Histological effects of Cu^{2+} to white shrimp *Litopenaeus vannamei* (Crustacea: Decapoda) juveniles at low salinities

Efectos histológicos del Cu^{2+} en juveniles del camarón blanco *Litopenaeus vannamei* (Crustacea: Decapoda) a bajas salinidades

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Resumen.- Juveniles del camarón blanco *Litopenaeus vannamei* fueron expuestos durante 25 días a salinidades de 1, 5, y 10 ups y a la concentración de Cu^{2+} que se usa comúnmente en las granjas camaronícolas de México, con el propósito de observar posibles alteraciones histológicas en la glándula antenal, intestino medio, branquias y hepatopáncreas. La supervivencia fue de 100% en controles y tratamientos y no se observaron histopatologías en branquias, intestino y glándula antenal. Después de 10 días de exposición al Cu^{2+} a la salinidad más baja, se observaron en el hepatopáncreas desprendimiento de las células epiteliales (< 75%), infiltración de hemocitos (< 75%), y una reducción de las células R y B (100%). Las mismas alteraciones se presentaron, aunque en menor grado, a las salinidades de 5 y 10 ups. Estos efectos deberían ser considerados para establecer criterios de calidad de agua para el cultivo de camarón en agua continentales, debido a que la interacción metal-salinidad puede inducir respuestas adversas en los camarones.

Palabras clave: Crustáceos, toxicidad del cobre, daños histológicos, salinidad

Abstract.- Juveniles of the white shrimp *Litopenaeus vannamei* were exposed during 25 days to the Cu^{2+} concentration commonly used in Mexican shrimp farms, at salinities of 1, 5, and 10 psu, in order to observe possible histological alterations to the antennal glands, midgut, gills and hepatopancreas. Survival was 100% in controls and treatments, and no histopathologies were observed in gills, midgut and antennal glands. Sloughing of epithelial cells (< 75%), infiltration of hemocytes (< 75%) and reduction in R and B cells (100%) were observed after 10 days in the hepatopancreas of shrimps exposed to Cu^{2+} at the lowest salinity and, to a lower degree, at salinities of 5 and 10 psu. This should be taken into consideration to establish criteria of acceptable water quality for inland aquaculture, because the interaction metal-salinity may induce adverse shrimp responses.

Key words: Crustaceans, copper toxicity, histological damage, salinity

Introduction

The white shrimp *Litopenaeus vannamei* (Boone, 1931) is the most important shrimp species in Latin American aquaculture, although in recent years it has been introduced to several Asiatic countries, which have become its main producers (FAO 2006). In Mexico, the estimated total yield of the 66,468 ha dedicated to its semi-intensive culture during 2007 was 114,317 ton, > 89% of which were produced in the NW states of Sinaloa and Sonora (CESA 2008).

The pollution of coastal waters, the outbreaks of shrimp diseases and the feasibility of shrimp culture in low salinity waters have led to rapid growth of inland shrimp farming; this has become a growing business in many areas of the world, because it allows expansion in disease-free areas, thereby reducing health risks (Fast & Menasveta 2000, Li *et al*. 2008).

L. vannamei is a hyper-hypo-osmoregulator; its isosmotic point is close to 24.7 psu (Castille & Lawrence 1981) and due to its ability to tolerate a wide range of salinities (0.5-40 psu), it has become a popular species for low salinity culture (Roy *et al*. 2007), which is a growing activity in some inland Mexican states.

Shrimp culture practices include the use of bioactive compounds such as disinfectants, therapeutics, feed additives, fertilizers and algaecides (Dias-Bainy 2000). Among these, copper sulfate is used to control macroalgae and bivalve growth in about 61% of the commercial shrimp farms of the State of Sinaloa, whereas close to 43.2% utilize some unidentified, probably Cu-based fungicide (Lyle-Fritch *et al*. 2006).

