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ARTICLE

Physiological responses of the red rocky crab *Cancer antennarius* exposed to different concentrations of copper sulfate

Respuestas fisiológicas en el cangrejo de roca *Cancer antennarius* expuesto a diferentes concentraciones de sulfato de cobre

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Resumen.- Se evaluaron en el cangrejo de roca *Cancer antennarius* las respuestas fisiológicas, metabólicas y hematológicas a diferentes concentraciones de sulfato de cobre (0,5, 1,0, 1,5, 2,0 mg L^{-1}). Las respuestas a determinar fueron: dosis letal media, capacidad osmorreguladora, consumo de oxígeno, excreción de amonio, relación atómica O:N, glucosa, el CTH y la concentración de hemocianina. En cuanto a la dosis letal media (LD_{50}) se determinó que la concentración fue de 1,6 mg L^{-1} de sulfato de cobre. El patrón observado en la mayoría de las respuestas medidas fue un aumento directamente proporcional a la concentración de sulfato de cobre; a excepción de la capacidad osmorreguladora, en la que el patrón de osmorregulación, de ser típicamente isosmótico en condiciones normales, se modificó a hiposmótico. En la relación atómica O:N se observó una disminución en los valores debido a cambios en sustrato metabólico como un resultado del estrés causado por la exposición al sulfato de cobre.

Palabras clave: Consumo de oxígeno, excreción de amonio, sulfato de cobre, *Cancer antennarius*

Abstract.- In this study we evaluated physiological, metabolic and hematological responses for the crab *Cancer antennarius* to different copper sulfate concentrations (0.5, 1.0, 1.5, 2.0 mg L^{-1}). The evaluated responses were: the median lethal dose, osmoregulatory capacity, oxygen consumption, ammonium excretion, atomic relation O:N, glucose, THC and hemocyanin concentration. The median lethal dose (LD_{50}) we found was 1.6 mg L^{-1} of copper sulfate. The pattern followed on most of the observed responses was an increase directly proportional to the amount of copper sulfate concentration, except for osmoregulatory capacity where the osmoregulation pattern, usually isosmotic in normal conditions, was modified to hyposmotic. In the atomic relation O:N we observed a decrease in values due to changes in metabolic substrate as a result of stress caused by exposure to copper sulfate.

Key words: Oxygen consumption, ammonia excretion, copper sulfate, *Cancer antennarius*

INTRODUCTION

Copper is an essential micronutrient required by all living organisms for a variety of physiological and biochemical processes. In crustaceans, copper is also part of the respiratory pigment, called hemocyanin, which participates in the transport of oxygen. Although copper is an essential element to aquatic organisms, it can be potentially toxic when its amount is above the usual standards (Gutierrez-Galindo *et al.* 1994, Martins *et al.* 2011). Even though there are important natural copper sources, multiple human activities have considerably increased the input of this metal in estuarine and marine environments around the world (Niencheski *et al.* 2006).

Organisms assimilate copper according to the proportion of this metal in the water; these assimilations occur throughout epithelia surfaces, where ions are also absorbed and excreted (Santore *et al.* 2001). Copper competes with other cations in order to bind with the uptake sites such as the gills, and once it is absorbed in various tissues it can become toxic (Engel 1993, Santore *et al.* 2001, Abolude 2009, Frias-Espericueta *et al.* 2011).

Different studies have shown that some metals such as copper have the ability to produce reactive oxygen species, resulting in lipid peroxidation, DNA damage, changes in sulfide groups and

altered calcium homeostasis (Stohs & Bagchi 1994, Barata *et al*. 2005). Sensitivity to copper can also depend on the homeostatic regulation of its uptake, storage and excretion (Depledge & Rainbow 1990). The metal concentrations were monitored since the 80's when it was observed that an increase of metals in the sea had begun due anthropogenic activities.

Buck *et al.* (2007) made a study in San Francisco Bay (North Pacific) where they found that the copper has reduced their concentrations of free hydrated copper ion, and the toxic species of copper, to below 1013M. However these copper-binding organic ligands, Cu_2+ would exist at levels toxic to most microorganisms living within the Bay.

In recent years there have been comparisons of these measurements and it has been determined that these metals are principally copper, cadmium, and zinc in the North Occident of Mexico (Gutierrez-Galindo *et al.* 1997, Frias-Espericueta *et al*. 2011).

Responses to stressors have traditionally been observed at the population, community or ecosystem level. Effects however are also manifested at the organismic level by impairing molecular, cellular and physiological functions (Hebel *et al.* 1997). Different histological, physiological, and molecular toxicants actions induce stress referred as biomarkers; they have been suggested and used to predict the stress effects and the animal response (Wedderburn *et al.* 1998, Sokolova & Lanning 2008). Osmoregulation maintaining osmotic homeostasis can be considered as a possible marker for crustaceans (Lignot *et al.* 2000). Osmoregulation, one of the most important regulatory functions an aquatic animal has to perform, has been extensively studied in many crustaceans that effectively osmoregulate, either as hyper-isosmoregulators or hyper-hypo-regulators; this occurring in marine, brackish and freshwater conditions (Mantel & Farmer 1983, Péqueux 1995).

