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EFFECT OF COPPER ON THE RNA/DNA RATIO AT DIFFERENT TEMPERATURES IN JUVENILES OF *PETENIA KRAUSSII* (PISCES: CICLIDAE)

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ABSTRACT**:** The effect of temperature and copper on the RNA/DNA and protein/DNA ratios of *Petenia kraussii* juveniles was evaluated. Juveniles were acclimated for 15 days at temperatures of 22°C and 30°C and later kept in aquaria containing copper concentrations of 0.2 and 1.2 mg/L for each temperature for 30 days. They were then transferred to water containing no metal to be detoxified. The bioassays were performed statically with water and metal renewal every 24 hours. RNA/DNA levels decreased in the organisms kept at 22°C, the decrease being more noticeable on the juveniles exposed to the higher copper concentration. The same response was evidenced for the protein/DNA ratio. Copper had no significant effect on the RNA/DNA and protein/DNA ratios of the specimens exposed to 1.2 mg/L concentrations at 30°C, whereas a slight increase of these parameters was detected on the specimens exposed to 0.2 mg/L during the first week. *P. krassii* juveniles, particularly those kept at 30°C, did not reach referent RNA/DNA and protein/DNA values after 30 days. These results demonstrate that temperature changes are determinant of copper toxicity in the juveniles of this species.

Key words: RNA/DNA ratio, copper, toxicity, temperature

RESUMEN: Se evaluó el efecto de la temperatura y el cobre sobre la relación ARN/ADN y proteínas/ADN en juveniles de *Petenia kraussi*. Los juveniles fueron aclimatados durante 15 días a temperaturas de 22 y 30ºC y posteriormente colocados primero en acuarios con dosis de 0,2 y 1,2 mg/L de cobre para cada temperatura durante 30 días y luego transferidos a agua sin metal para su depuración. Los bioensayos se realizaron de forma estática con renovación de agua y metal cada 24 horas. Los niveles de ARN/ADN disminuyeron en los organismos expuestos a 0,2 y 1,2 mg/L de cobre a la temperatura de 22ºC, siendo más pronunciado el descenso en la mayor dosis del metal. Este mismo comportamiento fue observado para la relación proteínas/ADN. No hubo un efecto significativo del metal sobre la relación ARN/ADN y proteínas/ADN en los ejemplares expuestos a la dosis de 1,2 mg/L de cobre y temperatura de 30ºC, mientras que a la dosis de 0,2 m/L de cobre se observó un ligero incremento de los parámetros durante la primera semana. Estos resultados demuestran que la temperatura determina la citotoxicidad del cobre en juveniles de *Petenia kraussi*. Los juveniles no llegaron a alcanzar valores de ARN/ADN y proteínas/ADN similares a los controles después de 30 días de depuración, particularmente los expuestos a 30ºC.

Palabras clave: relación ARN/ADN, toxicidad, cobre, temperatura

INTRODUCTION

The bioaccumulation and toxic effects of copper and other metals are directly affected by several environmental factors such as salinity, water hardness, pH and temperature (ABEL, 1990), the latter being one of the most important, capable by itself of affecting the metabolism (LEMUS *et al*. 1993) and distribution of organisms within the ecosystem (COUTANT, 1987).

Xenobiotics trigger defense mechanisms that require significant energy use by the organism being threatened, as these chemicals jeopardize organisms' life cycles, metamorphosis, reproduction, and particularly, growth, the latter requiring high consumption of energy and being susceptible to a variety of biotic and abiotic factors (MEEKAN *et al.* 2003). The growth of organisms is determined by their energy status; their DNA, RNA, and protein synthesis is modulated by thermal changes, the organisms thus becoming potential targets of toxic substances such as transition metals.

Determining growth through RNA/DNA ratios has been limited in toxicity research. However, it has been widely

used to evaluate the physiological conditions or growth ratios of white muscle of larvae and fish juveniles in natural environments (PECK *et al.* 2003). Growth is the result of cell multiplication or hyperplasia and cellular increase or hypertrophy. RNA is present in varying concentrations in the nucleus and cytoplasm, and it is directly related to protein synthesis (BULOW, 1987; CALDERONE *et al*, 2003). RNA/DNA is regarded as a metabolic ratio activity index, and protein/DNA as the protein synthesis ratio (BULOW, 1987). These indices have been used as toxic effect biomarkers of chemical compounds on fish instantaneous growth (BARRON & ADELMAN 1984; KNOWLES & MCKEE, 1987; CHOPITE & NUSETTI, 1989). However, their implication is limited in toxicological research of metals in organisms exposed to thermal changes.

