Comparative Biochemistry and Physiology, Part C 206–207 (2018) 41–47

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Exposure to a Brazilian pulp mill effluent impacts the testis and liver in the zebrafish

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ARTICLE INFO

Keywords:
Testis
Biochemistry
Lactate
Lactate dehydrogenase
P38 mitogen-activated protein kinase
Oxidative stress
Morphology

ABSTRACT

While many studies have shown that pulp mill effluents can affect ovarian physiology in fish, far fewer studies have considered the effects in males. We conducted a lab study to examine the effects of effluent from a Brazilian pulp and paper mill on hepatic and testicular morphology and various aspects of testicular physiology in the zebrafish *Danio rerio*. Males were exposed to lab water (control) or 4% effluent for 14 days. Effluent exposure did not affect testis size as measured by the gonadosomatic index, but contributed to morphological changes in the seminiferous tubules. The number of cysts with histopathological changes was elevated in effluent-exposed fish and the number of cysts containing spermatids was significantly reduced. The testis of effluent exposed fish had reduced levels of lactate, elevated lactate dehydrogenase activity, increased levels of reactive oxygen species and reduced levels of phosphorylated P38 mitogen-activated protein kinase (pP38 MAPK). Separate studies showed that the addition of lactate to testicular tissue incubated *in vitro* increased the activation of P38 MAPK. Effluent exposure also increased vacuolization, necrosis, apoptosis, hyperemia, and fat infiltration of the hepatocytes. Collectively, we provide evidence of short term effects of pulp mill effluent on testicular and hepatic physiology and biochemistry in the zebrafish.

1. Introduction

For > 30 years, there have been reports that effluents from some pulp and paper mills have the potential to negatively affect fish populations. Many of these studies have shown effects of pulp mill effluents on reproduction (*e.g.* Munkittrick et al., 1998; Hewitt et al., 2008; van den Heuvel, 2010). Studies involving wild fish, *in situ* experiments, and laboratory *in vivo* tests conducted on a world-wide basis have documented reductions in sex steroid hormone levels, gonad size and fecundity, alterations in secondary sex characteristics, and delayed sexual maturity associated with exposure to pulp mill effluents or its constituents (*e.g.* Tana and Nikunen, 1986; Van Der Kraak et al., 1992; Kovacs et al., 2013). Other studies have shown that the liver is also affected by exposure to pulp mill effluents as evidenced by changes in gene expression, metabolism and morphology (Khan, 2010; Orrego et al., 2011; Costigan et al., 2012).

The purpose of this study was to evaluate the potential of treated effluent from the Klabin pulp and paper mill in Santa Catarina State in Brazil to affect liver and testicular morphology and the biochemistry of the testis in the zebrafish (*Danio rerio*). This was interest because few studies have tested effluents from South American pulp mills, and Brazilian mills in particular, for effects on fish. As the specific chemicals responsible for the biological effects of pulp mill effluents are not known, it was of interest to determine if Brazilian tree species or the mill process used would contribute to the toxicity of this effluent. As well, relative to studies conducted with female fish, few studies examine the responses of male fish to pulp mill effluents. In this study, adult zebrafish were exposed *in vivo* to effluent for 14 days and effects on testicular and hepatic structure were determined by histological evaluation. As well, a suite of biochemical responses within the testis were determined including measurement of lactate content, lactate dehydrogenase activity, total reactive species, and the amounts of P38 mitogen-activated protein kinase (P38 MAPK) and phosphorylated P38 mitogen-activated protein kinase (pP38 MAPK).

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https://doi.org/10.1016/j.cbpc.2018.02.005
Received 29 November 2017; Received in revised form 22 February 2018; Accepted 25 February 2018
Available online 27 February 2018
1532-0456/

2. Material and methods

2.1. Chemicals

Histological resin was obtained from Leica Biosystems (Nussloch, Germany). The indicator of reactive oxygen in cells 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Acrylamide and bis-acrylamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-P38 (sc-728) and anti-pP38 (sc-17852-R) were obtained from Santa Cruz Biotechnology (Dallas TX, USA). Peroxidase conjugated anti-rabbit IgG and the Immobilion™ Western Chemoluminescent Horseradish Peroxidase (HRP) substrate were obtained from Millipore (St. Charles, MO; Temecula, CA, USA). The test kits for Lactate (Ref: 138) and LDH (Ref: 086) were purchased from Labtest (Lagoa Santa, Minas Gerais, Brazil). All other chemicals were of analytical grade.

2.2. Pulp and paper mill effluent

Effluent was collected from the Klabin pulp and paper mill located in Correia Pinto in Santa Catarina State, Brazil. Pinus is used as the feedstock for the mill which uses a conventional Kraft bleaching process with the following sequence - O, C1, E1, D1, E2, where O represents the extended delignification, C1 the molecular chlorine treatment, E1 the first alkaline extraction, D1 the first extraction using chlorine dioxide and E2 second alkaline extraction. The effluent was treated in an aerated lagoon system with oxygen application and a 7 day retention time. The lagoon is exclusively for the treatment of the effluents generated by the pulp mill. After treatment, the effluent was released in the Canoas river. Effluent was collected after the last step of treatment and immediately before release to the river.

