

Dietary L-Carnitine prevents histopathological changes in Tilapia (*Oreochromis niloticus*) exposed to Cylindrospermopsin

Remedios Guzmán-Guillén^{a*}, Ana I. Prieto Ortega^a, Rosario Moyano^b, Alfonso Blanco^c, Vitor Vasconcelos^{d,e}, Ana M. Cameán^a

^a Area of Toxicology. Faculty of Pharmacy. University of Sevilla. C/Profesor García González 2, 41012 Sevilla. Spain.

^b Department of Pharmacology, Toxicology and Legal and Forensic Medicine, University of Córdoba. Campus de Rabanales Carretera Madrid-Cádiz s/n, 14071 Córdoba. Spain.

^c Department of Anatomy and Comparative Pathology and Anatomy, University of Córdoba. Campus de Rabanales Carretera Madrid- Cádiz s/n, 14071 Córdoba. Spain.

^d Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), Porto 4050-123, Portugal.

^e Department of Biology, Faculty of Sciences of the University of Porto, Porto 4169-007, Portugal.

***Corresponding author:**

Remedios Guzmán Guillén

Area of Toxicology. Faculty of Pharmacy. University of Sevilla
C/ Profesor García González 2, 41012 Sevilla, Spain.

e-mail address: rguzman1@us.es

Tel: +34 954 556762

Fax: +34 954 233765

Running title: L-carnitine prevents CYN-induced histopathological changes in Tilapia

Abstract

Cylindrospermopsin (CYN) is a cytotoxin highly water-soluble which is easily taken up by several aquatic organisms. CYN acts as a potent protein and glutathione synthesis inhibitor, as well as inducing genotoxicity, oxidative stress and histopathological alterations. This is the first study reporting the protective effect of a L-carnitine (LC) pretreatment (400 or 880 mg LC/kg bw fish/day, for 21 days) on the histopathological alterations induced by pure CYN or *Aphanizomenon ovalisporum* lyophilized cells (400 µg CYN/kg bw fish) in liver, kidney, heart, intestines and gills of tilapia (*Oreochromis niloticus*) acutely exposed to the toxin by oral route. The main histopathological changes induced by CYN were disorganized parenchyma with presence of glycogen and lipids in the cytoplasm (liver), glomerulonephritis, glomerular atrophy and dilatation of Bowman's capsule (kidney), myofibrosis, loss of myofibrils, with edema and haemorrhage (heart), intestinal villi with necrotic enterocytes and partial loss of microvilli (gastrointestinal tract), and hyperemia and haemorrhage (gills). L-carnitine pretreatment was able to totally prevent those CYN-induced alterations from 400 mg LC/kg bw fish/day in almost all organs, except in the heart, where 880 mg LC/kg bw fish/day were needed. In addition, the morphometric study indicated that LC managed to recover totally the affectation in the cross sections of the proximal and distal convoluted tubules in CYN-exposed fish.

Keywords: Cylindrospermopsin, *Aphanizomenon ovalisporum*, L-carnitine, Histopathology, Tilapia.

1. Introduction

The eutrophication of lakes and reservoirs leads to water blooms of cyanobacteria in many countries throughout the world, decreasing the water quality by the release of off flavours, water discoloration and accumulation of surface scums (Ibelings and Chorus, 2007). Equally important is the ability of some bloom-forming species of cyanobacteria to produce secondary metabolites, namely the cyanotoxins (Moreira et al., 2012). Cylindrospermopsin (CYN) is recognized as one of the most globally important freshwater algal toxins. Structurally, CYN is a tricyclic guanidine moiety combined with hydroxymethyluracil first isolated and identified in 1992 (Ohtani et al., 1992) and highly soluble in water (Sivonen and Jones, 1999). Four naturally occurring analogues of CYN have been also identified: 7-epicylindrospermopsin (7-Epi-CYN), deoxy-cylindrospermopsin (7-deoxy-CYN) (Kinnear, 2010), 7-deoxy-desulfo-cylindrospermopsin and 7-deoxy-desulfo-12-acetylcylindrospermopsin (Wimmer et al., 2014). The ever-expanding distribution of CYN producers into temperate zones is heightening concern that this toxin will represent serious environmental and human health risks across many countries (Kinnear, 2010).

Several authors have showed that CYN causes damages in different organs (liver, kidney, lungs, thymus, spleen, adrenal glands, intestinal tract, the immune system and heart) in mammals, consistent with the concept of a more general cytotoxicity and its action as an inhibitor of protein and glutathione synthesis (Runnegar et al., 1994; Falconer et al., 1999; Hawkins et al., 1985; Humpage et al., 2000; Terao et al., 1994), as well as genotoxicity (Humpage, 2008; Bazin et al., 2010). Experiments from our laboratory have demonstrated the important role of oxidative stress in the mechanism of CYN-toxic action *in vivo* (Gutiérrez-Praena et al., 2011a; Puerto et al., 2011; Guzmán-Guillén et al., 2013a) and *in vitro* in different human and fish cell lines (Gutiérrez-Praena et al., 2011b; 2012a,b). Moreover, different studies have demonstrated that CYN can produce histopathological changes in several tissues of tilapia (*Oreochromis niloticus*) acutely exposed to pure CYN orally (Puerto et al., 2014) or by intraperitoneal injection (Gutiérrez-Praena et al., 2012c), or subchronically exposed to cyanobacterial cells containing CYN by immersion (Guzmán-Guillén et al., 2015a).

Furthermore, morphometric studies concerning CYN toxicity in several organs of tilapia have demonstrated changes in the average nuclear diameter of hepatocytes, as

well as in the cardiac fibers and capillaries diameters in the heart (Gutiérrez-Praena et al., 2012c; Guzmán-Guillén et al., 2015a,b). However, contradictory results have been described for the kidney, with decreased cross sections of proximal and distal convoluted tubules after a 5-day exposure to pure CYN (Gutiérrez-Praena et al., 2012c), whereas increases were found after 24 h-exposure to pure CYN (Guzmán-Guillén et al., 2015b) and after 7-days exposure to cyanobacterial cells containing CYN and deoxy-CYN (Guzmán-Guillén et al., 2015a).

Research into CYN has been performed with pure standard toxin, extracted toxin and cyanobacterial cells containing CYN. Several studies suggest that cell extracts – hence field populations of CYN-producing blooms – are likely to contain one or more bioactive compounds other than CYN, hence increasing the risk of toxic effects (Falconer et al., 1999; Hawkins et al., 1997; Norris et al., 1999; Seifert, 2007). In tilapia, exposure to cyanobacterial cells (*A. ovalisporum*) containing CYN and deoxy-CYN induced higher effects on several oxidative stress biomarkers in comparison to pure CYN (Gutiérrez-Praena et al., 2012d), but possible differences concerning histopathological changes have not been studied yet.

The protective effects of different antioxidants against the toxic action of cyanobacterial toxins, mainly Microcystins (MCs) and CYN, in aquatic organisms has been previously reported. In some previous papers we showed that selenium (Se), vitamin E and N-acetylcysteine (NAC) could restore tissue redox status and pathological alterations in fish following exposure to toxic cyanobacterial cells containing MCs (Atencio et al., 2009; Prieto et al., 2008, 2009; Puerto et al., 2009, 2010). Regarding CYN, to the extent of our knowledge, there are currently three substances that have demonstrated prevention of oxidative stress and/or histopathological lesions in fish exposed to this toxin: NAC (Gutiérrez-Praena et al., 2012d; 2014), vitamin E (Guzmán-Guillén et al., 2015b,c) and L-carnitine (LC) only against oxidative stress (Guzmán-Guillén et al., 2013b).

L-carnitine (β -hydroxy- γ -N-trimethyl ammonium-butyrate; LC) is a water-soluble quaternary amine synthesized from protein-bound lysine and methionine, with the assistance of vitamin C and other secondary compounds produced in animal bodies (Rebouche, 1991). LC plays an important role in fatty acid metabolism, as its major function is the transport of long-chain fatty acids into mitochondria for oxidation,

particularly in the heart and skeletal muscles (Bueno et al., 2005). LC also serves a protective role against reactive oxygen species (ROS) by exerting antioxidant properties (Derin et al., 2004; Gómez-Amores et al., 2007; Guzmán-Guillén et al., 2013b). Histopathological studies suggest that LC might have a protective effect against ischemia-reperfusion (IR) injury in rat liver (Canbaz et al., 2007), and against myocardium pathological alterations in dogs (Folts et al., 1978; Keene et al., 1991). Previous work has shown the beneficial effects of LC on fish and crustacean growth and on feed conversion in marine and freshwater species (Becker et al., 1995; Chatzifotis et al., 1995; Rodehutsord, 1995; Santulli and D'Amelio, 1986; Torrele et al., 1993; Yang et al., 2012). Furthermore, LC protects fish against high levels of ammonia and xenobiotics (Santulli and D'Amelio, 1986, Tremblay and Bradley, 1992; Schreiber et al., 1997), and alleviates the stress related to extreme water temperature changes (Harpaz et al., 1999). Specifically in tilapia fish, Jayaprakas et al. (1996) reported that LC supplementation (900 mg LC/kg diet) had a positive effect on their growth and reproductive performance, whereas Schlechtriem et al. (2004) showed that LC treatments had no effect on the growth or survival of hybrid tilapia, although it offered better resistance to xenobiotics. LC could also stimulate physiological regeneration and prevent pathological alterations of epithelial layers in gills and skin of guppy fish (Schreiber et al., 1997).