Monitoring the crustacean physiological condition during the process of osmoregulation could therefore have a potential use as a biomarker in coastal waters, estuaries and lagoons. In particular, osmoregulatory capacity (OC) can be used as a reliable biomarker to monitor the physiological condition and the effect of stressors in crustaceans (Lignot *et al.* 2000).

The metabolic rate measurement has been used as a tool to determine the impact that several environmental factors can have in the organism, such as temperature, salinity or exposure to pollutants. This allows us to determine the energetic costs that these combinations have in the organism (Altinok & Grizzle 2003, Manush *et al.* 2004, Brougher *et al.* 2005, Sokolova & Lanning 2008). Oxygen consumption (VO_2) is intimately associated with the metabolic work and the energy flow that an organism can use for homeostatic process. Hemocyanin respiratory pigment is also a protein responsible of transporting oxygen and carbon dioxide through the hemolymph that constitutes 60 to 95% of the total plasma protein-, and an efficient physiological indicator of stress conditions.

The ammonium excretion rate has been used to evaluate the effect of various environmental factors and contaminants in crustacean physiology (Jiang *et al.* 2000). Ammonium represents 40 to 90% of the total nitrogen excreted by the crustaceans and is continuously released through the branchial epithelium (Regnault 1987).

Cancer antennarius (Stimpson, 1856) is a crustacean that its defense mechanism is based in the defense response of the hemocytes. These are incorporated in the hemolymph and they release molecules such as the serum prophenoloxidase system. Regarding crustaceans their hemocyte classification is based on morphological, functional, ultrastructural characteristics and the presence or absence of granules. Generally they are represented in 3 groups: hyalinocytes, semi granulocytes and granulocytes (Vargas-Albores *et al.* 1993).

Physiological studies have been done in other economically important crustaceans such as white shrimp *Litopenaeus vannamei* (Sanchez *et al*. 2001) or the crab *Cancer magister* and *Callinectes sapidus* (Hutcheson 1974 in Sokolova & Lanning 2008) in Cd; however, in red rocky crab *C. antennarius* there has been only a study on larval nutrition (Re & Bückle 1983) and in relation to thermal biology (Padilla-Ramirez *et al*. 2015). Therefore, there are not available physiological studies that measure metabolic responses, glucose, lactate, total protein, cholesterol, triglycerides, lipids, hemocyanin, hemocytes and respiratory burst.

Cancer antennarius is an important crab in Baja California in the north and southern part of the peninsula; its distribution range extends from northern California up to Alaska (MacGinitie 1935, Re & Bückle 1983). It lives in rocky shores, sandy substrates, semi buried under stones, in bays and estuaries. Its sexual maturity is reached at 2 years of age and it can be 60-80 mm wide (Carroll & Win 1989). The commercial importance of rock crab lays in its chelae, which is similar to that of the Dungeness crab *Cancer magister*, *Cancer antennarius* that are fished both commercially and recreationally. The industry is smaller compared to the fishery of *Cancer magister*, and it is mostly captured in California (Carroll & Win 1989).

The aim of this study is to evaluate the effect that an injection of copper sulfate can have in the osmoregulatory capacity, oxygen consumption, ammonium excretion, glucose and total hemocyte count and hemocyanin concentration in *C. antennarius* adults.

MATERIALS AND METHODS

Cancer antennarius crabs were obtained from Ejido Erendira, municipality of Ensenada, Baja California, Mexico (31º16'19.52"N, 116º23'46.46"W) during the summer season in the weight range of 200-300 g wet weight (w.w.). In the local area distribution of red crab in Baja California, temperatures in an annual cycle vary between 15°C in winter and 20.5ºC in summer (Center for Atmospheric Sciences 2013).

These animals were acclimated one week to laboratory temperature conditions of 20ºC in a closed system with water replacement; they were also fed daily with chunky fresh fish. They were individually distributed in plastic baskets where each one of these contained 20 organisms. In total, 250 adult crabs were used. They were randomly selected before each experiment. 50 crabs were used as experimental controls, 25 of these were injected with saline solution 500 µL, and this group was called 'saline control'. Another group of 25 was considered as the controlled organisms: they were not injected. Both groups control and saline control- were exposed to the same experimental procedure. To determine the lethal dose (LD_{50}) 10 crabs (n total= 40) were injected with each of the concentrations of copper sulfate (0.0, 0.5, 1.0, 1.5, and 2.0 mg L^{-1}) (Fermont 63362) (Chemical Products Monterrey, Mexico, CAS7758-99-8), and survival was monitored for 96 h. The median lethal dose was determined by the Probit method.

Different copper sulfate concentrations were prepared for each experiment, and the number of experimental crabs was 25 for each concentration where (control C 0.0, CS control saline and 0.5, 1.0, 1.5, and 2.0 mg L^{-1}) of copper sulfate. All groups of crabs were injected with 500 ìL of the solution at the right side of their fifth pereipod.