2.3. Animal care, maintenance and exposure

Male zebrafish weighing between 200 and 300 mg were used in this study. They were obtained from a commercial producer (Botiquarium Enterprise, Florianópolis, SC, Brazil) and housed in aquaria supplied with dechlorinated water pH 7.4 at 28 °C under a 12L:12D photoperiod. The fish were fed once a day.

All experiments were conducted using protocols approved by the Brazilian College of Animal Experimentation (Protocol CEUA/PP00968). For the *in vivo* exposures, male zebrafish (6 animals per group) were exposed semi-statically to lab water or 4% effluent in a 15L-aquarium with aeration. The experiments were 14 days in duration and the water was changed every two days. Testing of the effluent at a 4% dilution was based on previous studies of the toxicity of effluent from this mill on tilapia (*Oreochromis niloticus*) and reflected local riverine (fluvial) concentrations of the effluent (Zunino and Soares, 2007).

2.4. Sample collection

Fish were immobilized in ice cold water, euthanized by spinal transection and weighed. The testes were removed, weighed and separated for histology studies or homogenized for biochemical or Western blot analysis. The gonadosomatic index (GSI) was calculated as the testis weight/whole fish body weight × 100.

2.5. Histological analysis

For histological analysis, the testes and livers from six zebrafish from both control and effluent-exposed treatments were fixed in Bouin's solution for 24 h at room temperature. The samples were then dehydrated through a graded series of ethanol solutions and embedded in historesin. Serial sections (4 μm thickness) were cut and 12 to 20 slides with eight sections per slide were prepared for each specimen. The slides were stained with Hematoxylin–Eosin–Phloxyne and examined using a light microscope. The histological slides were analyzed qualitatively and quantitatively. For the testes, the numbers of cysts with histopathological changes and the number of cysts containing spermatids were counted on four different randomly chosen areas for each specimen. Each analyzed area was 0.0943 mm^2. As well, the number of spermatozoa was counted in four sections on two slides per testis (0.000885 mm^2).

2.6. Measurement of total lactate content and lactate dehydrogenase (LDH) activity

Testes collected after the 14 day exposure period were homogenized in cold Cortland's Buffer (124 mM NaCl, 5 mM KCl, 1.7 mM $CaCl_2$·2H2O, 3.4 mM NaH_2PO_4, 2.1 mM $MgCl_2$, 1.91 mM $MgSO_4$, 11.9 mM $NaHCO_3$, pH 7.6), centrifuged (2 min at 2000 × *g*) and the supernatant was collected. The samples were then analyzed for total lactate content and lactate dehydrogenase (LDH) activity by spectrophotometry based on the methods of Bergmeyer, 1983 and Burtis et al., 2007, respectively.

2.7. P38 and pP38 MAPK measurement

The amounts of P38 and pP38 MAPK were determined by polyacrylamide gel electrophoresis and immunoblotting. Testes were obtained from animals exposed *in vivo* for 14 days to effluent. In other tests, testes were obtained from untreated zebrafish and incubated *in vitro* for 30 min in Cortland's buffer supplemented with 10 mM sodium lactate to analyze the direct effect of lactate on P38 activation. The testes were homogenized in a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl pH 6.8, 1 mM Na_3VO_4, 0.2% triton X-100, 0.5 mM dithiothreitol, 1 mM benzamidine. The total protein concentration was determined based on Lowry et al., 1951. For the electrophoresis analysis, samples were boiled for 3 min in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8. Equal protein concentrations (50 μg/μl) were loaded onto 12% polyacrylamide gels and separated by discontinuous SDS-PAGE. Separated proteins were transferred to nitrocellulose membranes for 1 h at 100 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol and 0.25% SDS). The nitrocellulose membranes were incubated for 2 h in blocking solution (TBS: 0.5 M NaCl, 20 mM Trizma, plus 5% bovine serum albumin) and then incubated overnight at 4 °C with anti-P38 (1:100) or anti-pP38 (1:200). Membranes were incubated for 2 h with anti-rabbit IgG (1:1000) and immunoreactive bands were visualized using the Immobilon™ Western Chemiluminescence HRP substrate kit (Zanatta et al., 2011). The images of the band were captured using the ChemiDoc MP system and the optical densities determined with Image Lab Software (Bio-Rad).

2.8. Determination of testicular ROS

The total ROS generation in testes was detected using the fluorogenic probe H_2DCFDA. Testes were collected from fish that had been exposed to control water or 4% effluent for 14 days. The testes were homogenized in sodium phosphate buffer (20 mM NaH_2PO_4 and 140 mM KCl; pH 7.4), centrifuged (960 × *g*, 10 min, 4 °C) and the supernatant incubated in the presence of 1 mM H_2DCFDA for 30 min at 37 °C. H_2DCFDA oxidation was measured by spectrophotometry (excitation in 485 and emission in 520 nm) using the methods described by Halliwell and Whiteman (2004). The results were expressed as fluorescence units (UF)/μg of protein.