The aim of the present study was to evaluate the effectiveness of a LC pretreatment (400 or 880 mg LC/kg body weight -bw- fish/day, for 21 days) on the pathological and ultrastructural changes induced by pure CYN or by *A. ovalisporum* cells (containing CYN and deoxy-CYN) in liver, kidney, heart, intestines and gills of tilapia (*O. niloticus*) acutely exposed to the toxin by oral route. To the extent of our knowledge, this is the first study concerning the prevention of these alterations by LC in fish intoxicated with CYN.

2. Materials and Methods

2.1. Chemicals

Pure CYN (purity > 95%) was supplied by Alexis Corporation (Lausen, Switzerland). LC was provided by Fragon Ibérica (Barcelona, España 32183-12). Chemicals for the different assays were provided by Sigma–Aldrich and VWR International Eurolab. HPLC-grade methanol, dichloromethane, formic acid, and

trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Deionized water ($>18 \text{ M}\Omega \text{ cm}^{-1}$ resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, USA). BOND ELUT[®] Carbon cartridges (500 mg, 6 mL) were supplied by Agilent Technologies (The Netherlands, Europe).

2.2. Collection of *Aphanizomenon ovalisporum* strain culture and determination of cyanobacterial toxins

Aphanizomenon ovalisporum (LEGE X-001) cyanobacterial CYN-producing strain (CYN+) was isolated from Lake Kinneret (Banker et al., 1997) and kindly supplied by Dr. Vitor Vasconcelos (Marine Research Centre, Porto, Portugal). A culture of this strain was maintained in Z8 medium at 25°C under continuous illumination with an intensity of $28 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ provided by cool white fluorescent tubes. After 33 days, cultures were harvested by decantation and filtration with plankton net (20 μm diameter). The biomass obtained was frozen at -80°C until lyophilization (Telstar Cryodos, Madrid).

CYN extraction and purification with graphitized carbon cartridges from the lyophilized culture of *A. ovalisporum* (CYN+) was performed according to Guzmán-Guillén et al. (2012), prior to its LC-MS/MS analysis. Chromatographic separation was performed using a Perkin Elmer Series 200 HPLC system (Wellesley, USA) coupled to an Applied Biosystems QTRAP LC/MS/MS system (Foster City, USA) consisting of a hybrid triple quadrupole linear ion trap (QqQ_{lit}) mass spectrometer equipped with an electrospray ion source. LC-MS/MS analyses were performed in the same conditions as in Guzmán-Guillén et al. (2013b), detecting CYN and deoxy-CYN in the sample culture.

2.3. Experimental setup and acclimation of fish

Seventy-two male *O. niloticus* (Nile tilapia, average weight: $50 \pm 8 \text{ g}$, length: $12 \pm 2 \text{ cm}$) were obtained from the fish hatchery “Aquaculture Valencia”, maintained and acclimatized at the laboratory for 15 days in the same conditions as Guzmán-Guillén et al. (2013b) before the beginning of the experiment.

2.4. Experimental exposure

After the acclimation period, 9 experimental groups (8 individuals per group) were established as shown in Table 1, according to Guzmán-Guillén et al. (2013b). Briefly, the groups considered were control group (aquarium 1), pure CYN control group (aquarium 2), lyophilized cells (containing CYN and deoxy-CYN) control group (aquarium 3), LC control groups (aquaria 4 and 7), and LC + CYN groups (aquaria 5, 6, 8 and 9) (Table 1). According to the treatment, each fish group was fed with a mixture of the corresponding components (fish food, pure CYN, toxic lyophilized cells, and different doses of LC), resulting in small sticky pellets. The amount of commercial fish food administered per fish was 0.5 g/day. Fish in aquarium 1 were daily fed with only commercial fish food. Fish in aquaria 2 and 3 were daily fed with commercial fish food for 21 days and, on day 21, together with the food they received a single oral dose of pure CYN or lyophilized cells containing CYN and deoxy-CYN (400 µg CYN/kg bw fish). Fish in aquaria 4 and 7 were daily fed with commercial fish food plus 400 or 880 mg LC/kg bw fish (equivalent to 20 or 44 mg LC/fish), respectively, for 21 days. Fish in aquaria 5, 6, 8 and 9 were daily fed with commercial fish food plus 400 or 880 mg LC/kg bw fish for 21 days and, on day 21, they received a single oral dose of pure CYN or lyophilized cells containing CYN and deoxy-CYN (400 µg CYN/kg bw fish). 24 h after exposure to the toxin, fish from all groups were anaesthetized in ice and euthanized by transection of the spinal cord. Organs (liver, kidney, heart, intestines and gills) were quickly removed, weighed, rinsed with ice-cold saline, frozen in liquid nitrogen, and kept at -80°C until further use. Doses of CYN and LC were selected according to Guzmán-Guillén et al. (2013b), who reported the beneficial antioxidant effects of LC dietary supplementation from 400 mg LC/kg bw fish/day, for 21 days, on the oxidative stress status of different organs in tilapia exposed to pure CYN or lyophilized *A. ovalisporum* cells (400 µg CYN/kg bw fish).

2.5. Light microscopy and electron microscopy

Tissue samples were taken from the liver, kidney, heart, intestines, and gills of control and exposed fish for histological examination. For light microscopy, samples were first fixed in 10% buffered formalin for 24 h at 4°C, and then immediately dehydrated in a graded series of ethanol, immersed in xylol and embedded in paraffin wax by using an automatic processor. Sections were processed according to Guzmán-Guillén et al. (2015b).

In case of electron microscopy (EM), samples were prefixed in 2% glutaraldehyde fixative (in pH 7.4 phosphate buffer for 10 h at 4°C) and postfixed in 1% osmium tetroxide fixative (in pH 7.4 phosphate buffer for 0.5 h at 4°C). Subsequently, they were dehydrated in a graded ethanol series and embedded in epon. Ultra thin sections (50-60 nm) were cut with a LKB microtome and were further processed according to Guzmán-Guillén et al. (2015b).

2.6. Morphometric study

For the structural quantifications, the fixed liver, kidney and heart were cut into three sections and each portion was then histologically processed, dehydrated in a graded series of ethanol, immersed in xylol and embedded in paraffin wax. Later, the fixed liver, kidney and heart were processed according to Guzmán-Guillén et al. (2015b). To quantify any alterations on hepatocytes size, the average diameter of the nuclei was employed; the average cross sections of the proximal and distal convoluted tubules and of cardiac fibers and capillaries were estimated in the case of the kidney and the heart, respectively.

2.7. Statistical Analysis

Data were analyzed by applying bivariate comparisons considering nonparametric methods. Differences among groups, with respect to hepatocyte nuclear diameters, convoluted tubules cross-sections, cardiac fiber and capillaries diameters values were tested using the Kruskal–Wallis test, whereas pair-wise differences were compared by the Tukey–Kramer test. The analyses were conducted using the Statistical software Statistica, version 6. Statsoft Inc. All reported *p* values were two-tailed, with $p < 0.05$ considered as significant.

3. Results

No fish died and no behavioural alterations were observed in any of the fish during the experiment. Moreover, no remarkable histopathological changes were observed in any of the studied organs of control fish (Figs. 1-5A, B) and fish treated only with L-Carnitine (Figs. 1-5C, D). Hence, only images from fish pretreated with one dose of LC was shown.

Regarding the liver of control fish, apparently normal hepatocytes with cytoplasmic organelles and mitochondria are observed (Figs. 1A, B), and this normal structure was also found in liver of fish pretreated with 400 mg LC/kg bw fish/day (Figs. 1C, D). The microscopic examination of the HE-stained liver sections of fish exposed to pure CYN revealed hepatic disorganization with presence of lipids (arrow) and glycogen (circle) in the cytoplasm (Fig. 1E). Ultrastructurally, hepatocytes showed a cytoplasm with large lipid vesicles (circle) displacing the cytoplasmic organelles, and granular glycogen (arrow) (Fig. 1F), being these alterations more accentuated than in the case of lyophilized *A. ovalisporum* cells. Fish exposed to lyophilized *A. ovalisporum* cells showed a disorganized parenchyma with great glycogen content in the cytoplasm (circle) and lipid droplets (arrow) (Figs. 1G, H). In fish pretreated with 400 or 880 mg LC/kg bw fish/day, and exposed to CYN, the parenchyma showed an apparently normal cord-like structure, with numerous cytoplasmic organelles, absence of lipids and glycogen and abundant mitochondria (circle) (Figs. 1I, J, K, L). Fish pretreated with 400 mg LC/kg bw fish/day and exposed to pure CYN presented cytoplasm with scarce glycogen content, without being a pathological process (figure not shown).