Hemolymph was extracted from 25 crabs for each experimental condition (0.5, 1.0, 1.5 and 2.0 mg L^{-1}), including the controlled organisms (C, CS). This was carried out by a hypodermic syringe with a solution of 1 ml heparin as anticoagulant. This syringe was inserted in the crab´s coxa and then 300 µL of hemolymph were extracted with an automatic pipette. After obtaining the hemolymph samples, these were placed on a sheet of parafilm, which was kept cold on a plate to 4°C. The hemolymph sample was divided into fractions of 50 µL for posterior analysis, and then it was diluted (1:1) with the solution of Vargas-Albores *et al.* (1993) for an analysis of the total count of hemocyte; 50 µL were diluted in Alsever solution for analysis glucose quantification.

To measure hemolymph osmolality 10 µL were taken. Hemolymph osmolality and of seawater were determined by the Wescor 550 vapor osmometer, and the data was expressed in mmol kg^{-1}. To assess the ability of the organisms to maintain osmoregulatory capacity from each treatment, this was calculated from the following formula: Osmoregulatory capacity OC= internal media concentration - concentration of the external medium (Lignot *et al.* 2000).

Oxygen consumption routine rate (OCRR, mg O_2 h^{-1} kg^{WW-1}) of the experimental organisms was quantified for each different experimental condition using a semi-open respirometer system (Díaz *et al*. 2007). The oxygen concentration was measured by an oxymeter (YSI 52B) with a polarographic sensor, which helped avoid contact with air. We also took an initial sample of oxygen before closing the respirometric chambers. Thirteen crabs were placed individually in respirometric chambers of 3000 mL, with an empty respirometric chamber to measure oxygen consumption by microorganisms present in the water (control). Water temperature was maintained at 20 ± 1ºC (Padilla-Ramirez *et al*. 2015).

Respirometric chambers remained closed for 40 min, as Thurberg *et al.* (1973). The respirometric chambers consisted of a complete airtight container that had a valve that allowed the aerated water to inlet and also to outlet water with a low oxygen concentration. After this time the final measurement was made and the aerated water flow was reopened to allow water exchange in the chambers. Measuring of OCRR exposed to different copper concentrations was calculated by the following equation according to Cerezo-Valverde *et al.* (2006) and Zheng *et al*. (2008):

$$OCRR = (Ct - Co)\ V / (WW \times T),$$

where Ct and Co represent the variation regarding the oxygen content (mg O_2 h^{-1} kg^{WW-1}), V is the volume of the respirometric chamber before and after measuring the tests; *ww* was the wet weight of *C. antennarius* (g) and T (h) the time duration.

To determine the ammonium excretion rate (AER) we followed the procedure described above for oxygen consumption. It took a 10 mL water sample from each chamber to measure ammonia concentration using the method of phenol indo blue (Rodier 1981 modificated by Medina-Romo 2015). The tubes were covered with parafilm and left to incubate for one hour. After the reaction, the samples were read in a spectrophotometer at an absorbance of 640 nm (DR/4000U Spectrophotometer). Ammonia concentration was determined by a calibration curve, with ammonium chloride J.T. Baker 0660-01 (Phillisbur, N.J., USA). The calculation for AER was carried out with the following equation:

$$AER = (Ct' - Co')\ V / (WW \times T)$$

where Ct' and Co' are the change in ammonia excretion initial and final in mg NH_4^+ h^{-1} kg^{WW-1}, V is the bottle volume control

before and after the tests, ww was the wet weight of *C. antennarius* (g) and T (h) the duration time.

The atomic relation oxygen:nitrogen (O:N) was calculated using the values obtained from crabs oxygen consumption and nitrogen excretion when exposed to different concentrations of copper sulfate. The calculation was made as follows:

$$O:N = \text{atomic weight } (NH_4^+) \,/\, \text{atomic weight } (O_2) * [QO_2] / [NH4+]$$

where QO_2 is the oxygen consumption and NH_4+ is the total ammonia excreted by organisms.

The samples of hemolymph were centrifuged at 800 g for 3 min at 4ºC, and the supernatant was removed for glucose quantification, using the kit called 'Scientific Pointe, Inc.' (with a sensitivity of 1 mg dL^{-1}). These samples were read at 500 nm on a Hatch spectrophotometer; the glucose concentration was calculated according to the fabricant instructions.

Quantifying hemocyanin was obtained immediately after the extraction, samples were taken from 10 µL of hemolymph without heparin from organisms from all experimental conditions, and the samples were diluted with 990 µL of distilled water in the cells of the spectrophotometer and read at 280 nm (Engel & Brouwer 1987). Hemocyanin concentration was determined by calculation based on a subunit of 74,000 Da for crustaceans (Hagerman 1986).

The total hemocyte count was carried out with an automated cell counter device TC10 Gemstar USA BIORAD in which hemolymph was introduced with the anticoagulant according to Vargas Albores *et al*. (1993) and placed in the appropriate counting chamber for reading. To perform the total hemocyte count (THC), 10 µL of hemolymph from each control and experimental group were placed in a TC10. Each plate was calculated with a dual chamber and then these were inserted in a TC10 cell counter for the hemocytes sequential counting.

