither the salt concentration nor the exposure time of fish to salt exhibited a statistically significant relationship.

Thus, there is sufficient evidence to affirm that none of the changes observed in the livers of the analysed fish can be unequivocally correlated with salt exposure. However, the hypothesis that epithelial lifting, oedema formation and vascular congestion of the gills are caused by the exposure of fish to salt cannot be entirely ruled out.

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