2.9. Effluent chemistry

Total phenols were determined using the colorimetric method based on the phenol and 4-aminoantipyrine (4-AAP) reaction. This approach uses 0.21 M sodium persulfate as a catalyst at pH 10 in ammonium

buffer. The concentration of phenols is proportional to the absorbance measured at 505 nm (Clescerl et al., 1999). Resin acids were measured by the methods described by Merilainen et al. (2007). Chemical Oxygen Demand (COD) was measured using the methods of (Clescerl et al., 1999). The sterols stigmasterol and β-sitosterol were measured by Gas Chromatography-Tandem Mass Spectrometry using the methods described in Milestone et al., 2012.

2.10. Statistical analysis

Data were expressed as mean ± S.E.M. or % of control of three independent experiments, with six samples in each group. ANCOVA was used to compare the GSI in control and effluent treated fish. An unpaired Student's *t*-test was used to determine significant differences between control and effluent treated groups in terms of biochemical measures, and morphological data in terms of the numbers of cysts with pathological changes or containing spermatids or the number of spermatozoa. Statistical analyses were performed using Graph Pad Prism6 software. Differences were considered significant at $p \leq 0.05$.

3. Results

3.1. Effluent chemistry

The final effluent used in this study contained high amounts of total phenols (3.8 mg/L) and resin acids (9.3 mg/L) and had a significant COD (210 mg/L). Sterols such as stigmasterol and β-sitosterol were below detection limits.

3.2. Effects of effluent exposure on GSI and testis histology

There were no differences in the GSI of control and effluent exposed fish as determined by ANCOVA ($p > 0.05$; $N = 26$ for controls and $N = 18$ for effluent exposed fish pooled across several studies; data not shown). In contrast, histological analysis revealed marked differences in testicular development of control and effluent-treated fish. The seminiferous tubules from control fish contained cysts at different phases of the spermatogenic wave. Cysts with spermatogonia, primary or secondary spermatocytes, and cysts with spermatids were found. The nucleus of Sertoli cells was evident in some cysts. The tubular lumen was filled with spermatozoa containing a head and tail (Fig. 1A). Each seminiferous tubule was surrounded by a thin layer of connective tissue, the intertubular region, with the interstitial or Leydig cells, blood vessels and collagen fibers (Fig. 1B). Testis from effluent-exposed fish exhibited a number of histopathological changes including disruptions in the germinal epithelium and large spaces in the intertubular region. There were cysts with cellular debris and apoptotic cells (Fig. 1C, D, E, and F). The number of cysts with histopathological changes was significantly elevated in effluent-exposed fish (Fig. 2A). The number of cysts containing spermatids was significantly reduced in effluent-exposed fish (Fig. 2A) but no changes were detected in the number of spermatozoa with effluent exposure (Fig. 2B).

3.3. Effects of effluent exposure on liver histology

The histological study of the liver tissue showed changes between animals of control and effluent-exposed groups (Fig. 1G, H, and I). In the control fish, the hepatocytes were arranged in rows containing two or more cells separated by sinusoids. Portal tracts were absent and biliary epithelial cells were located in the parenchyma of the liver. The hepatocytes were polyhedral with small vacuoles in cytoplasm (Fig. 1G). In the effluent-exposed fish there were different levels of histopathology among the animals analyzed. Two animals presented low, one medium and the others high levels of alterations. There was evidence of micro and macro-vacuolization of the hepatocytes, signs of necrotic cells in small or large areas, apoptotic cells, hyperemia, and low and elevated levels of fat infiltration (Fig. 1H, and I). The results of the histopathology analyses are presented in Table 1.

3.4. Effects of effluent exposure on testicular biochemistry

Fish exposed to effluent for 14 days had significantly reduced (66%) testicular lactate content compared to the control fish (Fig. 3A). LDH activity in the testes of exposed fish was increased 2.8-fold compared to the controls (Fig. 3B). Exposure to the effluent had no effect on P38 MAPK content as determined by immunoblotting (Fig. 4A). However, there was a significant (39%) reduction in the amount of pP38 MAPK in effluent exposed fish (Fig. 4B). The testis of fish exposed to the effluent for 14 days had increased total levels of ROS (61.9%), suggesting high oxidant activity in this tissue (Fig. 5).

To determine if the decreased amount of pP38 MAPK may be related to the reduction of total lactate, testes from untreated zebrafish were incubated *in vitro* in presence of lactate (10 mM) for 30 min and the amount of pP38 MAPK was analyzed by Western blot. Addition of lactate induced a significant increase in the amount of pP38 MAPK (Fig. 6).