The physiological response data obtained by the crab exposure to the copper sulfate were processed through an exploratory data analysis (Tukey 1977). A one-way analysis of variance was used following the tests to show the normality (Shapiro-Wilk test) and homoscedasticity (Bartlett test) of the data. A Kruskal-Wallis test was used to determine whether there were differences in the oxygen consumption OCRR, ammonium excretion rate AER, atomic relation O:N, glucose concentration, hemocyanin, and THC between the different concentration of copper sulfate. The effect of the exposition to copper sulfate on of the crab was then analyzed using Sigma Stat version 3.1 and plotted with Sigma Plot version 12.

Results

We found that the LD_{50} was 1.6 mg L^{-1} of copper sulfate for *Cancer antennarius*. The control group showed a 100% survival, whereas those injected with saline solution (concentration of 0.5 mg $L^{-1)}$ had a 95% survival. Organisms at a concentration of 1.0 mg L^{-1} had a survival of 66.6%. Whereas those exposed to 1.5 mg L^{-1} its survival decreased down to 58.30%. As for the ones with a concentration of 2.0 mg L^{-1}, their survival decreased down to 41.6% (Fig. 1, Table 1).

In experimental organisms, crabs injected with different copper sulfate concentrations showed an adverse effect in relation to their osmoregulatory capacity (OC). Crabs OC had a range from -1 ± 3.08 to -3 ± 3.58 for the control ones with a copper concentration of 0.5 mg L^{-1}. At a higher concentration the range was -29 ± 4.10 to -64 ± 4.11 (Fig. 2); a change was observed in *C. antennarius* hemolymph regulation pattern, changing from being isosmotic to hyposmotic.

Oxygen consumption routine rate and nitrogen excretion of *C. antennarius* increased as the copper dose also augmented. The value ranges for the OCRR were 46.06 ± 9.08 to 84.00 ± 10.1 mg $O_2 h^{-1} kg^{-1}$; and for the AER from 1.43 ± 0.21 to 0.26 ± 0.02 mg $NH^+_4 h^{-1} kg^{-1}$, being significantly different ($P < 0.05$) (Figs. 3 and 4).

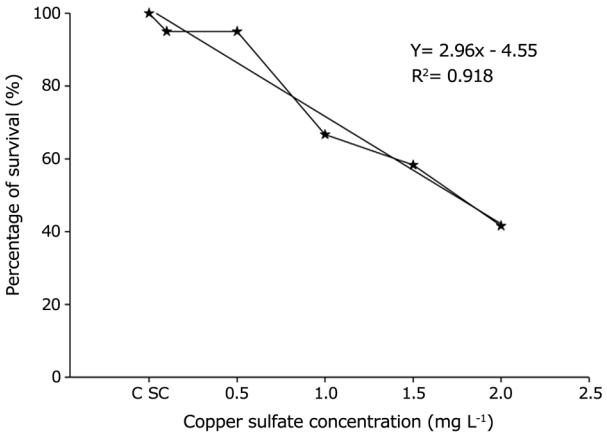

Figure 1. Percentage of survival (%) of *Cancer antennarius* exposed to different concentrations of copper sulfate (10 organisms per unit concentration). C control, SC saline control / Porcentaje de supervivencia (%) de *Cancer antennarius* expuesto a diferentes concentraciones de sulfato de cobre (10 organismos por concentración). C control, SC control salino

Table 1. Physiological responses of organisms exposed to different concentrations of copper 0.5; 1.0; 1.5, and 2.0 and two controls (C) 0.0 and saline control (CS) lowercase letters set significance (*P* < 0.001) / Respuestas fisiológicas obtenidas de los organismos expuestos a las diferentes concentraciones de cobre 0,5; 1,0; 1,5; y 2,0 y dos controles (C) 0,0 y control salino (CS), las letras minúsculas establecen la significancia (*P* < 0,001)

Physiological responses	Copper sulfate concentrations (mg L^{-1})					
	Control (C)	Control (CS)	0.5	1.0	1.5	2.0
OC (mmol kg^{-1})	-1 ± 3.08^a	-3 ± 3.93^a	-3 ± 3.58^a	-29 ± 4.10^b	-57 ± 5.32^c	-64 ± 4.11^c
Atomic ratio O:N	54.56 ± 4.28^a	46.75 ± 4.61^a	35.45 ± 3.22^a	26.25 ± 4.2^b	15.50 ± 3.2^c	11.25 ± 4.2^c
OCR (mg O_2 h^{-1} kg^{-1})	46.06 ± 9.08^a	47.87 ± 9.93^a	49.79 ±10.58^a	65.36 ± 9.1^b	74.58 ±10.3^c	84.00 ±10.1^c
AER (mg NH_4 h^{-1} kg^{-1})	0.26 ± 0.02^a	0.28 ± 0.018^a	0.31 ± 0.022^a	1.20 ± 0.1^b	1.24 ± 0.08^c	1.43 ± 0.21^c
THC (1 x 10^6)	3.00 ± 0.78^a	4.87 ± 0.71^a	5.68 ± 0.62^a	6.98 ± 0.72^b	8.28 ± 0.92^c	9.36 ± 0.51^c
Hemocyanin (mg L^{-1})	2.50 ± 0.58^a	2.53 ± 0.61^a	2.70 ± 0.62^a	3.7 ± 0.7^b	4.56 ± 0.42^c	5.34 ± 0.51^c
Glucose (mg L^{-1})	25.72 ± 4.23^a	27.45 ± 4.61^a	31.24 ± 3.22^a	44.59 ± 4.2^b	60.41 ± 3.22^c	74.63 ± 4.2^d