Copper is an essential element for living organisms: it is a key component of enzymes that act as catalysts in several metabolic processes, and it is required for the synthesis of the main crustacean respiratory pigment (hemocyanin), of oxidation-reduction enzyme systems, as well as of other compounds, which are essential for normal growth and development (Davis & Mertz 1987, Li *et al*. 2007a).

However, when present in relatively high concentrations, Cu^{2+} becomes toxic and has harmful effects at the cellular, systemic or whole organism level (Yang *et al*. 2008). This might be the case in semi-intensive shrimp farms, since the low water exchanges used in most of these farms may cause copper accumulation in the pond water and sediments (Lacerda *et al*. 2006). This accumulation might affect the survival and growth of cultured shrimp especially in inland waters, because an important factor for metal toxicity is their chemical species (Erk *et al*. 2008), and it is generally accepted that toxicity is inversely related to ambient salinity (Wright 1995).

The aim of this study was to examine the histological alterations caused to the midgut, gills and hepatopancreas of *L. vannamei* kept at a range of low salinities, and exposed to the Cu^{2+} concentration used in Mexican shrimp farms.

Material and methods

Sampling and acclimation of test organisms

Seawater-grown juveniles of *L. vannamei* (1.5-2 g), not exposed previously to $CuSO_4$, were obtained from a commercial shrimp farm, transferred to the laboratory, kept under observation during three days in a 100 L plastic holding tank and moved later for one additional week to 30 L plastic containers, at a density of 15 shrimp per container, with continuous aeration and a 12:12 h light-dark photoperiod.

Throughout this stage, the organisms were fed *ad libitum* twice daily with pelleted 35% protein shrimp food. Uneaten food was removed after 1 h and water exchanges with seawater obtained from Mazatlan Bay and filtered (10, 5 and 1 μm and 0.5 μm-mesh activated charcoal,were 100% every 24 h. The average background dissolved Cu^{2+} concentration, determined by atomic absorption spectrophotometry (Spectra® AA, Varian) was 2.0 μg L^{-1}. The accuracy and precision of the method were assessed using reference material MA-M-2/TM. Throughout the experiment the mean water temperature, pH, DO and total ammonia concentrations were 28 ± 1°C, 8.11 ± 0.08, 5.8 ± 0.15 mg L^{-1} and 3.7 ± 0.7 μg L^{-1}, respectively.

After this first stage, salinity was decreased with dechlorinated tap water at a rate of 0.5 psu h^{-1} from the initial 35 psu to 25 psu, which is the isosmotic point of *L. vannamei* (Castille & Lawrence 1981). After one day at this salinity, salinities were changed progressively during one week to the experimental values (10, 5 and 1 psu). Shrimp were kept at these salinities for one week (habituation period: McGraw & Scarpa 2004), during which the conditions were as in the previous days, but water was renewed every 48 h.

Experimental procedure

After the habituation period, the shrimp of three containers for each salinity were exposed to 0.101 mg L^{-1} of Cu. This corresponded to the Cu^{2+} concentration in 1 m-deep ponds treated with the amount of copper sulfate used by Mexican farmers to reduce macroalgae growth (4 kg ha^{-1} of $CuSO_4 \cdot 5H_2O$). Each Cu treatment had its respective control. Uneaten food was removed daily with a siphon. Observations on survival, behavior and exuviae were performed every 12 h. The solutions were renewed every 48 h and other experimental conditions were as for the habituation period.

The copper content of the pelleted food was not determined, but the shrimp of all treatments and of the respective controls were fed the same food and daily ration. Therefore, any histological alteration in comparison to the control groups was taken as indicative of a Cu^{2+} effect in the challenge treatment (Odendaal & Reinecke 2007, Frías-Espericueta *et al*. 2008a).

Histological procedures

The experiment lasted 25 days. Every 5 days, the cephalotorax and the abdominal region of three shrimps, chosen at random in each container, were injected with Davidson solution and left in the same solution for 24 h to ensure tissue fixation.