4. Discussion

The current study showed that 14 day exposure to effluent from the Klabin pulp and paper mill negatively impacts testicular physiology in the zebrafish. This included changes in the biochemistry of the testis including effects on lactate content, LDH activity, pP38 MAPK content and ROS as well as spermatogenesis and development of the testis and liver These findings were consistent with early lab studies in which female tilapia exposed to 4% Klabin effluent for 28 days had altered plasma cholesterol, testosterone and 17β-estradiol levels, transitory changes in ovary and liver size and an increased incidence of intersex (Zunino and Soares, 2007; Soares unpublished).

Testicular morphology is widely used as means of detecting the impacts of anthropogenic chemicals in fish. Surprisingly few studies have considered the impacts of pulp mill effluents on testicular morphology as most of these studies have evaluated effects on testicular size (GSI) or secondary sex characteristics (*e.g.* Hewitt et al., 2008). Many of these studies showed little effect of pulp mill effluent exposure on testicular morphology. A study evaluating wild sucker (*Catostomus commersoni*) collected at reference and pulp mill exposed sites reported no differences in the proportions of spermatogonia, spermatocytes, spermatids, and spermatozoa in the testis (Parrott et al., 2010). Other studies with mosquitofish (*Gambusia affinis*), reported no histologic abnormalities in the testes collected at either at reference or pulp mill exposed sites in the Dengcun River in China (Hou et al., 2011). Like the previous studies we found no changes in the number of spermatozoa in zebrafish exposed to pulp mill effluent. However, there was a decrease in the number of spermatid cysts in the pulp mill effluent exposed fish and these fish had an increased incidence of morphological anomalies including apoptosis. These results were consistent with some previous studies showing testicular degeneration and individual or clustered apoptotic germ cells in adult zebrafish and fathead minnow testes following exposure to a range of endocrine disruptors including bisphenol A and ethinylestradiol (Johnson et al., 2009; Silva et al., 2012; Lora et al., 2016).

Intersex which includes the presence of testicular and ovarian tissues in one individual is often seen as an outcome of exposure to endocrine active compounds. For example, Chiang et al. (2015) showed intersex characteristics in the testis of juvenile rainbow trout which had been exposed to a Chilean pulp and paper mill effluents from 11 to 31 days post hatch. In studies with juvenile zebrafish exposed to pulp mill effluent between days 10–38 post-hatch and sampled at day 60 there was a higher percentage of males group exposed to the highest concentration of pulp mill effluent but no effects on spermatogenesis or the incidence of intersex (Orn et al., 2006). In our experimental