OC: Osmoregulatory capacity, OCR: Oxygen routine consumption, AER: Ammonium excretion, THC: Total hemocyte count

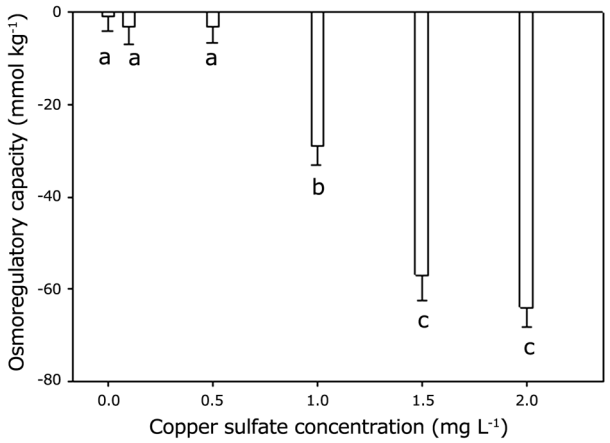

Figure 2. Osmoregulatory capacity (mmol kg^{-1}) of *Cancer antennarius* exposed to different concentrations of copper sulfate (25 organisms per unit concentration. Different letters indicate significant differences between concentrations of copper sulfate (α= 0.05). Media ± SE / Capacidad osmorregulatoria (mmol kg^{-1}) de *Cancer antennarius* expuesto a diferentes concentraciones de sulfato de cobre 25 organismos por concentración). Diferentes letras indican diferencias significativas entre las concentraciones de sulfato de cobre (α= 0,05). Media ± ES

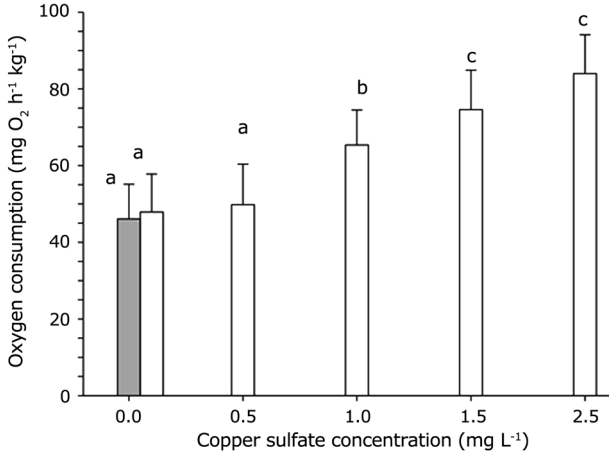

Figure 3. Oxygen routine consumption (mg O_2 h^{-1} kg^{-1}) of *Cancer antennarius* exposed to different concentrations of copper sulfate. Different letters indicate significant differences between concentrations of copper sulfate (α= 0.05). Media ± SE / Consumo de oxígeno de rutina (mg O_2 h^{-1} kg^{-1}) de *Cancer antennarius* expuesto a diferentes concentraciones de sulfato de cobre. Diferentes letras indican diferencias significativas entre las concentraciones de sulfato de cobre (α= 0.05). Media ± ES

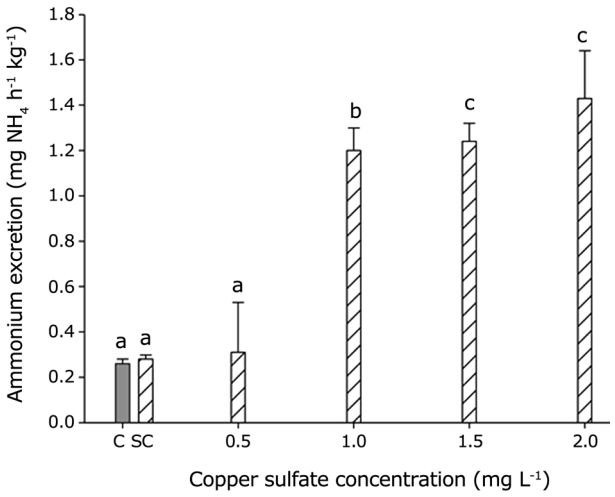

Figure 4. Ammonium excretion (mg NH_4 h^{-1} kg^{-1}) of *Cancer antennarius* exposed to different concentrations of copper sulfate (25 organisms per unit concentration). C control, SC saline control. Different letters indicate significant differences between concentrations of copper sulfate (α= 0.05). Media ± SE / Excreción de amonio (mg NH_4 h^{-1} kg^{-1}) de *Cancer antennarius* expuesto a diferentes concentraciones de sulfato de cobre (25 organismos por concentración). C control, SC control salino. Diferentes letras indican diferencias significativas entre las concentraciones de sulfato de cobre (α= 0,05). Media ± ES