The kidney of control fish showed apparently normal glomeruli and tubules (Figs. 2A, B), as well as the structure presented by fish pretreated with 400 mg LC/kg bw fish/day (Figs. 2C, D). Fish exposed to pure CYN or to lyophilized *A. ovalisporum* cells showed glomerular atrophy (circle), dilatation of Bowman's capsule (arrow) and tubulonephrosis (star) under light microscopy (Figs. 2E, G). Electron microscopy revealed hyalinized cells (arrow) and swelling with loss of microvilli of the proximal convoluted tubules (PCT) (circle) (Figs. 2F, H). Fish pretreated with 400 or 880 mg LC/kg bw fish/day prior to CYN intoxication presented normal renal structure with normal podocytes, abundant microvilli and mitochondria (circle) (Figs. 2I, J, K, L).

Apparently normal muscle fibers were observed in heart of control fish and fish pretreated with 400 mg LC/kg bw fish/day (Figs. 3A, B, C, D). Fish exposed to both pure CYN and lyophilized *A. ovalisporum* cells showed myofibrosis, loss of myofibrils (circles), abundant edema (star) and some haemorrhage (arrow), without significant differences among both treatments (Figs. 3E, F). Fish pretreated with LC and exposed to CYN presented a cardiac parenchyma with an apparently normal structure, with some haemorrhage (circle) in case of pretreatment with 400 mg LC/kg bw fish/day

(Fig. 3G, H), whereas normal myofibrils and abundant mitochondria (circle) were observed with the highest dose assayed (880 mg LC/kg bw fish/day) (Figs. 3I, J).

Intestine samples from control fish exhibited apparently normal villi and enterocytes with abundant microvilli (circle) (Figs. 4A, B), as well as fish pretreated only with LC, which presented abundant caliciform cells (arrows) (Figs. 4C, D). Fish exposed to CYN revealed enteritis processes with abundance of necrotic enterocytes (circle) by light microscopy (Fig. 4E) and partial loss of microvilli (circle) under electronic microscopy (Fig. 4F). These lesions are very similar in treatments with pure CYN and lyophilized *A. ovalisporum* cells. Light microscopy of samples from fish pretreated with both doses of LC and exposed to the toxin exhibited normal structure and enterocytes, with abundant caliciform cells (arrows) (Figs. 4G, I). Ultrastructurally, the tissue showed abundant microvilli (circles), as well as caliciform cells with mucous granules and very active mitochondria (arrow) (Figs. 4H, J).

Gills of control fish showed an apparently normal structure (Figs. 5A, B), and LC pretreatment at both doses assayed did not alter this status (Figs. 5C, D). Injuries observed by light microscopy in gills of fish intoxicated with pure CYN were characterized by hyperemia in the secondary lamellae (arrows) and haemorrhage (circles) (Figs. 5E, G). The ultrastructural study showed gill arches with loss of continuity and cell infiltrates (circle) in the case of pure CYN (Fig. 5F) and with tumefaction (circle) when fish were exposed to lyophilized *A. ovalisporum* cells (Fig. 5H), inducing more accentuated effects than pure CYN. Fish pretreated with the antioxidant and later exposed to CYN showed an apparently normal structure (Figs. 5 I, J).

Regarding the results obtained in the morphometric study in the liver, average hepatocyte nuclear diameters were not significantly augmented in fish exposed to pure CYN or to lyophilized *A. ovalisporum* cells (Fig. 6). LC administration did not induce any significant changes in this parameter either. In the kidney, the data concerning the average cross sections of the proximal convoluted tubules showed a significant increase in fish exposed both to pure CYN and to lyophilized *A. ovalisporum* cells (1.5-fold in both cases), in comparison to control fish, with no differences regarding the type of CYN employed. Pretreatment with LC in fish intoxicated with the toxin reduced these diameters, showing a total recovery of this parameter at both doses of LC assayed (Fig.

7a). The average cross-sections of distal convoluted tubules showed significant increases in fish exposed to pure CYN (1.3-fold), which were restored to basal levels from 400 mg LC/kg bw fish/day (Fig. 7b), in comparison to their respective groups of fish not supplemented with LC. Results from the morphometric study in heart revealed that toxin administration, regardless of the type of toxin, did not induce any significant changes in the cardiac fibers or capillaries diameters compared to their respective control groups. Moreover, LC *per se* did not alter these parameters either (Figs. 8a, b).

4. Discussion

To date, two substances have proved to be useful chemoprotectants for prevention of histopathological alterations in CYN-exposed fish. First, Gutiérrez-Praena et al. (2014) demonstrated that dietary NAC supplementation was able to reduce (22 mg NAC/fish/day) or totally prevent (45 mg NAC/fish/day) histopathological alterations in tilapia exposed to CYN. Then, we showed the beneficial effects of vitamin E (25 mg vitamin E/fish/day) on preventing similar lesions in the same organs of tilapia exposed to CYN (Guzmán-Guillén et al., 2015b). The main histopathological alterations found in several organs of CYN-exposed tilapia in the present work are in accordance with previous studies carried out in our laboratory with fish intoxicated with CYN (Gutiérrez-Praena et al., 2012c, 2014; Puerto et al., 2014; Guzmán-Guillén et al., 2015a). The novelty of the present work lies in the fact that LC was able to prevent the pathological changes induced in fish by CYN. In this sense, the mechanism underlying the protective effect of LC against histopathological damages induced by CYN in fish still remains unexplored, although LC antioxidant activity seems to be involved, as the results of histopathological scoring were compatible with the improved results of different oxidative stress parameters obtained by administration of this substance (Guzmán-Guillén et al., 2013b).

In the present study, pretreatment with LC reduced the alterations induced in liver of CYN-exposed fish, although a non-pathological glycogen accumulation still remained only with the lowest dose of 20 mg LC/fish/day in fish intoxicated with pure CYN. However, a total recovery was obtained in fish pretreated with the highest dose of LC (44 mg LC/fish/day), in a similar way as Gutiérrez-Praena et al. (2014) demonstrated in fish pretreated with NAC and intoxicated with CYN. L-carnitine has been associated with a protective effect in experimental IR injury models regarding the

liver, kidney, heart, skeletal muscle, spinal cord, and skin flaps (Canbaz et al., 2007; Mister et al., 2002; Xie et al., 2006; Patel et al., 2012). Demirdag et al. (2004) demonstrated that CCl₄-induced steatosis, inflammation and necrosis were significantly reduced in rats which received subcutaneous LC. Moreover, its administration significantly reduced the degree of pathologic alterations induced by acetaminophen in liver of mice (necrosis, hyperemia, sinusoidal congestion and haemorrhages) (Yapar et al., 2007). Li et al. (2012) demonstrated that human hepatocyte cells exposed to H₂O₂ showed decreased expressions of peroxisome proliferator-activated receptor alpha (PPAR- α), carnitine palmitoyl transferase 1 (CPT1) and acyl-CoA oxidase (ACOX), leading to attenuated lipid metabolism which would be significantly improved by LC, due to its role in fatty acids β -oxidation.

Regarding the kidney, LC pretreatment managed to prevent completely the lesions induced in this organ by exposure to CYN, from the dose of 20 mg LC/fish/day, obtaining the same protection with the highest dose of 44 mg LC/fish/day. This is in agreement with other studies that obtained similar protection against CYN toxicity conferred by NAC with 22 and 45 mg/fish/day (Gutiérrez-Praena et al., 2014) and by vitamin E with 25 mg/fish/day (Guzmán-Guillén et al., 2015b). However, in the present study LC was effective in preventing the adverse effects of twice the CYN dose assayed by Gutiérrez-Praena et al. (2014) (400 versus 200 μ g CYN/kg bw fish). Furthermore, in a previous study carried out with NAC and MCs, this antioxidant showed to have pro-oxidant properties at the highest dose assayed (96.8 mg NAC/fish/day, for 7 days) (Puerto et al., 2010), whereas in the present work, 44 mg LC/fish/day supplied for 21 days did not show any pro-oxidant effects *per se*, and prevented the adverse effects of the toxin. This comparison may lead to think of better protective properties of LC as antioxidant, compared to NAC. Previous works have described the antioxidant properties of L-carnitine and propionyl-L-carnitine (PLC) in preventing renal function deterioration due to IR (Mister et al., 2002; Gorur et al., 2005; Rabie et al., 2012). According to Rabie et al. (2012), the favourable effects of LC are probably mediated by virtue of its antioxidants and anti-inflammatory effects or its ability to increase the intracellular carnitine content, with a consequent improvement in mitochondrial oxidative phosphorylation and energy production. The antioxidant capacity of LC has been demonstrated by prevention of nephrotoxicity induced in rats by exposure to cisplatin (Sayed-Ahmed et al., 2004). Xiang et al. (2013) recently suggested that

inhibition of tubulointerstitial inflammation by LC in cyclosporine-exposed rats occurred via the suppression of macrophage influx.