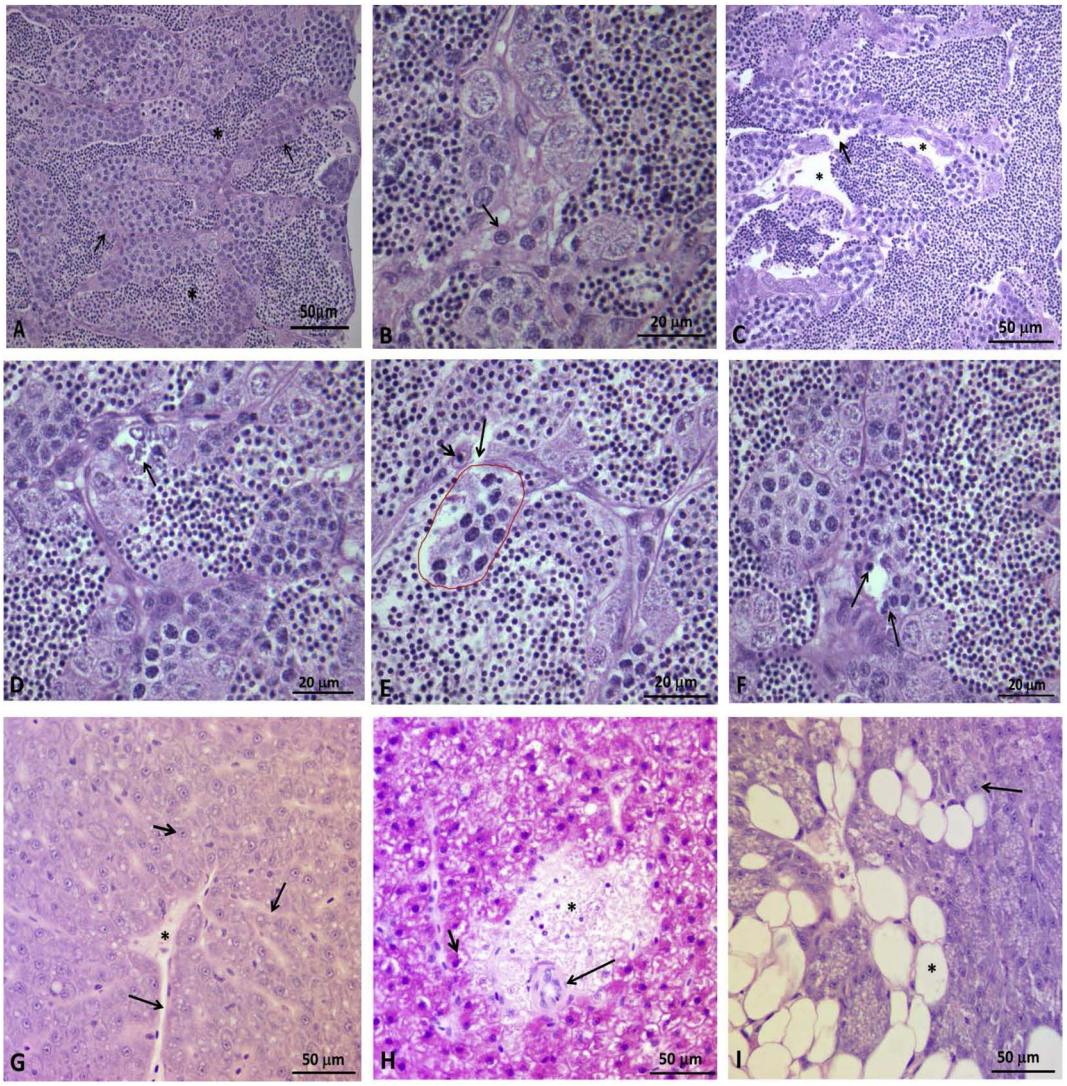

Fig. 1. Histology of testis and liver tissues stained with Hematoxylin-Eosin-Phloxyne. (A, B) Histological sections of control testis. (A) Segments of spermatogenic tubules with different germ cells cysts (arrows), and the lumen filled with spermatozoa (asterisk). (B) Intertubular region showing Leydig cells (arrow). (C, D, E, F) Spermatogenic tubules of effluent-exposed group. (C) Tubules with morphological alterations, such as disruption of the germinal epithelium (arrow), and large spaces in the intertubular region (asterisk). (D) Modified cysts with cellular debris, necrotic cells and apoptotic cells (arrow). (E) Tubule with ruptured basal lamina (large arrow), histiocytic cell (small arrow) and cyst with cellular debris (red line). (F) Apoptotic cells (arrows). (G, H, I) Liver tissues. (G) Control group liver with central venule (asterisk), hepatic sinusoids (large arrow), and hepatocytes (small arrow). (H, I) - Liver of effluent-exposed group. (H) Hepatocytes with vacuolization (small arrow), area of necrotic cells (asterisk), and hepatic arteriole (large arrow). (I) Liver tissue with fat infiltration (asterix) and hepatocytes with vacuolization (arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

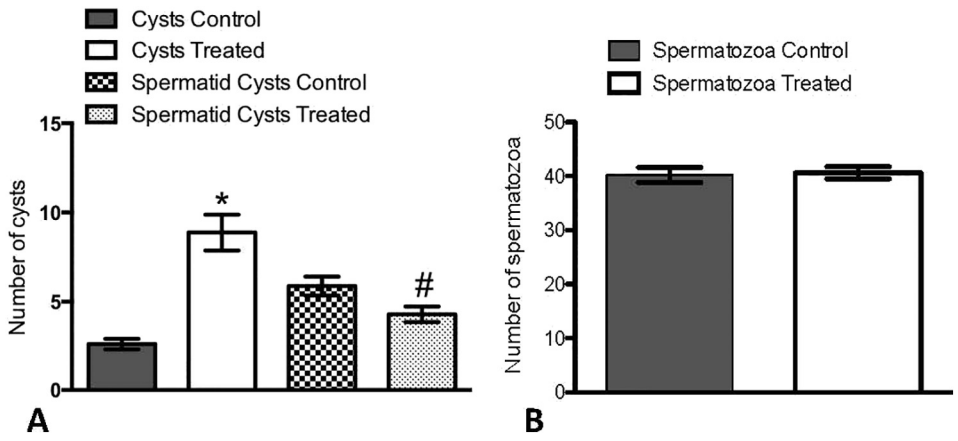

Fig. 2. Effects of pulp mill exposure on spermatogenesis. The numbers of modified cysts and cysts containing spermatids (A) and spermatozoa (B) in testes obtained from control and pulp mill exposed zebrafish. * significantly higher than in controls as determined using a *t*-test.

Table 1
Summary of the histopathological changes in the liver of zebrafish exposed to water (Control) or 4% pulp mill effluent for 14 days. Each fish was scored for the incidence of histopathological changes[a] and the median score for each response is reported. The results are based on the analysis of 3 control and 5 effluent treated zebrafish.

Histopathological finds	Control group	Effluent group
Hydropic degeneration	0	4.4
Necrosis	0	2.4
Apoptosis	0.3	2.2
Fat infiltration	0.3	2.2
Hyperemia	0.3	2.0

[a] (0) = no histopathology; (1) = histopathology in < 20% of fields; (2) = histopathology in 20–40% of fields; (3) = histopathology in 40–60% of fields; (4) = histopathology in 60–80% of fields; and (5) = histopathology in 80–100% of fields.