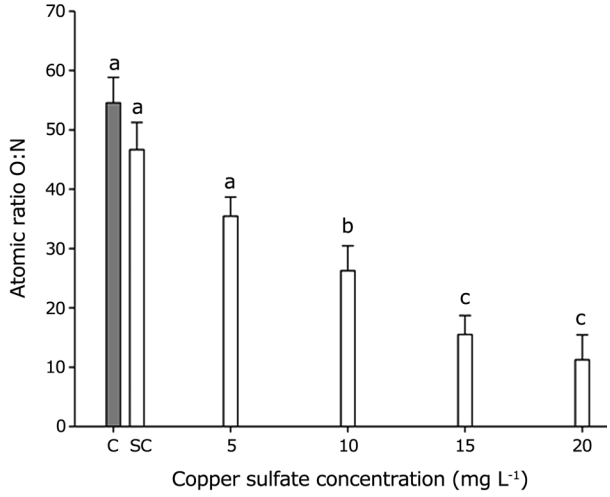

Figure 5. Atomic ratio oxygen:nitrogen O:N of *Cancer antennarius* exposed to different concentrations of copper sulfate. C control, SC saline control. Different letters indicate significant differences between concentrations of copper sulfate (α= 0.05). Media ± SE / Relación atómica oxígeno:nitrógeno O:N de *Cancer antennarius* expuesto a diferentes concentraciones de sulfato de cobre. C: control, SC: Control salino. Diferentes letras indican diferencias significativas entre las concentraciones de sulfato de cobre (α= 0,05). Media ± ES

The values of the O:N in each of the different concentrations had an interval of 11.25 ± 4.2 to 54.56 ± 4.28 indicating a change in the metabolic substrate type used by the crab (Fig. 5).

At the lowest concentration of copper the glucose in hemolymph (25.72 ± 4.23 mg L^{-1}) was not different for control organisms, crabs in the saline group and those exposed to 0.5 mg L^{-1} of $CuSO_4$. Though we did find significant differences when the copper concentration incremented glucose levels increased from 44.59 ± 4.2 to 74.63 ± 4.2 ($P < 0.05$) (Fig. 6).

The concentration of hemocyanin in the hemolymph of the organisms exposed to copper sulfate had an interval of 2.50 ± 0.58 to 5.34 ± 0.51 mmol L^{-1}. Controls showed no significant differences ($P > 0.05$) in their levels of hemocyanin compared to saline control organisms. There were no significant differences ($P > 0.05$) between the values of hemocyanin in the crabs at control, saline control conditions, and the concentration of 0.5 mg L^{-1} $CuSO_4$. Crabs injected with high concentrations of copper sulphate, did show meaningful differences ($P < 0.05$) in the levels of hemocyanin (Fig. 7).

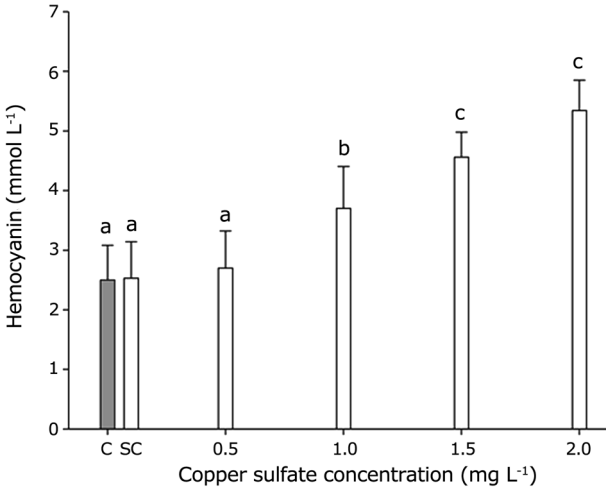

Figure 6. Hemocyanin concentration (mmol kg^{-1}) in hemolymph of *Cancer antennarius* exposed to different concentrations of copper sulfate. C control, SC saline control. Different letters indicate significant differences between concentrations of copper sulfate (α= 0.05). Media ± SE / Concentración hemocianina (mmol kg^{-1}) en hemolinfa de *Cancer antennarius* expuesto a diferentes concentraciones de sulfato de cobre. C control, SC control salino. Diferentes letras indican diferencias significativas entre las concentraciones de sulfato de cobre (α= 0,05). Media ± ES

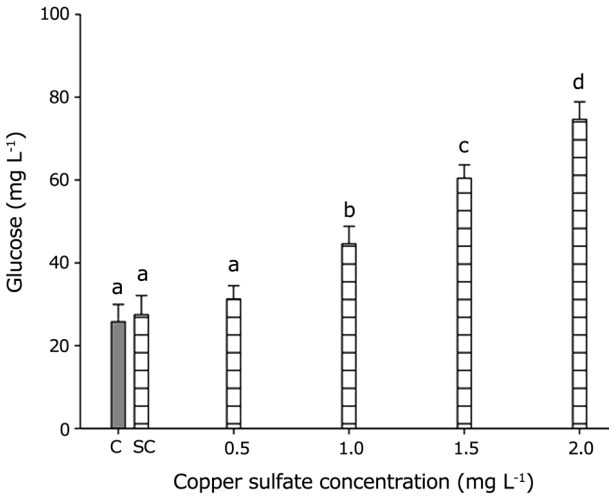

Figure 7. Glucose concentration (mg L^{-1}) in hemolymph of *Cancer antennarius* exposed to different concentrations of copper sulfate. C control, SC saline control. Different letters indicate significant differences between concentrations of copper sulfate (α= 0.05). Media ± SE / Concentración de glucosa (mg L^{-1}) en la hemolinfa de *Cancer antennarius* expuesto a diferentes concentraciones de sulfato de cobre. C control, SC control salino. Diferentes letras indican diferencias significativas entre las concentraciones de sulfato de cobre (α= 0,05). Media ± ES