In this research, the cytotoxic effect of copper at different temperatures on the physiological conditions and on the RNA/DNA and protein/DNA ratios of *Petenia kraussii* juveniles was determined under laboratory conditions.

MATERIALS AND METHODS

Collection of specimens

Specimens of *Petenia kraussii* with an average weight of 3.78 ± 0.98 g were collected in La Aguá lagoon, near Chiguana, state of Sucre, Venezuela (10° 30' N, 63° 41' W), using a 3m high, 17m wide, 3mm mesh fishing net. The samples were kept in buckets and carried to the laboratory, where they remained for 5 days, after which they were divided in two groups to be acclimated, one at 22° and the other at 30°C for 15 days. During this period organisms were fed with a commercial diet, Universal, thrice a day *ad libitum*.

Experimental design

The experimental design was carried out according to the methodology standardized by Lemus *et al*. 1993. The specimens previously acclimated at 22 and 30°C were exposed to 0.20 and 1.20 mg/L copper concentrations prepared from $CuSO_4 \cdot 5H_2O$. The organisms were randomly selected and placed in 40L aquaria. Each experimental and control group was duplicated. Additionally, these experiments were performed again to accomplish detoxification tests. From each aquarium, 4 specimens were taken after 1, 2, 3, and 4 weeks of exposure, with their own replicate, totalizing 8 organisms for each time interval. The metal was added daily with a 100% water renewal. Water pH had an average value of 8.1 ± 0.15, water hardness was 55 ¼g/L of $CaCO_3$, and the temperatures of the two sets of aquaria were 22.00 ± 0.5°C and 30.0 ± 0.75°C, respectively.

Sampling

Eight specimens, 4 from each aquarium, were killed by decapitation every week. 300 mg of white ephaxial muscle was immediately dissected from the upper dorsal area in the back of the operculum of each fish. A portion of muscle was also taken to determine dry weight.

RNA and DNA determination

To determine nucleic acids, a modification of Bentle's method was used (WESTERMAN & HOLT, 1988). 30 mg of muscle was homogenized in the cold with 800 ¼L of 1 mol/L NaCl to which 50 ¼L of 1mg/mL K proteinase was subsequently added (Type V *Streptomices griseus*), allowed to stand for 30 minutes, and centrifuged at 3000 rpm for 30 min. Duplicate 100 ¼L aliquots were taken from the supernatant, treated with 1 mL of 5 ¼g/mL ethidium bromide and 400 ¼L of distilled water, and subsequently incubated at 37°C for 1 hour, after which they were added Buffer 80 mmol/L Tris acetate, 4 mmol/L $MgCl_2$, and 3.2 mmol/L Ca Cl_2, and their fluorescence values measured in a Turner fluorometer model 405. The filters used were 360 nm for excitation and 590 for emission.

After the reading took place, 20 ¼L of 5mg/mL RNase (EC 3.1.27.5, type 1-A from bovine pancreas) was added and the aliquots were incubated for one hour at 37°C to read their fluorescence again. Finally, each sample was treated with 20 ¼L of 1mg/mL DNase (EC 3.1.21.1, from bovine pancreas) and incubated for 1 hour at 37°C before a final fluorescence reading was taken.

RNA and DNA calculations

RNA fluorescence was determined by subtracting the first reading from the second one, and that of DNA by subtracting the second value from the third. These values were compared to the RNA pattern curve (Sigma type XXI of *E. Coli,* 0.15 mg/ml), and to that of DNA (Type I highly polymerized thymus cortex, 0.1 mg/mL). The pattern curves used varied between 0.2 and 2 µg/mL and between 0.2 and 4 µg/mL for RNA and DNA, respectively.

All reagents used to determine nucleic acids belonged to the commercial brand SIGMA, and determinations were expressed in ¼g/mg of dry weight.

Protein determination

A hundred microliters of the homogenate prepared to determine nucleic acids was centrifuged in ependorf tubes at 3000 for 15 min. Duplicate aliquots of 25¼L were taken and treated with 1.25 mL of 800 mmol/L NaCl and 400 ¼L of Bradford's reagent (BRADFORD, 1976). Absorbance was read at 590 nm after 30 min in a Gilford spectrophotometer model 250. The pattern curve was plotted using 1 mg/mL of Bovine Serum Albumin Standard (BSA).