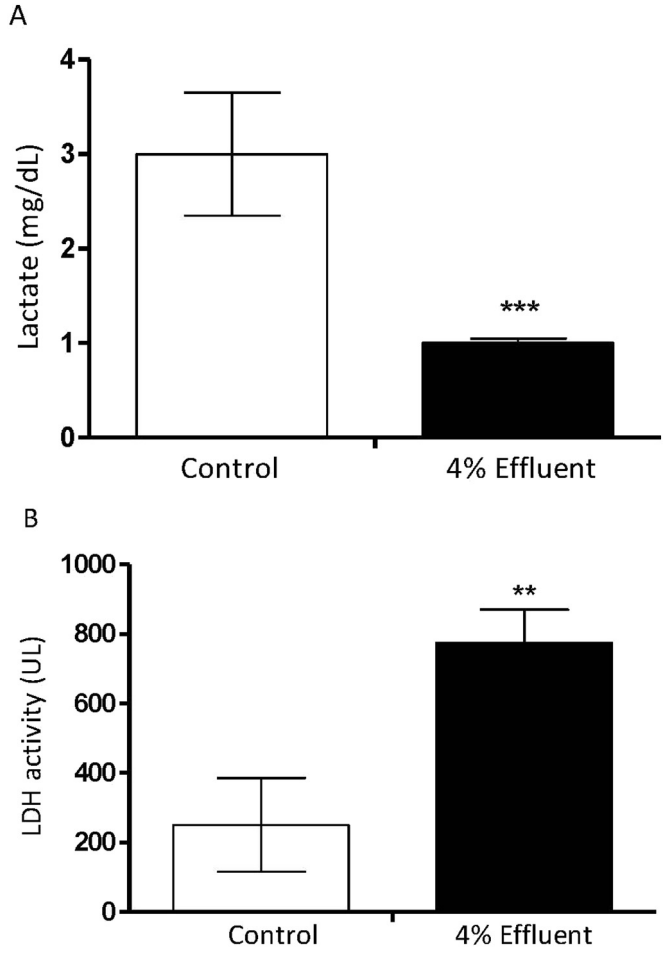

Fig. 3. Effects of pulp mill exposure on testicular lactate content and lactate dehydrogenase (LDH) activity. Lactate content (A) and LDH activity (B) in the testes from zebrafish exposed to reference water (control) or 4% pulp mill effluent for 14 days. Values were pooled from two experiments $N = 9$ fish. **Significant at $p < 0.01$ and ***$p < 0.001$ compared to the control, as determined using an unpaired *t*-test.

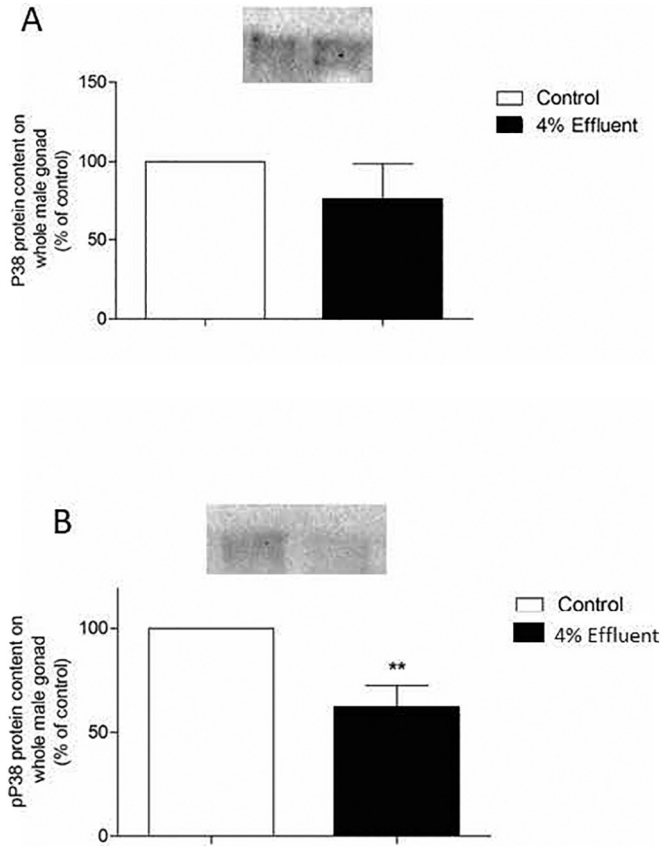

Fig. 4. The testicular mitogen-activated protein kinase (MAPK) system response to pulp mill effluent exposure. Testicular P38 (A) and phospho-P38 (pP38) MAPK content (B) in the testes from zebrafish exposed to reference water (control) or 4% pulp mill effluent for 14 days. The inserts show a representative Western blot for P38 and pP38 experiments. Values were pooled from two experiments $N = 9$ fish. **Significant at $p < 0.01$ and ***$p < 0.001$ compared to the control, as determined by an unpaired *t*-test.