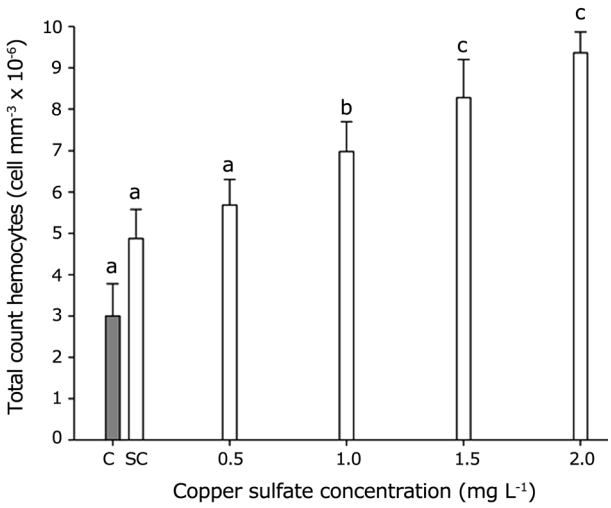

Figure 8. Total hemocyte count (THC cell mm^{-3} x 10^6) of *Cancer antennarius* exposed to different concentrations of copper sulfate. C control, SC saline control. Different letters indicate significant differences between concentrations of copper sulfate (α= 0.05). Media ± SE / Conteo total de hemocitos (células THC mm^{-3} x 10^6) de *Cancer antennarius* expuesto a diferentes concentraciones de sulfato de cobre. C control, SC control salino. Diferentes letras indican diferencias significativas entre las concentraciones de sulfato de cobre (α= 0,05). Media ± ES

As for the total hemocyte count there were not significant differences (THC) between the control, the saline control and the organisms exposed to 0.5 mg L^{-1} of $CuSO_4$. However, there were significant differences ($P < 0.05$) in the THC (6.98 ± 0.72 to 9.36 ± 0.511 x 10^6) at high concentrations of $CuSO_4$ (Fig. 8).

Discussion

The injection of copper sulfate had major consequences on survival and general physiological performance of *Cancer anntenarius*. Mendoza-Rodriguez (2009) in *Caementarus cryphiops* observed the effect of copper sulfate in a concentration of 0.0078 and 0.0126 mg L^{-1}, determining a survival of 10 and 0%, respectively. In the case of the commercial shrimp *Artemesia longinaris*, a median lethal dose was obtained at 72 h of 0.212 mg L^{-1} (Scelzo 1997). For commercial crab *Lithodes santolla*, Amin & Comoglio (2010) obtained a median lethal copper concentration of 0.002985 mg L^{-1} at 96 h. In the shore crab *Carcinus maenas*, Boitel & Truchot (1989) they conducted acute toxicity studies with copper obtaining the average lethal concentration of 1 and 2 mg L^{-1} in the water, after 96 h of exposure.

These studies show that the median lethal dose and survival of *C. antennarius* compared to other crustacean species may differ due to exposure to this type of metal, as it can be direct (injected into the circulatory system of crustacean) or indirect (the copper dissolved in the seawater). Estuarine and coastal areas are often the most polluted areas in the ocean, due to urban development and human industrial and agricultural activities even though the concentration has a range of 1.5-60 µg g^{-1}. In most of the work when the exposure was indirect the concentrations used were lower (0.05 µg g^{-1}) compared to those ones regarding the direct use (1.5 mg L^{-1}) or mixed due of synergetic effects or antagonistic (Cu/Zn or Zn/Cu, Frias-Espericueta *et al.* 2011). In this study, *Cancer antennarius* had a direct exposure to copper and its median lethal concentration (LD_{50}) was in proportion to the volume internal medium (VD) resulting in 1.6 mg L^{-1}.

According to Jones (1941), *Cancer antennarius* is a strict osmoconformer when it is exposed to different salinities, maintaining an osmolality concentration of 998 mmol kg^{-1} in their hemolymph. Similar results were found by us in *C. antennarius* from the control group: organisms exposed to higher concentrations of copper had a change in their osmoregulatory pattern from isosmotic to hyperosmotic, a possible damage in the gills, causing disruption of the mechanisms used for the maintenance of water and ions balance in the red

rocky crab *C. anntenarius*. In *Cancer irroratus* and *Carcinus maenas*, Thumberg *et al*. (1973), reported a reduction of 90-100% of their hemolymph osmotic pressure. Bambang *et al.* (1995) in *Penaeus japonicus* obtained a change in hypo and hyper-osmoregulatory capacity due to an exposure to a higher copper concentration indicating that an imbalance in plasma ions occurred. The effect of copper on osmoregulatory capacity was therefore dependent on the dose. The mechanism responsible for the variation in crustacean's osmoregulatory capacity seems correlated to the reduction or increasing in ion concentration in the hemolymph and inhibition of the gill Na+/ K+ ATPase activity, however we did not estimate the activity of this pump in the crabs. In the present study, osmoregulatory capacity proved to be a useful tool to monitor the toxic effect of copper, since the osmoregulatory capacity can be used as a physiological marker condition of *Cancer antennarius* to detect sublethal stress effects and the effect of pollutants in seawater as copper.