Longitudinal body sections of shrimps were dehydrated in 70, 80, 96 and 100% ethanol, cleared with xylene, embedded in paraffin and 5-μm sections were obtained with a conventional microtome. Each sample was re-hydrated, stained with hematoxylin-eosin (Yang

et al. 2007, Frías-Espericueta *et al*. 2008b) and examined under a microscope.

The longitudinal body sections allowed a complete observation of the tissues of each shrimp. The degree of histological damage was scored according to the percentage of fields with histological damage, out of the total number of fields observed in the samples of each treatment, using the scale suggested by Zodrow *et al*. (2004) (severe, moderate, mild and none = 100%, < 75%, < 25 % and 0% of the fields with histological damage, compared to the control sections).

The number of fields observed varied from 90, to 60, 45 and 13 for hepatopancreas, gills, midgut and antennal glands, respectively depending on the size of the tissue, and covered the whole longitudinal section of each tissue. This gave a total of 270, 180, 135 and 39 fields observed every fifth day for each treatment.

Results

No mortalities occurred in controls and treatments, and no histological alterations were observed in gills, midgut and antennal glands. Salinity had an initial effect on the hepatopancreas structure, since after 5 days in hypo-osmotic conditions we observed a decrease of B and R cells in the treatments and in the control organisms. The degree of this alteration was inversely related to salinity, and no alteration was observed after the first 5 days.

There was also a low degree (< 25% of the fields observed) of infiltration of hemocytes in the spongy connective tissue layer of the peripheral sheath and the apical region, and sloughing of epithelial cells was observed in the hepatopancreas of the controls throughout the experiment (Figs. 1a and 2a).

After 25 days of exposure to Cu^{2+} and 1 psu salinity, the hepatopancreas showed 75% of the fields with sloughing of epithelial cells and infiltration of hemocytes (Fig. 1b, c). Reductions in R and B cells were observed after 10 d of exposure in about 75% of the fields in shrimp exposed to Cu^{2+} at the lowest salinity (1 psu). The percentage decreased to 25% after 15 and 20 days, but reached 100% by the end of the experiment (Fig. 1b). This effect was observed also in 25% of the fields after 20 days, at salinities of 5 psu (Fig. 2b) and 10 psu (Table 1).

Table 1

Time-dependent (days) histological damages in the hepatopancreas (compared to the control sections) of *Litopenaeus vannamei* after exposure to 0.101 mg L^{-1} of Cu^{2+} and different salinities

Daños histológicos en función del tiempo (días) en el hepatopancreas de *Litopenaeus vannamei* (con respecto al control) después de exponerlo a 0,101 mg L^{-1} de Cu^{2+} y a diferentes salinidades

Damage	1 psu					5 psu					10 psu				
	5	10	15	20	25	5	10	15	20	25	5	10	15	20	25
Reduction of R and B cells	--	xx	x	x	xxx	--	--	--	x	x	--	--	--	x	x
Infiltration of hemocytes	--	--	--	--	xx	--	--	--	--	--	--	--	--	--	--
Sloughing of epithelial cells	--	--	--	--	xx	--	--	--	--	--	--	--	--	--	--

Severe (xxx), moderate (xx), mild (x) and no damage (--) = 100%, < 75%, < 25 % and 0% of the fields with histological damage, compared to controls (Zodrow *et al*. 2004)