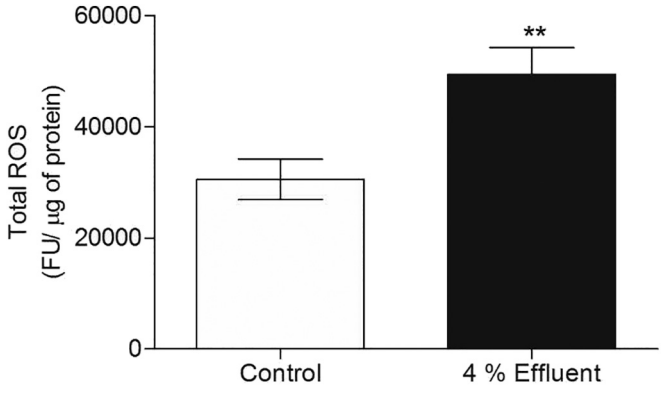

Fig. 5. Reactive oxygen species in the testis in response to pulp mill effluent exposure. Total reactive oxygen species (ROS) as determined by oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (H2-DCFDA) in testes from zebrafish exposed to reference water (control) or 4% pulp mill effluent for 14 days. Values are based on a $N = 6$ per treatment. **Significant at $p < 0.01$ when compared to the control as determined by unpaired *t*-test.

conditions, which included a shorter period of treatment (14 days), lower pulp mill effluent concentration (4%) and the treatment of adult fish, there was no evidence of oocytes in the testis.

It is well established that both the metabolism and morphology of the liver are sensitive to a range of environmental toxicants. In the current study, we used histology to evaluate the potential for pulp mill effluent to affect hepatic development. The changes in liver histology observed in this study were consistent with earlier work conducted in the western mosquitofish (*Gambusia affinis*). In that study, hypertrophy of hepatocytes, a significant increase in Kupffer cells, circulatory disturbances, widespread nuclear pycnosis, focal necrosis, and fatty degeneration were observed in the liver of fish collected downstream of a pulp and paper mill in China (Hou et al., 2011). These authors also reported cellular vacuolization in the liver. In rainbow trout exposed to polluted river water, the liver showed hepatocytes with numerous lipid vacuoles (Vigano et al., 2010).

To our knowledge this is the first study to examine the effects of

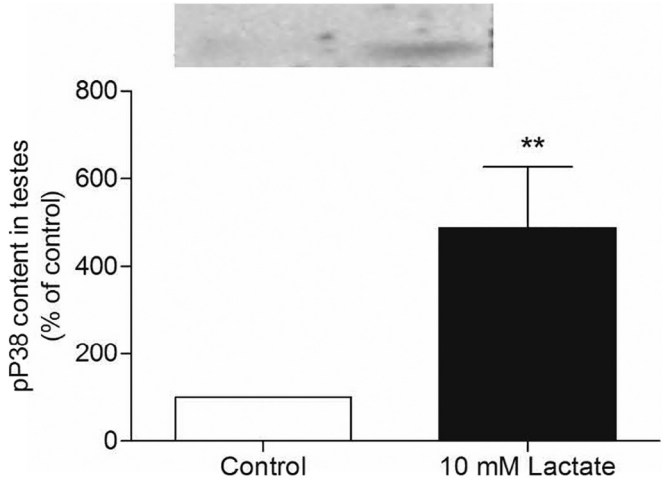

Fig. 6. Acute effects of pulp mill effluent on the mitogen-activated protein kinase (MAPK) system in the testis. Amount of pP38 MAPK as determined by Western blotting in testes from untreated zebrafish incubated *in vitro* for 30 min with either buffer alone or sodium lactate (10 mM). The insert shows a representative Western blot for pP38. $N = 5$ for buffer and lactate-treated testes.** Significant at $p < 0.01$ compared to the buffer treated testes.

pulp mill effluents on testicular lactate content in fish. The amount of lactate in the testis is considered an important indicator of testicular physiology/biochemistry since lactate production by Sertoli cells is essential for development and viability of germ cells in both fish and mammals (Crespo et al., 2016; Zanatta et al., 2011). Lactate produced by Sertoli cells by the action of lactate dehydrogenase is the main source of energy of germ cells and disruption of this pathway negatively affects normal reproductive physiology (Schulz et al., 2010; Zanatta et al., 2011). The exposure of zebrafish to treated effluent from the Klabin pulp and paper mill resulted in significantly lower levels of lactate in the testes and an associated increase in LDH activity. We assume that the elevated LDH activity in the pulp mill exposed fish is part of the compensatory mechanism in response to the low lactate levels.

Beyond its role as a cellular nutrient, lactate can indirectly act as a signaling molecule in pathways related to apoptosis and oxidative balance in mammals. Both low levels of lactate and high consumption of lactate can affect cell function. For example, the absence of lactate availability into germ cells leads to increased apoptosis (Erkkilä et al., 2002; Bustamante-Marín et al., 2012). The increased turnover of lactate can contribute to the generation of free radicals and oxidative stress. Lactate is known to activate protein kinases including Akt and p38 MAPK in germ cells from rats indirectly, by increase of ROS levels (Galardo et al., 2014).