Statistical analyses

Multifactorial variance analyses were performed for RNA, DNA, proteins and RNA/DNA and protein/DNA rates with the purpose of establishing the individual and overall effect of temperature, metal concentrations and time of exposure (HICKS, 1973). The analyses were carried out for the accumulation and detoxification process.

RESULTS

RNA, DNA, and protein levels and RNA/DNA and Protein/DNA ratios were significantly affected by the copper dose, temperature, and time of exposure, though RNA and RNA/DNA ratios were not affected by time of exposure alone. The interaction of factors such as temperature, time of exposure, and metal concentrations was also determined.

DNA values varied significantly depending on time of exposure to the metal, temperature, and metal concentration. In those organisms not exposed to the metal and kept at a temperature of 30°C during the four weeks, DNA average values (1.62 ± 0,08 and 2.04 ± 0.17) were higher than those exposed at 22°C (1.07 ± 0.5 and 1.58 ± 0.08) during the same period. At 22°C, DNA concentrations were similar between Cu-treated and control individuals, while a DNA decrease was evident in those exposed to copper at 30°C when compared to their control counterparts (Table 1).

Organisms kept at 22°C without metal showed higher RNA concentrations than their counterparts kept at 30°C. At 22ºC RNA concentration varied between 5.30 ± 0.61 and 6.65 ± 0.46 during four weeks of exposure to Cu, while at 30ºC it varied between 2.70 ± 0.90 and 3.12 ± 0.37 for the same period (Table 2).

In organisms exposed to two copper concentrations at 22°C, RNA levels decreased depending on the metal dose. In those exposed to 0.2 mg/L, the average value was 3.65

TABLE 1. DNA contents (µg/mg of dry weight) in ephaxial muscle from juveniles *Petenia kraussii* through four weeks of Cu-exposure and Cu-depuration at 22 and 30°C. N=8 organisms.

Cu-exposed and Cu-depurated fish at 22ºC					
Weeks	Control	Cu-exposure		Cu-depuration	
		mg/l Cu			
		0.2	1.20	0.2	1.20
1	1.07±0.15	1.52±0.05	1.61±0.08	1.51±0.08	1.48±0.05
2	1.58±0.08	1.46±0.02	1.66±0.07	1.52±0.04	1.49±0.09
3	1.55±0.11	1.52±0.13	1.58±0.13	1.48±0.04	1.58±0.04
4	1.52±0.10	1.58±0.07	1.52±0.07	1.47±0.04	1.49±0.05
Cu-exposed and Cu-depurated fish at 30ºC					
Weeks	Control	Cu-exposure		Cu-depuration	
		mg/l Cu			
		0.2	1.20	0.2	1.20
1	1.77±0.13	1.33±0.11	1.47±0.06	1.55±0.05	1.55±0.10
2	2.04±0.17	1.56±0.08	1.55±0.09	1.50±0.08	1.56±0.11
3	1.62±0.08	1.59±0.05	1.54±0.05	1.46±0.10	1.57±0.08
4	1.86±0.12	1.55±0.10	1.58±0.06	1.52±0.06	1.51±0.05

TABLE 2. RNA contents (µg/mg of dry weight) in ephaxial muscle from juveniles *Petenia kraussii* through four weeks of Cu-exposure and Cu-depuration at 22 and 30°C. N=8 organisms.

Cu-exposed and Cu-depurated fish at 22ºC					
Weeks	Control	Cu-exposure		Cu-depuration	
		mg/l Cu			
		0.2	1.20	0.2	1.20
1	5.30±0.61	3.65±0.10	1.91±0.12	2.83±0.41	1.20±0.08
2	5.47±0.22	3.39±0.16	2.62±0.16	3.64±0.14	2.95±0.19
3	5.35±0.23	3.28±0.14	2.61±0.05	4.38±0.13	1.53±1.58
4	6.65±0.46	3.04±0.13	1.94±0.11	5.36±0.27	3.61±0.30
Cu-exposed and Cu-depurated fish at 30ºC					
Weeks	Control	Cu-exposure		Cu-depuration	
		mg/l Cu			
		0.2	1.20	0.2	1.20
1	3.12±0.37	2.68±0.20	1.88±0.08	2.28±0.13	1.65±0.17
2	3.06±0.20	1.85±0.19	1.95±0.11	2.94±0.15	1.81±0.09
3	2.39±0.11	2.70±0.38	2.13±0.13	2.80±0.12	1.57±0.09
4	2.70±0.19	2.39±0.24	2.48±0.11	3.66±0.18	2.73±012