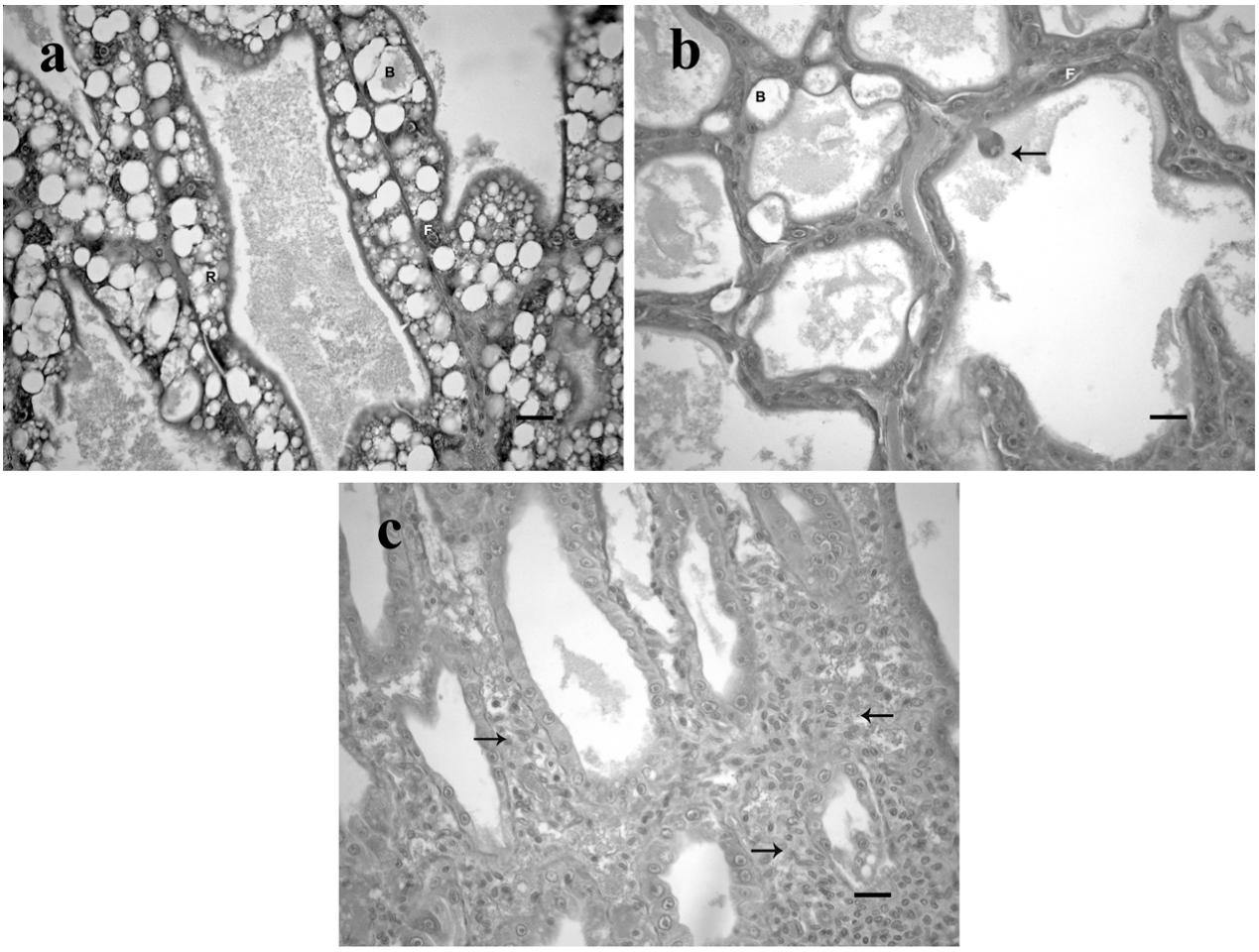

Figure 1

a) Hepatopancreas tissue of control shrimp exposed to 1 psu after 25 days. Normal distribution of F, B and R cells in the tubular epitelium. Scale bar 20 μm. b) Hepatopancreas tissue of shrimp exposed to Cu^{2+} and 1 psu after 25 days. Severe reduction of B and absence of R cells, sloughing of epithelial cells (arrow) causing modification of normal structure. Scale bar 20 μm. c) Hepatopancreas tissue of shrimp exposed to Cu^{2+} and 1 psu after 25 days. Hemocytic infiltration in the connective tissue (arrow) and reduction of B and R cells. Scale bar 20 μm