In the present study, the fact that morphometric alterations were only visible in the kidney could be reflecting the more susceptible nature of this organ by exposure to CYN. LC pretreatment, from the lowest dose assayed (20 mg LC/fish/day), was effective in preventing the increases induced by CYN in the cross sections of the proximal and distal tubules. This protection observed is in accordance with that obtained by Guzmán-Guillén et al. (2015b), where fish were supplemented with vitamin E for 7 days before 24h-exposure to pure CYN.

The heart was the only organ which needed the highest dose of LC (44 mg/fish/day) to totally prevent the histopathological lesions induced by CYN in it, obtaining a partial amelioration of the effects with the lowest LC dose (20 mg/fish/day). The same pattern was observed in tilapia pretreated with NAC and vitamin E and intoxicated with pure CYN (Gutiérrez-Praena et al., 2014; Guzmán-Guillén et al., 2015b). Our results are in accordance with the protection shown by LC in cisplatin-exposed rats which also had developed myofibrolysis, edema and haemorrhage, with a marked decrease in ATP levels in heart tissues that would render the cardiac cells vulnerable to damage by cisplatin (Al-Majed et al., 2006). This is explained because LC plays a protective role in metabolic disorders and a critical role in the energy metabolism of tissues that derive a considerable part of their metabolic energy from fatty acid oxidation, such as heart, skeletal muscle, liver and placenta (Shekhawat et al., 2007). Thus, in our study it seems that LC supplementation prevented CYN-induced decrease in ATP by replenishing the myocardium with adequate carnitine for its energy production. Moreover, LC could have ameliorated CYN effects in the heart due to its participation in fatty acid β -oxidation, as cardiac hypertrophy is associated with decreases in this process (Bernardo et al., 2010). LC and PLC possess an anti-ischemic action after IR injury and improve endothelial dysfunction in hypertensive rats, enhancing the recovery of myocardial function and various metabolic parameters. This is partly due to their antioxidant activity, prevention of energy loss and increased NO participation in endothelium-dependent relaxations in hypertensive rats (Packer et al., 1991; Paulson et al., 1995; Xie et al., 2006; Bueno et al., 2005). However, only LC was able to increase the release of the vasodilator PGI₂ and even enhanced TXA₂ production in normotensive rats (Bueno et al., 2005). LC ability to increase NO

production (De Marchi et al., 2012) and to restore myocardial energy reserves (Dayanand et al., 2011) were also the interpretation of Strilakou et al. (2013) for the prevention of cardiac interstitial edema and dysfunction in choline-deficient rats by administration of 200 mg LC/kg bw/day, for 4 weeks. Whereas these authors hypothesized about the possible carnitine-induced heart toxicity at higher doses than the assayed of 200 mg LC/kg bw, in our study we did not find that 400 or 800 mg LC/kg bw administered to fish produced any damage itself.

Supplementation with LC was able to decrease totally the enteritis and necrosis induced by CYN in the gastrointestinal (GI) tract in this work. This prevention was offered from the lowest dose of 20 mg LC/fish/day, in agreement with the protection shown by NAC and vitamin E against CYN-intoxication (Gutiérrez-Praena et al., 2014; Guzmán-Guillén et al., 2015b). A similar protection by LC was observed in the gastric mucosa of rats exposed to ethanol (Dokmeci et al., 2005) or with small intestinal IR injury, by inhibition of proinflammatory cytokines secretion (Yuan et al., 2011), partly due to LC capacity to enhance mitochondrial energy metabolism, in agreement with the activation of mitochondria in fish pretreated with LC in our study. The role of carnitine in the GI tract became a topic of discussion, and some authors suggested that fatty acid oxidation facilitated by carnitine may be obligatory for the maintenance of normal gut morphology and function in mice (Shekhawat et al., 2007).

In this work, LC pretreatment prevented completely the lesions induced in gills by exposure to CYN, even the tumefaction induced by lyophilized cells, from 20 mg LC/fish/day, in agreement with the protection offered by NAC and vitamin E against CYN toxicity (Gutiérrez-Praena et al., 2014) and by vitamin E with 25 mg/fish/day (Guzmán-Guillén et al., 2015b). Schreiber et al. (1997) showed that 1100 mg LC/kg diet protected gills and skin of reproducing guppies (*Poecilia reticulata*) from anionic xenobiotics under heat stress, probably due to LC interaction with cardiolipin, resulting in protection of mitochondrial membranes and functions, with enhanced stability and impermeability of epithelial layers in the gills presumably by regeneration of damaged foci and stimulation of cellular energetic metabolism. A similar protection was provided by a lower level of carnitine supplementation (150 mg LC/kg diet) in gills of hybrid tilapia reared under intensive pond culture conditions (Schlechtriem et al., 2004). This mechanism could also explain the protective effects of LC against the injuries induced by CYN in gills in our work.

Likewise in other organs and tissues, acetyl-L-carnitine resulted in improvement of mitochondrial function in rats after spinal cord injury, due perhaps to the membrane stabilization conferred by the synthesis of lipids/phospholipids (Patel et al., 2012). Mitochondrial alteration and damages as a consequence of CYN exposure were also observed in Caco-2 cells (Gutiérrez-Praena et al., 2012b). Furthermore, the mitochondrial membrane potential (MMP) was increased in CYN-treated HepG2 cells (Štraser et al., 2013), and it is known that a high MMP is positively correlated with the production of ROS (Suski et al., 2012). According to Sieroslawska and Rymuszka (2015), the relationship between ROS level and mitochondrial status in CYN-treated cells has not been studied yet. Thus, taking into account the previous works demonstrating the amelioration by LC of mitochondrial damage by different xenobiotics and/or injuries, we assume that LC was able to prevent the lesions in the mitochondrial proteins target of CYN in our study. This could be evidenced by the very active mitochondria observed in LC-pretreated fish before exposure to the toxin. This is important due to the implications that the possible injuries in mitochondrial proteins may have in production of structures and/or in metabolic processes. In general, the protection offered by LC in the present work against the lesions found seems to be a result of its role in β -oxidation, its superoxide scavenger and antioxidant capacity, anti-ischemic action, and stabilizing effect on mitochondrial function and cell membranes (Muthuswamy et al., 2006; Canbaz et al., 2007).

Our results present an advantage of LC application compared to the other antioxidants previously tested against cyanotoxins-intoxication. In general, NAC offered only a partial protection against histopathological changes induced by MCs or CYN with 20 or 22 mg NAC/fish, needing normally the highest dose (44 or 45 mg NAC/fish) for a total prevention (Puerto et al., 2010; Gutiérrez-Praena et al., 2014). Moreover, NAC showed pro-oxidant properties at the highest dose assayed (Puerto et al., 2010). However, in the present work, the lowest dose tested (20 mg LC/fish) was generally sufficient for a complete prevention of CYN-induced histopathological lesions in all organs, except in the heart, where 44 mg LC/fish were needed to achieve it. Regarding vitamin E, its pro-oxidant activity at high doses *in vivo* has been demonstrated (Pearson et al., 2006), although supplementation of fish with 25 mg vitamin E/fish did not show this activity *per se* (Guzmán-Guillén et al., 2015a,b). In the present work, LC high water-solubility leads to think that toxicity associated with its

excessive administration would not be so plausible in fish and the excess carnitine would be degraded, so the negative impact would be mainly on the cost of the feed. According to Harpaz (2005), the need for high levels of LC may not be very effective because of its cost; thus, the fact that we have found protection at the lowest dose assayed is another advantage for its use.

5. Conclusions

This work demonstrates for the first time that LC pretreatment (400 or 880 mg LC/kg bw fish, equivalent to 20 or 44 mg LC/fish/day), is able to prevent or ameliorate the histopathological alterations induced in liver, kidney, heart, intestines and gills of tilapia (*O. niloticus*) acutely exposed to pure CYN or to *A. ovalisporum* cells containing CYN and deoxy-CYN (400 µg CYN/kg fish bw). Therefore, LC might be used in advance as a preventive measure to reduce histopathological and morphometric changes in fish resulting from the exposure to CYN.