± 0.10 during the first week, reaching 3.04 ± 0.3 during the fourth week of exposure. In organisms exposed to 1.2 mg/L, during the first week, this RNA average value decreased to 1.91 ± 0.12, followed by an increase during the next two weeks and a subsequent decrease for the final week with an average value similar to that of the first. In organisms exposed to copper at 30°C, the decrease in RNA levels was not as evident as the one shown by specimens exposed to the metal at 22 °C (Table 2).

Regarding protein contents, a decrease caused by the metal in both doses was observed. Protein levels in the control group at 22°C varied between 440.76 ± 32.55 and 538.68 ± 46.73, while for those exposed to 0.2 mg/L they varied between 287.44 ± 9.37 and 322.63 ± 5.92 and decreased even more in those exposed to 1.20 mg/L of copper (Table 4). In organisms kept at 30°C, protein levels also diminished as compared to the control group, although the decrease was smaller than that of organisms kept at 22 °C (Table 3).

The RNA/DNA ratio in juveniles kept at 22 °C decreased in organisms exposed to copper, those subject to 1.2 mg/L being the more affected. No effect by either concentration was observed on this parameter in organisms kept at 30ºC (Fig. 1). The protein/DNA ratio showed a response similar to the one indicated for the RNA/DNA ratio (Fig. 2).

These results demonstrate that copper decreases the RNA/DNA and protein/DNA ratios in organisms kept at 22°C from the first week of exposure, those ratios remaining low during the whole period of exposure, while organisms kept at 30°C did not show a decrease of these parameters, their values staying similar to those of the control group.

During the detoxification period it was evident that all parameters but DNA levels were significantly affected, DNA showing variation with respect to temperature and concentration though not to exposure time. During this period, it was manifest that organisms kept at 22°C tended to increase the values of RNA, DNA, proteins, protein/DNA and RNA/DNA after 30 days of detoxification.

The protein/DNA ratio in organisms detoxified at 22 °C and exposed to both doses of the metal reached values of 297.52 ± 41.11 and 219.43 ± 11.56 for organisms exposed to 0.2 and 1.2 mg/L of Cu, respectively. These figures did not reach the average values of the control group. Organisms detoxified at 30°C followed the same trend observed during the accumulation period. However it is important to point out that in those organisms exposed to the 0.2 mg/L dose, the protein/DNA ratio was above that of the control groups and even the RNA/DNA ratio reached an average value higher than that of control groups during the fourth week of detoxification. The RNA/DNA ratio showed a response pattern similar to that of the protein/DNA ratio.

TABLE 3. Protein contents (µg/mg of dry weight) in ephaxial muscle from juveniles *Petenia kraussii* through for weeks of Cu-exposure and Cu-depuration at 22 and 30°C. N=8 organisms.

Cu-exposed and Cu-depurated fish at 30ºC					
Weeks	Control	Cu-exposure		Cu-depuration	
		mg/l Cu			
		0.2	1.20	0.2	1.20
1	440.76±32.5	322.63±5.92	227.28±14.4	267.14±9.51	178.27±14.8
2	459.56±7.53	311.64±12.6	254.36±7.88	303.63±26.7	232.81±15.2
3	444.59±26.3	302.72±7.95	259.29±5.06	371.32±12.6	268.98±9.58
4	538.68±46.3	287.44±9.37	227.57±11.3	436.14±41.0	325.84±15.2
Cu-exposed and Cu-depurated fish at 30ºC					
Weeks	Control	Cu-exposure		Cu-depuration	
		mg/l Cu			
		0.2	1.20	0.2	1.20
1	305.56±44.7	267.04±11.23	220.54±7.17	244.07±14.01	194.57±24.19
2	291.34±29.1	212.61±11.31	223.38±5.52	259.50±49.31	212.48±8.64
3	243.26±14.3	261.82±34.91	233.46±10.69	264.38±14.43	241.67±8.01
4	269.54±24.9	264.01±17.97	253.21±4.14	359.57±7.54	266.01±10.03

DISCUSSION

Temperature is perhaps one of the most important environmental factors affecting the growth rate of larvae and fish juveniles (CALDERONE *et al.* 2003; PECK *et al*. 2003). In previous research carried out with *Petenia kraussii* acclimated to different temperatures, it was demonstrated that an increase in temperature from 22°C to 30°C caused an increase in DNA levels, together with a marked decrease of RNA and protein levels, bringing about a reduction of RNA/DNA and protein/DNA ratios (LEMUS *et al.* 1993). The reduction of the last two parameters indicated that a temperature increase of 8°C changed the organism's physiological state, thus having implications in growth, manifested as a decrease of current growth when the thermal environment was modified.