a) Tejido del hepatopáncreas de camarones del control expuestos a 1 ups durante 25 días. Distribución normal de células F, B y R en el epitelio tubular. Tamaño de la barra: 20 μm. b) Tejido del hepatopáncreas de camarones expuestos a Cu^{2+} y 1 ups durante 25 días. Reducción severa de células B y ausencia de células R, alteración de células epiteliales (flecha) que causan una modificación de la estructura normal. Tamaño de la barra: 20 μm. c) Tejido del hepatopáncreas de camarones expuestos a Cu^{2+} y 1 ups durante 25 días. Infiltración hemocítica en el tejido conectivo (flecha) y reducción de células B y R. Tamaño de la barra: 20 μm

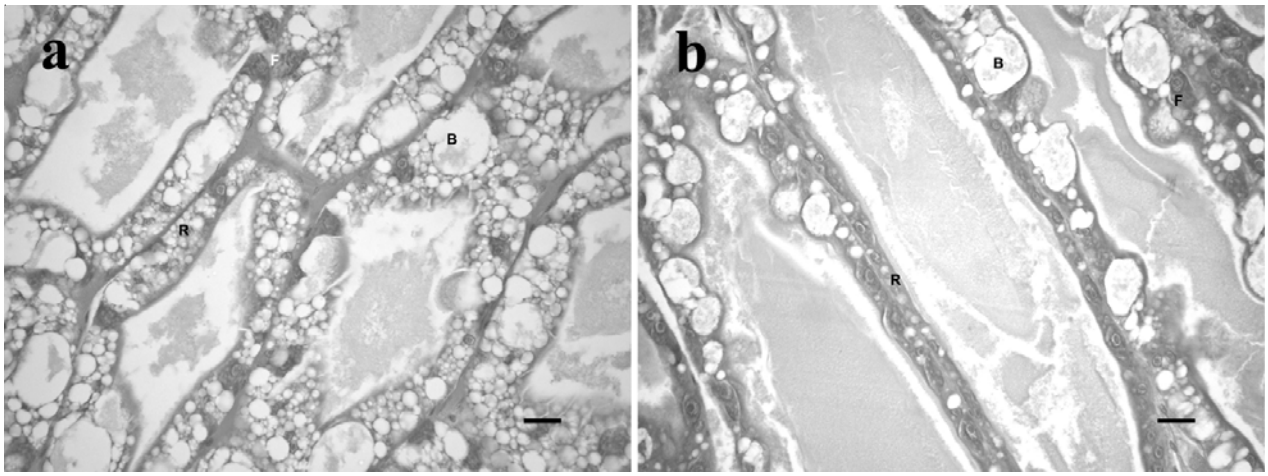

Figure 2

a) Hepatopancreas tissue of control shrimp exposed to 5 psu after 25 days. Normal distribution of F, B and R cells in the tubular epitelium. Scale bar 20 μm. b) Hepatopancreas tissue of shrimp exposed to Cu^{2+} and 5 psu after 20 days. Reduction of B and R cells. Scale bar 20 μm

a) Tejido del hepatopáncreas de camarones del control expuestos a 5 ups durante 25 días. Distribución normal de células F, B y R en el epitelio tubular. Tamaño de la barra: 20 μm. b) Tejido del hepatopáncreas de camarones expuestos a Cu^{2+} y 5 ups durante 20 días. Reducción de células B y R. Tamaño de la barra: 20 μm

Discussion

The optimum salinity for *L. vannamei* growth is close to 20 psu, and high deviations from this value affect growth and survival because of the high-energy expenditure for osmoregulation (Li *et al*. 2007b). Our habituation period (one week) probably allowed ion equalization between shrimp hemolymph and the surrounding medium (McGraw & Scarpa 2004), thereby reducing osmoregulatory stress and allowing a relative tolerance to the Cu-induced stress, as indicated by the lack of mortality observed in the Cu^{2+} treatments.