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Figure legends

Fig. 1. Histopathological changes in liver of Tilapia (*Oreochromis niloticus*) exposed to a single dose of 400 µg pure CYN/kg fish bw, and pretreated with 400 or 880 mg LC/kg fish/day for 21 d. (A, C, E, G, I, K): HE-stained liver sections. Bars: 10 µm. (B, D, F, H, J, L): Ultrastructural observations. Bars: 10 µm. (A, B) Control fish: (A) Normal hepatic cords, hepatocyte with an apparently normal morphology; (B) Detail of apparently normal hepatocytes with cytoplasmic organelles and mitochondria. (C, D) Tilapia pretreated with 400 mg LC/kg fish/day, for 21 days: (C) Parenchyma with an apparently normal morphology, with hepatocytes arranged in cords and normal pancreatic area; (D) Detail of apparently normal hepatocytes with cytoplasmic organelles and absence of lipids and granular glycogen. (E, F) Tilapia exposed to a single dose of 400 µg pure CYN/kg fish bw: (E) Disorganized hepatic parenchyma, hepatocytes with presence of glycogen (circle) and lipid vesicles (arrow); (F) The hepatocyte cytoplasm appears with a few organelles but full of lipids (circle) and slight presence of granular glycogen (arrow). (G, H) Tilapia exposed to lyophilized *A. ovalisporum* cells containing CYN (400 µg CYN/kg fish): (G) Disorganized hepatic parenchyma, hepatocytes with presence of glycogen (circles); (H) Disorganized parenchyma with great glycogen content in the cytoplasm (circle) and lipid droplets (arrow) (I, J) Tilapia pretreated with 400 mg LC/kg fish/day for 21 days and exposed to lyophilized *A. ovalisporum* cells containing CYN (400 µg CYN/kg fish): (I) Apparently normal parenchyma showing the normal cord-like structure of hepatocytes; (J) Detail of apparently normal hepatocytes with cytoplasmic organelles and no lipids and granular glycogen. (K, L) fish pretreated with 880 mg LC/kg fish/day for 21 days and exposed to lyophilized *A. ovalisporum* cells containing CYN (400 µg CYN/kg fish): (K) Apparently normal hepatic parenchyma; (L) Detail of apparently normal hepatocytes, with abundant mitochondria (circle).

Fig. 2. Histopathological changes in kidney of Tilapia (*Oreochromis niloticus*) exposed to a single dose of 400 µg pure CYN/kg fish bw, and pretreated with 400 or 880 mg LC/kg fish/day for 21 d. (A, C, E, G, I, K): HE-stained liver sections. Bars: 10 µm. (B, D, F, H, J, L): Ultrastructural observations. Bars: 10 µm. (A, B) Apparently normal

glomeruli and tubules. **(C, D)** Tilapia pretreated with 400 mg LC/kg fish/day, for 21 days: **(C)** Apparently normal glomeruli and tubules; **(D)** Apparently normal proximal convoluted tubule (PCT). **(E, F)** Tilapia exposed to a single dose of 400 µg pure CYN/kg fish bw: **(E)** Glomerulonephritis, glomerular atrophy (circle), dilation of Bowman's capsule (arrow) and tubulonefrosis (star); **(F)** Tubulonefrosis of proximal convoluted tubules (PCT) with thickened and hyalinized cells (arrow). **(G, H)** Tilapia exposed to lyophilized *A. ovalisporum* cells containing CYN (400 µg CYN/kg fish): **(G)** Glomerulopathy and glomerular atrophy (circle), dilation of Bowman's capsule (arrow) and slight tubulonefrosis (star); **(H)** Tumefaction and loss of microvilli from the proximal convoluted tubules (PCT) (circle). **(I, J)** Tilapia pretreated with 400 mg LC/kg fish/day for 21 days and exposed to 400 µg pure CYN/kg fish bw: **(I)** Apparently normal glomeruli and tubules; **(J)** Proximal convoluted tubule (PCT) apparently normal, with abundant microvilli. **(K, L)** Tilapia pretreated with 880 mg LC/kg fish/day for 21 days and exposed to 400 µg pure CYN/kg fish bw: **(K)** Apparently normal hepatic parenchyma; **(L)** Detail of apparently normal distal convoluted tubule (DCT) with abundant mitochondria (circle).

Fig. 3. Histopathological changes in heart of Tilapia (*Oreochromis niloticus*) exposed to a single dose of 400 µg pure CYN/kg fish bw, and pretreated with 400 or 880 mg LC/kg fish/day for 21 d. **(A, C, E, G, I)**: HE-stained liver sections. Bars: 10 µm. **(B, D, F, H, J)**: Ultrastructural observations. Bars: 10 µm. **(A, B)** Apparently normal muscle fibers. **(C, D)** Tilapia pretreated with 400 mg LC/kg fish/day, for 21 days: **(C)** Apparently normal muscle fibers; **(D)** Detail of apparently normal myofibrils with perfectly arranged and normal bands. **(E, F)** Tilapia exposed to a single dose of 400 µg pure CYN/kg fish bw or to lyophilized *A. ovalisporum* cells containing CYN (400 µg CYN/kg fish): **(E)** Myofibrosis, loss of myofibrils (circles), abundant edema (star) and certain haemorrhage (arrow); **(F)** Loss and disintegration of myofibrils (circle). **(G, H)** Tilapia pretreated with 400 mg LC/kg fish/day for 21 days and exposed to lyophilized *A. ovalisporum* cells containing CYN (400 µg CYN/kg fish): **(G)** Apparently normal muscle fibers but some haemorrhage (circle); **(H)** Detail of normal myofibrils without loss of contractile material. **(I, J)** Tilapia pretreated with 880 mg LC/kg fish/day for 21

days and exposed to lyophilized *A. ovalisporum* cells containing CYN (400 µg CYN/kg fish): **(I)** Apparently normal muscle fibers; **(J)** Normal myofibrils and abundant mitochondria (circle).

Fig. 4. Histopathological changes in gastrointestinal tract of Tilapia (*Oreochromis niloticus*) exposed to a single dose of 400 µg pure CYN/kg fish bw, and pretreated with 400 or 880 mg LC/kg fish/day for 21 d. **(A, C, E, G, I)**: HE-stained liver sections. Bars: 100 µm. **(B, D, F, H, J)**: Ultrastructural observations. Bars: 10 µm. **(A, B)** Control fish: **(A)** Apparently normal intestinal villi and enterocytes; **(B)** Enterocytes with abundant microvilli apparently normal and caliciform cells (circle). **(C, D)** Tilapia pretreated with 400 mg LC/kg fish/day, for 21 days: **(C)** Apparently normal villi and enterocytes with abundant caliciform cells (arrow); **(D)** Enterocytes with apparently normal and abundant caliciform cells (arrows). **(E, F)** Tilapia exposed to a single dose of 400 µg pure CYN/kg fish bw or to lyophilized *A. ovalisporum* cells containing CYN (400 µg CYN/kg fish): **(E)** Detail of intestinal villi with necrotic enterocytes (circle); **(F)** Detail of enterocytes with partial loss of microvilli (circle). **(G, H)** Tilapia pretreated with 400 mg LC/kg fish/day for 21 days and exposed to 400 µg pure CYN/kg fish bw or to lyophilized *A. ovalisporum* cells containing CYN (400 µg CYN/kg fish): **(G)** Apparently normal villi with normal enterocytes and abundant caliciform cells (arrow); **(H)** Apparently normal enterocytes with abundant microvilli (circle). **(I, J)** Tilapia pretreated with 880 mg LC/kg fish/day for 21 days and exposed to 400 µg pure CYN/kg fish bw or to lyophilized *A. ovalisporum* cells containing CYN (400 µg CYN/kg fish): **(I)** Apparently normal villi with apparently normal enterocytes and abundant caliciform cells (arrow); **(J)** Enterocytes with abundant microvilli (circle) and abundant very active mitochondria (arrow).

Fig. 5. Histopathological changes in gills of Tilapia (*Oreochromis niloticus*) exposed to a single dose of 400 µg pure CYN/kg fish bw, and pretreated with 400 or 880 mg LC/kg

fish/day for 21 d. **(A, C, E, G, I)**: HE-stained liver sections. Bars: 10 μm . **(B, D, F, H, J)**: Ultrastructural observations (SEM). Bars: 10 μm . **(A, B)** Control fish. **(C, D)** Tilapia pretreated with 400 mg LC/kg fish/day, for 21 days: **(C)** Detail of gills with a normal structure; **(D)** Apparently normal structure of lamellae. **(E, F)** Tilapia exposed to a single dose of 400 μg pure CYN/kg fish bw: **(E)** Detail of gill filament with hyperemia in secondary lamellae (arrow) and haemorrhage (circle); **(F)** Gill arch with loss of continuity, erosion and cell infiltrate (circle). **(G, H)** Tilapia exposed to lyophilized *A. ovalisporum* cells containing CYN (400 μg CYN/kg fish): **(G)** Detail of gill filament with presence of hyperemia (arrow) and haemorrhage (circle) in secondary lamellae; **(H)** Gill arch surface with erosion and tumefaction (circle). **(I, J)** Tilapia pretreated with 400 mg LC/kg fish/day for 21 days and exposed to a single dose of 400 μg pure CYN/kg fish bw or to lyophilized *A. ovalisporum* cells containing CYN (400 μg CYN/kg fish): **(I)** Apparently normal gill filament; **(J)** Gill arch apparently normal.