The results of this research demonstrate that DNA levels in juveniles of *P. kraussii* exposed to copper at 22°C were not affected, while organisms exposed to the metal at 30°C showed a decrease compared to those not exposed to it (Table 1). This indicates that this parameter remains stable under copper exposure at 22°C, while with copper at 30ºC a decrease could be associated to a modification in cell proliferation. The inhibition of DNA synthesis could result from an adjustment in replicating genetic material to help forward or promote RNA synthesis and thus satisfy the need of some proteins to keep viability under the new experimental condition.

A temperature of 22°C caused greater copper toxicity than 30°C. Organisms not exposed to the metal at 22°C showed RNA concentrations varying between 5.35 ± 0.23 and 6.65 ± 0.46 during the four weeks of exposure, and falling to 3.04 ± 0.13 and 1.94 ± 0.11 on the fourth week of exposure to 0.2 and 1.2 mg/L of copper, respectively. This reduction came along with a reduction in protein levels and in protein/DNA and RNA/DNA ratios, suggesting a decrease in protein synthesis and in the growth rate of white muscle.

Although a reduction in the protein/DNA and RNA/DNA ratios of organisms exposed to the metal at 30°C was not observed during the last three weeks, it has been evidenced that *P kraussii* under these conditions shows a higher metal incorporation rate at 30°C than at 22°C (Lemus & Chung, 1999), indicating the important influence of temperatures on copper toxicity and on the growth of juveniles. The 30°C temperature may induce a greater efficiency in the neutralization and metabolization processes of copper, without risking the energy requirements needed for the species to grow, since it has been proved that an increase in temperature increases membrane fluidity and the activity of a variety of enzymes (Wright & Welbourm, 2002), which could be involved in copper metabolization.

There is little information regarding the effect of temperature on metal toxicity as a whole, and especially on the growth rate of juvenile fish related to the RNA/DNA ratio. Wright & Welbourm (2002) pointed out that the interpretation of data regarding temperature/toxic interactions obtained under laboratory conditions is very complex, since there may be sound differences concerning toxic tolerance and temperature at different levels of cell organization; that is why an interpretation of temperature

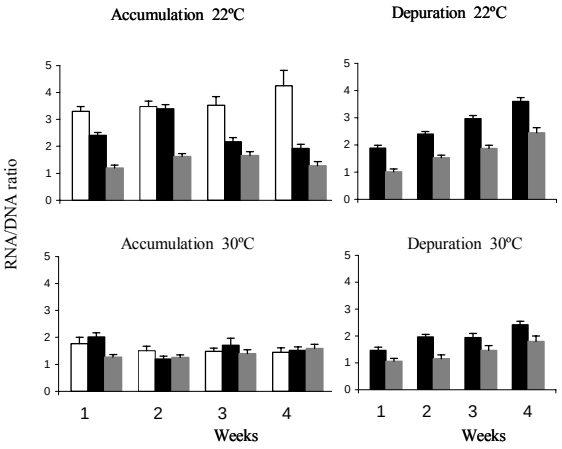

Fig. 1. RNA/DNA ratio in ephaxial muscle from juveniles *Petenia kraussii* exposed at 22 and 30°C and copper concentrations of 0.20 and 1.20 mg/l for four weeks of accumulation and detoxification. Bars indicate the average value of 8 determinations and the line, the standard deviation. White bars= control; black bars= 0.2 mg/l of Cu; and grey bars 1.2mg/l of Cu.