Cancer anntenarius oxygen consumption routine rate was affected by the copper concentration used. We observed a direct relationship: as the copper concentration increased, oxygen consumption also increased. Amin & Comoglio (2010) reported in *Lithodes santolla* a similar increment in the oxygen consumption routine rate, when organisms were exposed to different copper concentrations. In the fresh water crab *Potamonautes warren*, when it was exposed to sub lethal effects of copper, the rate of oxygen consumption increased up to the 14th day of exposure (Vosloo *et al*. 2002). When an organism homeostasis is disturbed, it leads to compensatory adaptive processes that in general, try to compensate the higher energy demand produced by the contaminant, in this case copper. Therefore, the metabolic rate of an organism should increase under toxic stress. Due to the limited energy resources of organisms, the additional metabolic costs result in a reallocation of energy resources (Beyers *et al*. 1999). Increased of oxygen consumption rate in *C. antennarius* was due to an increase investment of energy to balance the osmoregulatory change.

The ammonium excretion rate was also directly related to the process of osmoregulation since excretion of ammonia by the crabs when exposed to higher copper concentrations increased according to the osmoregulation-impaired ability produced by the stressful conditions. There have been studies on ammonia excretion in crustaceans, as in the case of *P. chinensis* in which Chen & Lin (1994) found increased levels of ammonia excretion when external medium variations occurred. This is because organisms in diluted media are used to balance their internal medium uptake of sodium by increasing their ammonium excretion through Na^+/ NH^+_4 pump to adjust their osmoregulatory capacity (Spaargaren *et al.* 1982, Regnault 1987, Jiang *et al.* 2000).

The O:N atomic ratio in red rocky crab was affected when it was exposed to higher concentrations of copper, reflecting the change of metabolic substrate type. When experimental organisms were exposed to lower concentrations of copper, metabolic energy came from a mixture of lipids and proteins. As for organisms were exposed to higher concentrations of copper, the crabs begin to catabolize proteins as a fuel metabolic substrate. Amin & Comoglio (2010) reported for *Lithodes santolla* that when it was exposed to the highest concentration of copper (1.61 mg L^{-1}) a 117% decrease occurred in the rate of O:N; similar to the results observed in the present study. This change in metabolic substrate was due to the stress to which they were exposed (Mayzaud & Conover 1988).

In this study, organisms showed an increase of glucose concentration in hemolymph in all copper concentrations tested. Due to stress caused by energy expenditure it derived to a metabolic rate increase in order to obtain energy and to channel the osmoregulation process. Racotta & Palacios (1998) demonstrated that glucose in the hemolymph of *L. vannamei* increases in response to stress caused by sampling hemolymph.

As for the group or organisms in control (C) and those in saline control (CS) of *C. antennarius*, THC was observed with an interval of 3 ± 0.78 to $4.87 \pm 0.74 \times 10^6$ in studies conducted with *Carcinus aestuarii*. Matozzo & Marin (2010) obtained in the control organisms a THC with a range of 1.04- to 12.21 x 10^6 cell mL^{-1} and an average of 6.4 x 10^6 cells mL^{-1}, which were close to those obtained in this study for the controlled organisms. Truscott & White (1990) reported that *Carcinus maenas* had concentrations of 14 to 32 x 10^7 for crab hemocyte. Smith & Ratcliffe (1978) obtained similar values of 25 x 10^7 and 16 x 10^7 for the same species. THC concentrations are higher because these organisms were exposed to different tidal rhythms. In *C. antennarius* we observed an increase in the total hemocyte count in relation to the concentration of copper sulfate. This could be due to the fact that crabs defense mechanism has no specificity. Organisms reacted to the copper injection as if it were a foreign agent, but since hemocytes did not find any bacteria, hemocytes did not attack; therefore when hemocytes were counted, the number increased directly proportional to the amount of copper.

Copper is a necessary component to hemocyanin, but excessive amounts lead to crustacean death (Raymont & Shields 1962, Eisler *et al.* 1972). In this study, as copper increased, hemocyanin also did in the hemolymph of the crabs. There was a direct correlation between the increase of oxygen consumption rate and the concentration of hemocyanin. That is because this

protein transports oxygen and in order to increase it requires a greater demand of hemocyanin. Pascual *et al.* (2003) mention that hemocyanin is a multifunctional protein that can be used as a storage protein, oxygen carrier and osmolytes.

The physiological, metabolic and hematological responses determined in *C. antennarius* allowed us to quantify the effect of the injection of different concentrations of copper, causing modifications of the physiological steady state of crabs and stress caused by exposure to the pollutant, increasing susceptibility to pathogens.

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