Fig. 6. Hepatocyte nuclear diameters values (μm) of Tilapia (*Oreochromis niloticus*) exposed to a single dose of 400 μg pure CYN/kg fish bw or to lyophilized *A. ovalisporum* cells containing CYN (400 μg CYN/kg fish), and pretreated with 400 or 880 mg LC/kg fish/day for 21 d. The values are expressed as mean \pm sd ($n=5$).

Fig. 7. Proximal (a) and Distal (b) convoluted tubules cross sections (μm) of Tilapia (*Oreochromis niloticus*) exposed to a single dose of 400 μg pure CYN/kg fish bw or to lyophilized *A. ovalisporum* cells containing CYN (400 μg CYN/kg fish), and pretreated with 400 or 880 mg LC/kg fish/day for 21 d. The values are expressed as mean \pm sd ($n=5$). The significance levels observed are $*p < 0.05$ or $***p < 0.001$ in comparison to control group values, and $\&p < 0.05$ or $\&\&p < 0.001$ when CYN-exposed fish are compared with or without LC pretreatment.

Fig. 8. Cardiac fibers (A) and Capillaries (B) diameters (μm) of Tilapia (*Oreochromis niloticus*) exposed to a single dose of 400 μg pure CYN/kg fish bw or to lyophilized *A. ovalisporum* cells containing CYN (400 μg CYN/kg fish), and pretreated with 400 or 880 mg LC/kg fish/day for 21 d. The values are expressed as mean \pm sd ($n=5$). The significance levels observed are $p < 0.05$ when CYN-exposed fish are compared with or without LC pretreatment.