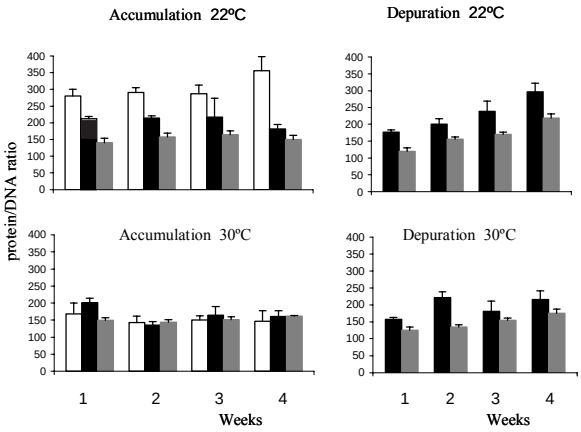

Fig. 2. Protein/DNA ratio in ephaxial muscle from juveniles *Petenia kraussii* exposed at 22 and 30°C and copper concentrations of 0.20 and 1.20 mg/l for four weeks of accumulation and detoxification. Bars indicate the average value of 8 determinations and the line, the standard deviation. White bars= control; black bars= 0.2 mg/l of Cu, and grey bars 1.2 mg/l of Cu.

and toxic interaction is still unwise. Hodson & Sprague (1975) demonstrated low levels of zinc toxicity in *Salmo salar* at 3°C as compared to that at 19°C when determining LC_{50}. Paradoxically, when the bioassay was extended for two weeks, the LC_{50} at 19°C remained unaltered, but at 3°C it decreased about 6-fold, the effect of temperature being reverted.

It is also important to point out that in bioassays on the copper-temperature interaction in the invertebrate *Carcinus maenas*, it was demonstrated that temperatures 10°C below and above the average value of its normal habitat caused a decrease and an increase of the cardiac frequency at the temperatures of 5 and 25°C, respectively. The researchers pointed out that a decrease of the cardiac frequency should be construed as a physiological response to copper use, and an increase as one to higher toxicity (CAMUS *et al*. 2004). On the other hand, this mechanism may be associated with the effect of temperature on membrane fluidity, as stated previously.

The particular effect of temperature and metals has been evaluated in natural and controlled conditions. In specimens of *Daphnia magna* during chronic exposures to cadmium concentrations of 0.4, 0.8, 2.1, 4.3, and 7.2 ¼g/L for 21 days, a growth reduction ensued after 7 days of exposure (KNOWLES & MCKEE, 1987). Also, in juveniles of *Sebastes schegleli* kept on a diet of copper ranging between 0 and 500 mg/kg of weight for 60 days, it was demonstrated that the RNA/DNA ratio in both the muscle and the liver was affected by the metal (KIM & KANG, 2004).

The results shown in this research evidence a significant decrease of protein levels in muscle tissue, the reduction being more marked in organisms exposed to copper at 22°C. A 50% protein reduction was observed in the sample exposed to 1.2 ppm of copper compared with their controls (Table 3). As DNA levels were not affected under such condition, the protein/DNA rate clearly reflects that there was a reduction of protein synthesis (Fig. 2) caused by a drop in RNA levels. On the other hand, in organisms exposed to the metal at 30°C, protein synthesis was not at risk.

SMITH *et al* (2001) evaluated protein synthesis and its required energy consumption in different tissues of *Orechromis mossambicus* exposed to copper for three weeks, and found that there was a reduction of protein synthesis in the skin together with a high requirement of energy for protein synthesis. They also found that there was no reduction in protein synthesis in gill and hepatic tissue, the energy use remaining unaltered. Cells or tissues having a high rate of protein synthesis are able to avoid an increase in energy consumption for protein synthesis when they are exposed to copper. In tissues in which the protein synthesis is smaller, any disturbance caused by copper probably induces an increase for potential damage and a higher requirement of energy for protein synthesis.

The organisms exposed to the metal at 22°C tended to recover their growth rate after 30 days of detoxification; however, they did not reach values similar to those of controls.

These results demonstrate that temperature is a key factor in copper toxicity and its effect on growth. Juveniles of *Petenia kraussii* reduced their growth rate depending on the concentration in organisms kept at 22°C, while in specimens exposed to 0.2 mg/L at 30°, a growth stimulation was produced during the first week. When exposed to 1.2 mg/L there was no effect. All this raises the question about the complexity of the effect of copper/temperature interaction on *Petenia kraussii* as a pattern dose-response is observed in bioassays carried out at 22°C, while a response that does not fit this pattern is present in those exposed to 30°C. This in turn raises the possibility that there are other cellular mechanisms involved in the toxicity process that must be evaluated.

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