Several studies have shown that exposure of fish to pulp and paper mill effluent is associated with increased oxidative stress and ROS formation (Oakes et al., 2005). For example, exposure of zebrafish to effluent from bleached sulfite or bleached kraft mills contributed to the increased production of 2-thiobarbituric acid reactive substances, lipid hydroperoxides, and the induction of ethoxyresorufin-*O*-deethylase activity (EROD) and fatty acid oxidase (FAO) activity. The oxidative effects of the effluent can be attributed to both its composition and origin, as well as the presence of metals with redox activity, such as copper (Oakes and Van Der Kraak, 2003). Our studies showed that total ROS was elevated in the testes of zebrafish exposed to 4% pulp mill effluent for 14 days. It will be interesting to determine if the Klabin effluent has elevated iron or copper content that may contribute to the increased oxidative stress.

P38 is a protein of the MAPK family that responds to extracellular stress signals including oxidative, physical or inflammatory stressors (Roux and Blenis, 2004). Several isoforms are expressed in various tissue types, including germ and Sertoli cells in mammals (Rossitto et al., 2015; Zhu et al., 2015) and its functions are thought to be conserved across the vertebrates including fish (Li et al., 2011; Zhu et al., 2015). The activated p38 MAPK (pP38) performs functions that are dependent on the cell type and the physiological/biochemical context (Petersen et al., 2005) including the activation and expression of genes related to oxidative defenses (Gutiérrez-Uzquiza et al., 2012). In Sertoli cells, P38 MAPK mediates actions such as cytoskeleton reorganization leading to phagocytic functions of the cell (Hai et al., 2014) and in germ cells is involved in the promotion of sperm motility, being also present in the acrosome (Zhu et al., 2015) The testicular microenvironment is considered highly vulnerable to oxidative stress due to the abundance of unsaturated fatty acids and ROS-generating system by mitochondrial metabolism and the actions of xanthine oxidase and NADP oxidase as well as cytochrome *p*450 (Rossi et al., 2016). Thus, the importance of P38 activation in response to oxidative changes provides a measure of cell protection.

Zebrafish exposed to pulp mill effluent for 14 days had decreased levels of activated pP38 MAPK in the testis. This can be occurring by a direct effect of effluent compounds inhibiting P38 MAPK activation or by an indirect effect on the extracellular and/or intracellular oxidant environment. Galardo et al. (2014) described that lactate metabolism to pyruvate in germ cells can activate the NAD(P)H oxidases, generating ROS. Based on the data from incubation of whole testis in presence of lactate, we detected an increase of pP38 MAPK total content, similar to that described by Galardo et al., 2014. So we suggest that the decreased lactate may be partly responsible for decreasing pP38 MAPK content in the testis of fish exposed to the effluent. In addition, the low lactate content, increased total ROS and reduced pP38 MAPK may all contribute to the impairment of ongoing spermatogenesis in the zebrafish.

The chemical composition of pulp mill effluents is complex and as a result it has been difficult to identify the causative agent(s) responsible for reproductive effects in fish. Defining these agents is complicated by the presence of a myriad of compounds in effluents and that their levels may vary with the type of wood being processed, the chemicals used and the products formed during pulp and paper mill production and treatment (Ratia et al., 2013; Lopes et al., 2013). As for the effluent from the Klabin mill there were low levels of sterols such as stigmasterol and β-sitosterol but high levels of phenols and resin acids. It is not clear if these contribute to the reproductive responses measured in this study. High levels of resin acids can be indicative of in-plant losses of treatment chemicals, wood furnish freshness as well as reduced effluent treatment efficiencies, and these have been associated with reproductive impairment in fish (Kovacs et al., 2013). In the case of kraft mills and mills using mechanical pulping, wood furnish, water usage and the type of bleaching and effluent biological treatment were not distinguishing factors concerning potential effluent-related effects on fish reproduction (Kovacs et al., 2011). While in general the organic content of pulp mill effluents is predictive of effects on fish reproduction (Kovacs et al., 2013), it is necessary to test individual mills and their constituents for their potential to affect reproduction.

In summary, this study describes a short term *in vivo* test with the zebrafish that was used to demonstrate that effluent from a Brazilian pulp mill affects testicular and hepatic function and that these responses in the testis are associated with effects on energy reserves (lactate content, lactate dehydrogenase activity), cell signaling (pP38 MAPK) and oxidative stress. Future studies should determine if this effluent represents a hazard to native species and if biochemical markers of testicular function are affected at other mills and thereby will have utility in identifying the constituents within pulp mill effluent that contribute to these responses.

Declarations of interest

None.

Acknowledgements

This project was supported by a grant from the CNPq/PVE (#401440/2014-1). We thank the Klabin Pulp and paper mill for providing the effluent used in this study. The authors are grateful to Prof. Rodrigo Otávio Alves de Lima of the Departamento de Patologia - CCS - UFSC for providing input on the analysis of the liver histology. Allisson J G Castro (CNPq/PVE 401440/2014-1 and CNPq/PDJ 438246-2016-0) and Ivana I E Baptista (CNPq/PVE 401440/2014-1) were supported by postdoctoral fellowships.

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