The histological effects observed in the hepatopancreas of the organisms grown in hypo-osmotic conditions (controls) are similar to those reported by Li *et al*. (2008), who found that the number of B and R cells was altered at a salinity of 3 psu. B cells are the main site for synthesis of digestive enzymes, which accelerate the mobilization of nutrients in the hepatopancreas tubules when there is an increased demand of energy to adapt to environmental stress. Thus, the decrease of these and of R cells might be due to utilization of their nutrient reserves, because of the increased energy demand for osmoregulation (Al-Mohanna & Nott 1989).

Due to their physiological functions (gas exchange, osmoregulation and nitrogen excretion), gills are the initial site of impact when aquatic organisms are exposed to waterborne pollutants: Cu-related gill damages were described by Soegianto *et al*. (1999), Yang *et al*. (2007) and Li *et al*. (2007a) in *Penaeus japonicus*, *Eriocheir sinensis* and *Macrobrachium rosenbergii*, respectively. Frías-Espericueta *et al*. (2008a) reported loss of regular structure, multifocal necrosis, and absence of pillar cells of the secondary filaments in the gills of *L. vannamei* exposed for 6 weeks to 0.877 mg Cu^{2+} L^{-1} and 25 psu salinity, whereas with the Cu concentration used in this work there was infiltration of hemocytes only after nine weeks. This infiltration was observed at the three salinities in shrimp exposed to Cu^{2+} and in the controls, which indicates that in this case it is not related to the presence of Cu^{2+}, but to the experimental hypo-osmotic conditions.

Ghate & Mulherkar (1979) observed gill necrosis in *Macrobrachium kistnensis* and *Caridina* sp. exposed in seawater to 0.1 mg Cu^{2+} L^{-1}, which indicates that, even at low salinities, *L. vannamei* is more tolerant to Cu^{2+} than these crustaceans, possibly because of interspecific differences, or because of the widespread use of Cu-based antimycotic compounds in Mexican hatcheries (Frías-Espericueta *et al*. 2008a).

The hepatopancreas is of interest in ecotoxicological research, since it has different physiological functions (secretory, absorptive, digestive and excretory) and serves as the main metal storage organ. Exposure to Zn^{2+} caused histological damage in the hepatopancreas of isopods (Odentaal & Reinecke 2007) and Manisseri & Menon (1995) observed alterations of the hepatopancreas tubular structure of *Metapenaeus dobsoni* exposed during 15 days to 0.105 μg Cu^{2+} L^{-1}. In addition, Yang *et al.* (2007) and Li *et al.* (2007a) also determined structural alterations and necrosis in *Eriocheir sinensis* and *Macrobrachium rosembergii*, respectively.

With a higher salinity and the same concentration of Cu^{2+} used in this study, Frías-Espericueta *et al.* (2008a) found minor alterations of the hepatopancreas of *L. vannamei*, but only after an exposure of nine weeks. This indicates an inverse relation between salinity and Cu^{2+} toxicity, and is probably due to the reduced formation of Cu-Cl complexes and the consequent higher concentration of the free metal ion at low salinities (Verslycke *et al.* 2003).

At low salinities shrimp actively maintain an hyper-osmotic internal environment, and the associated water fluxes necessary to maintain the homeostasis of the organisms (Roast *et al.* 2001) may facilitate metal uptake and a corresponding increase of the internal metal concentrations, due to the increased activity of the ionic pumps (Erk *et al*. 2008). This may cause metal homeostasis breakdown, which in turn affects important physiological and biological functions at the molecular and cellular level, causing damage to the cell micro-and ultrastructure and to different organelles, such as mitochondria, endoplasmic reticulum and nucleus membrane (Yang *et al.* 2008). This interaction metal-salinity should be considered to establish criteria of acceptable water quality for inland shrimp culture, because it may induce adverse shrimp responses.

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