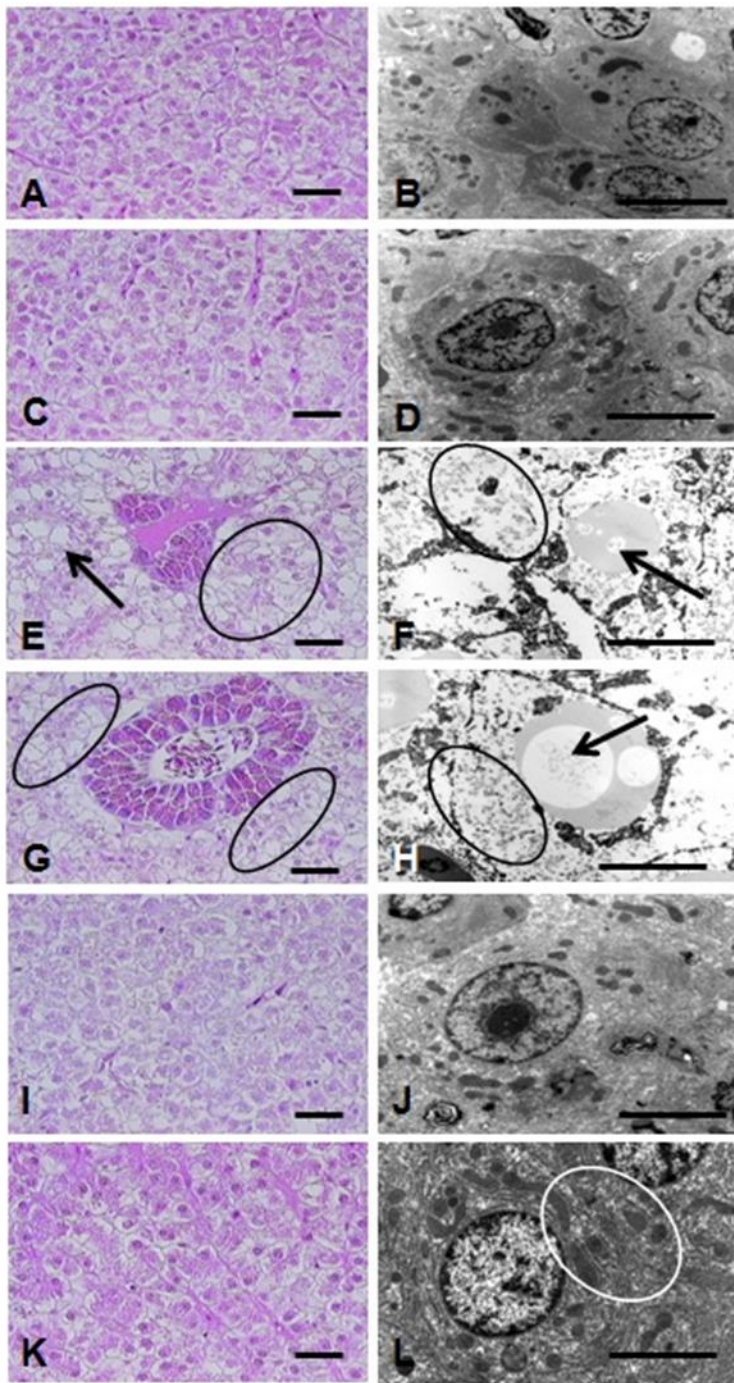


Figure 1

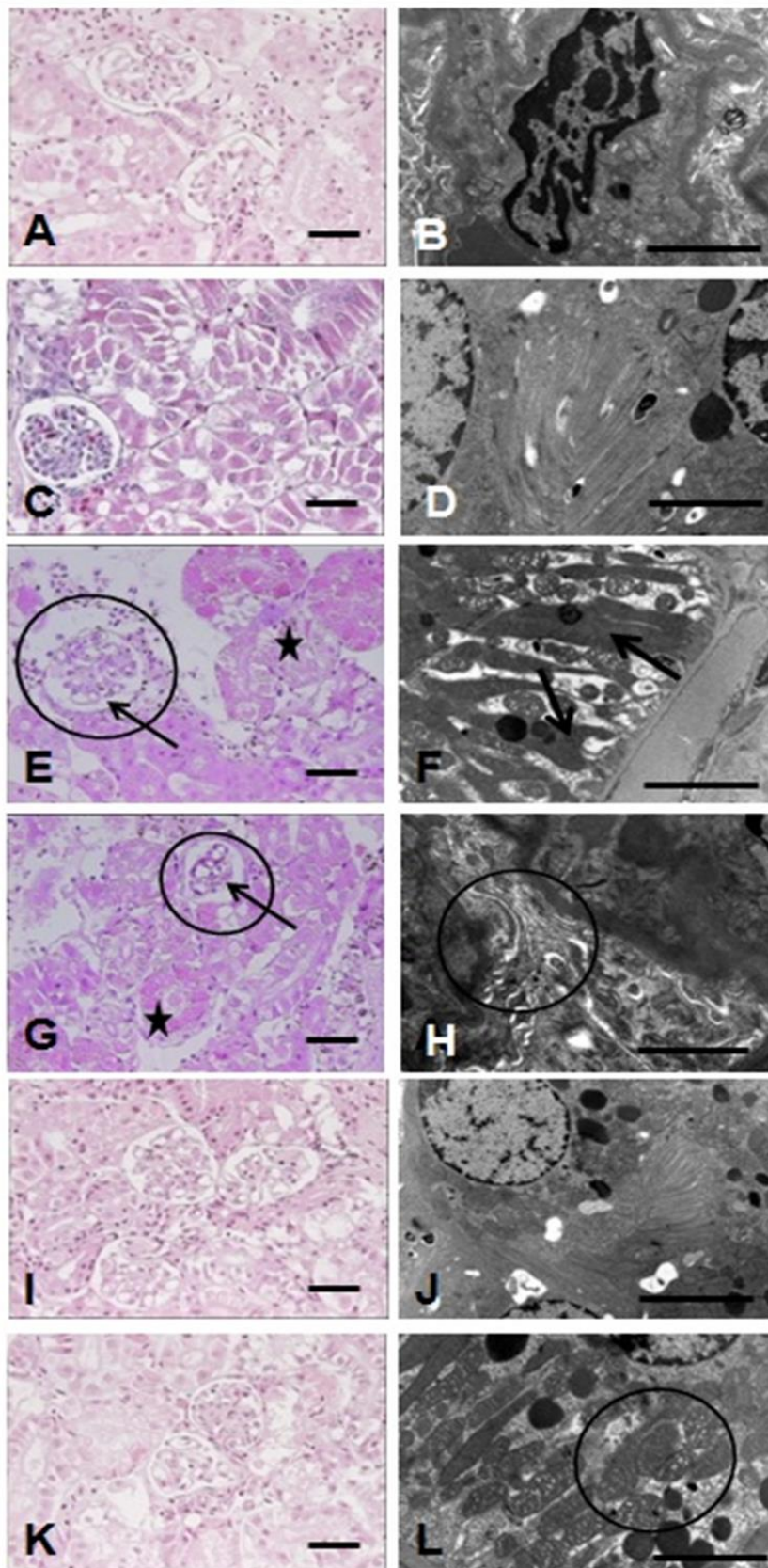


Figure 2

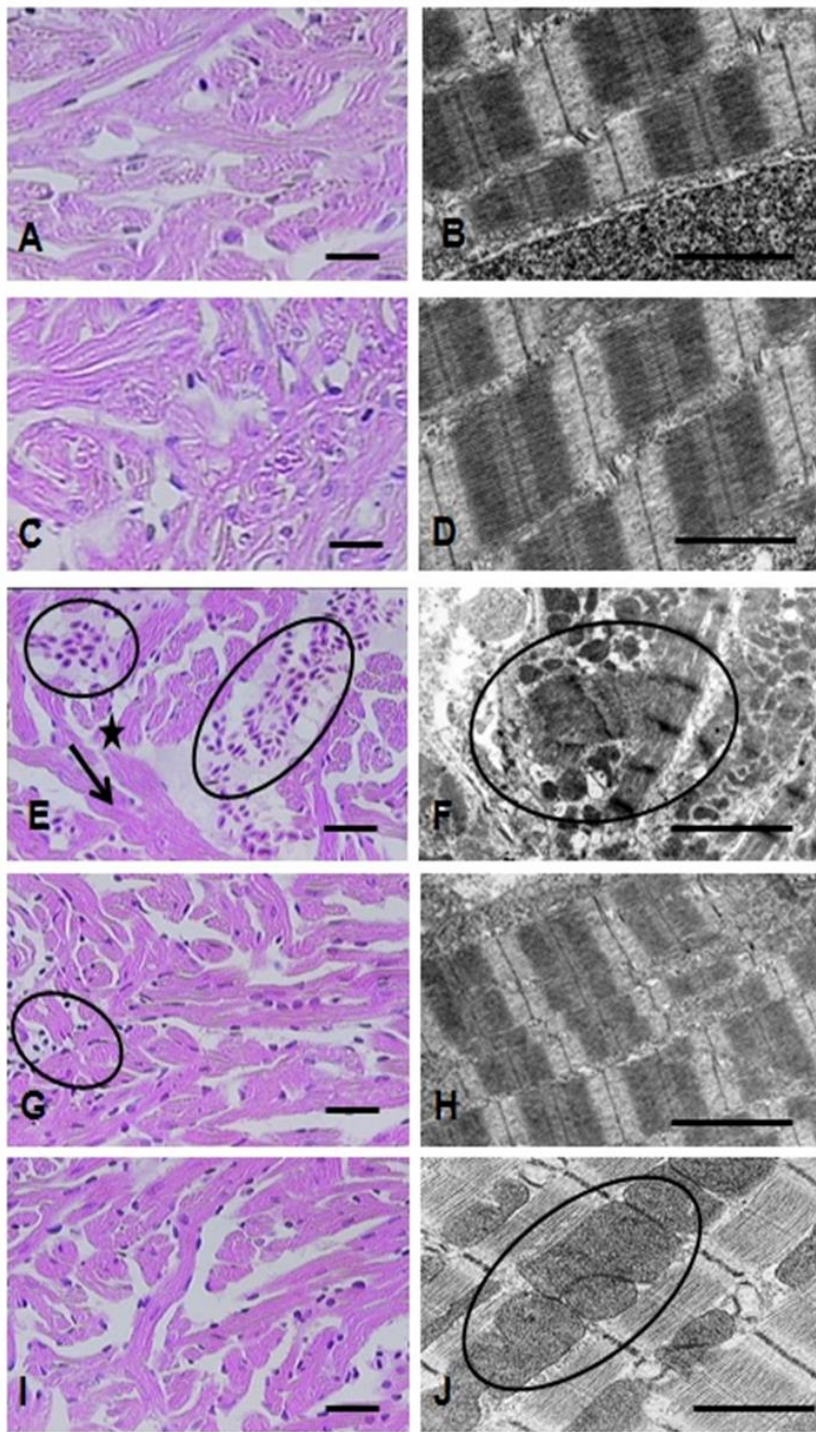


Figure 3

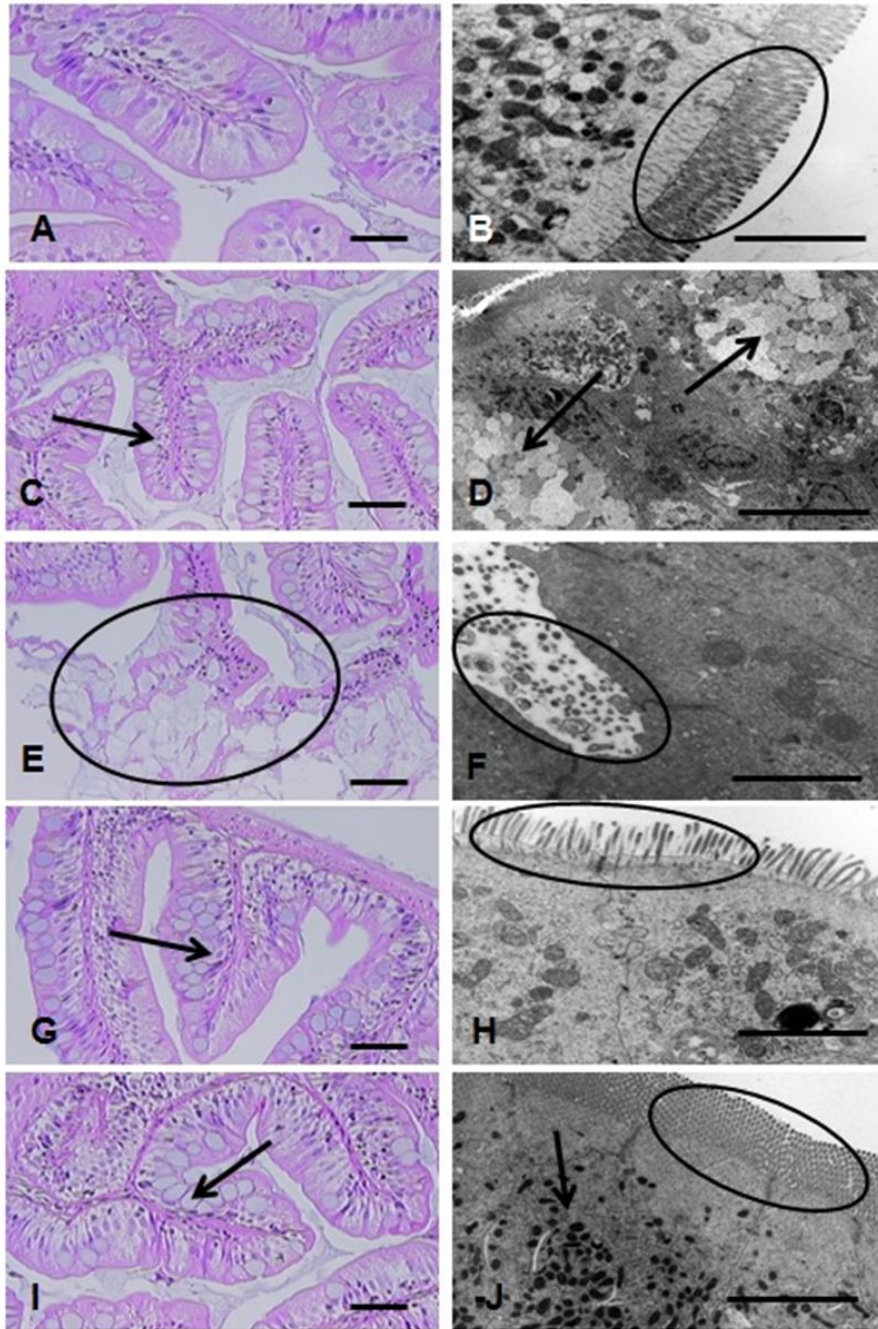


Figure 4

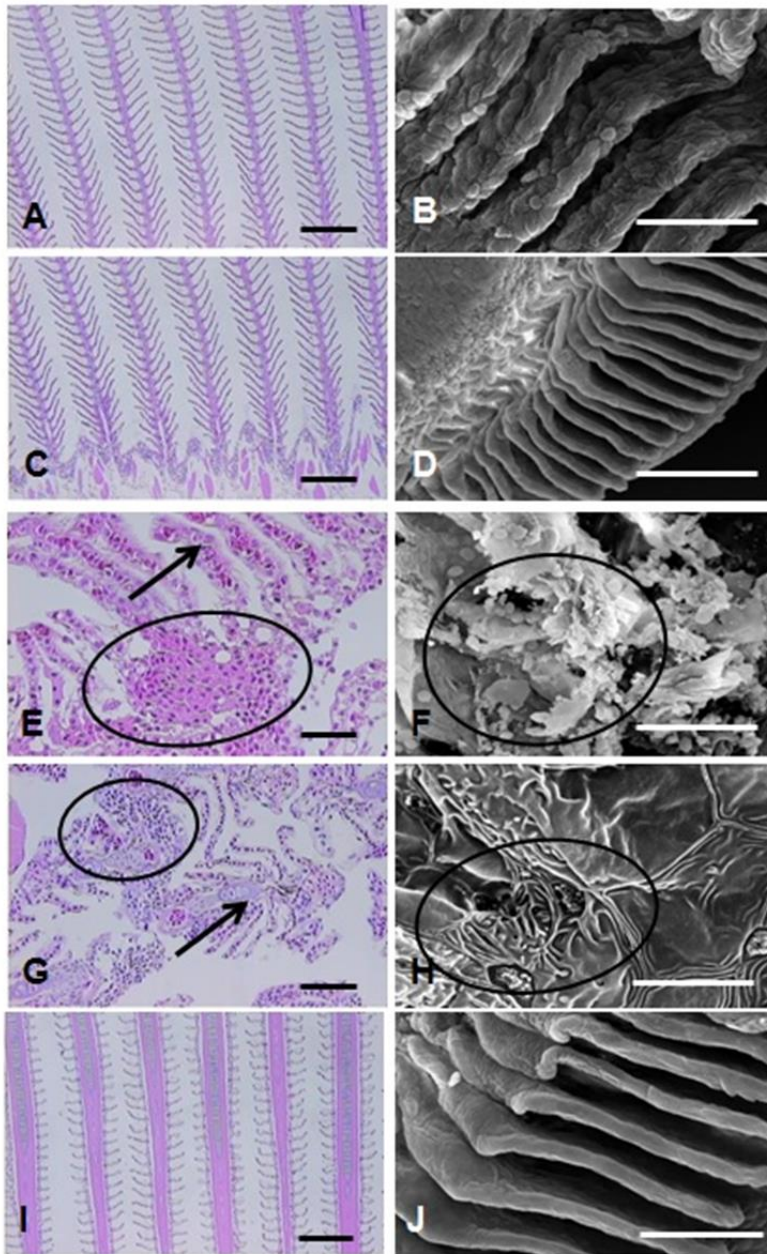


Figure 5

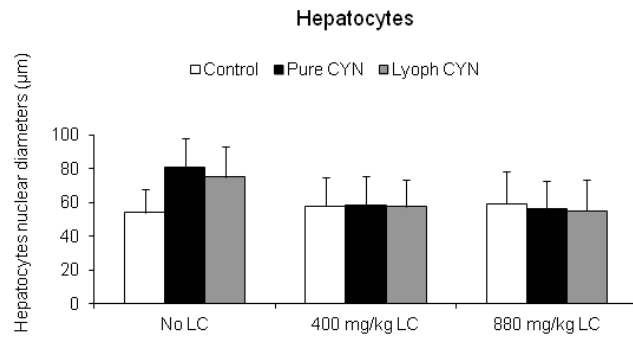


Figure 6

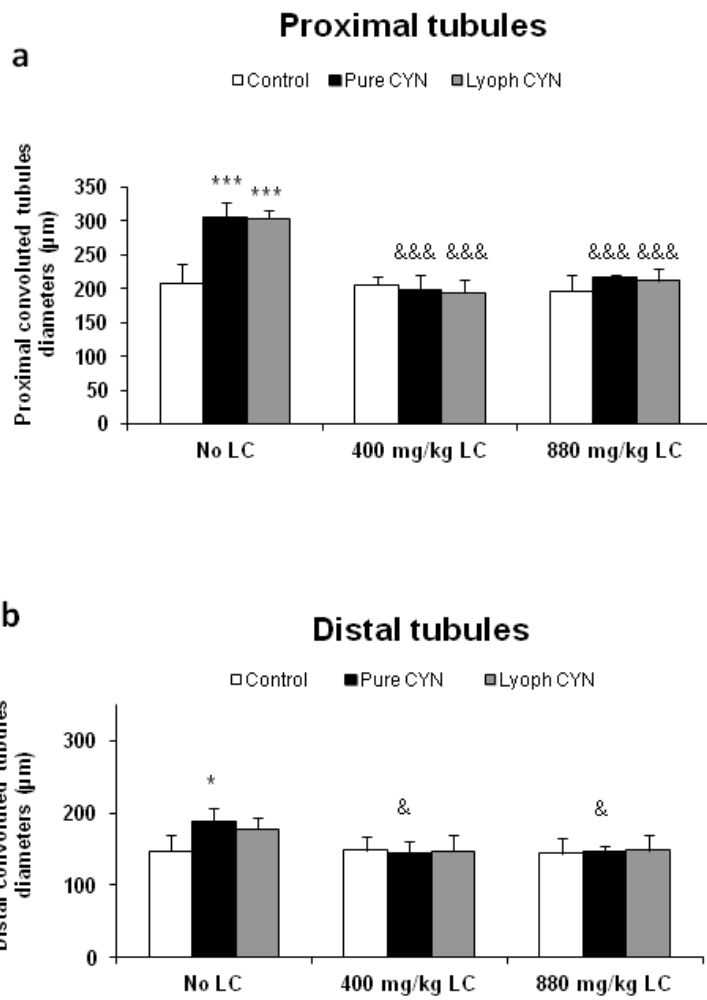


Figure 7

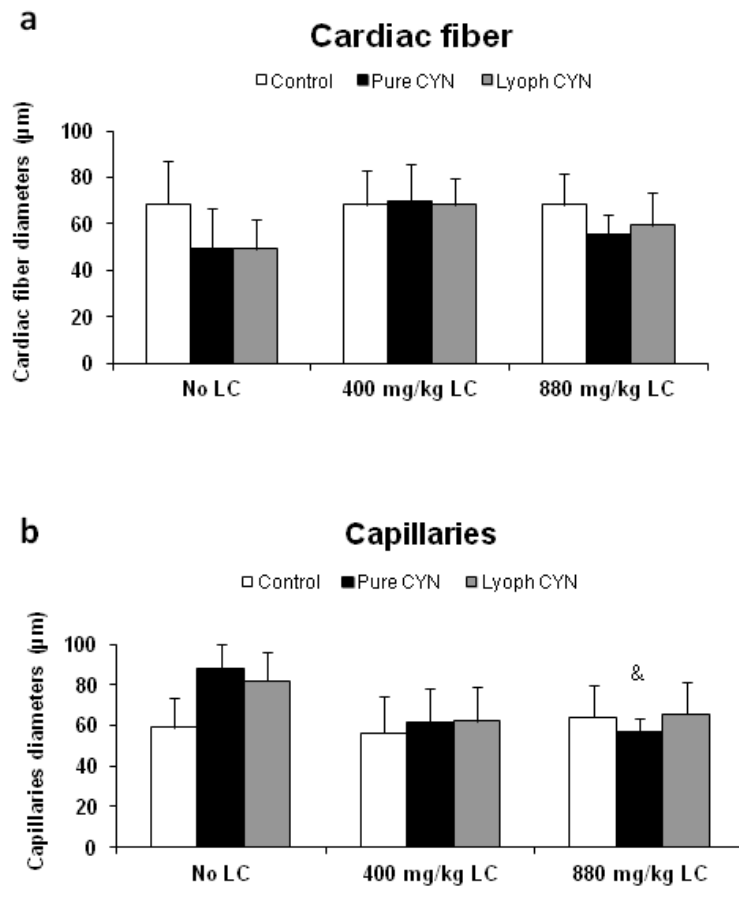


Figure 8

Table 1. Feeding conditions^a of *O. niloticus* and exposure conditions to Cylindrospermopsin (CYN). Numbers from 1 to 9 correspond with the aquaria where the different treatment conditions were carried out.

Treatment	1	2	3	4	5	6	7	8	9
Pure CYN	-	+	-	-	+	-	-	+	-
Lyophilized cells with CYN and deoxy-CYN	-	-	+	-	-	+	-	-	+
400 mg LC/kg bw fish/day (21 days)	-	-	-	+	+	+	-	-	-
880 mg LC/kg bw fish/day (21 days)	-	-	-	-	-	-	+	+	+

^a With (+) or without (-) L-carnitine supplementation (400 or 880 mg LC/kg fish/day, for 21 days).