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Toxicokinetics of heavy metals from a mining spill using *Carcinus maenas*

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Abstract

The knowledge of the reproduction and growth background related to the shore crab *Carcinus maenas* promotes the use of this crab as a model crustacean to asses the potential for endocrine disruption in crustaceans. In addition, an enzyme linked inmunosorbent assay (ELISA), sensitive to the shore crab vitellogenin in serial hemolymph samples allows determination of the extent of disruption of the process of vitellogenesis in female crabs and its likely impact on reproductive output. Intermoult females *Carcinus maenas* were exposed to concentrations of Cd: 3 $\mu g l^{-1}$, Cu: 15 $\mu g l^{-1}$ and Zn: 700 $\mu g l^{-1}$ determined at the Guadalquivir estuary after the Aznalcóllar mining spill, during 21 days. Crab hemolymph samples, were taken every seven days, and analyzed through an ELISA for *Carcinus maenas* vitellogenin. Vitellogenin concentration along the time was fitted to a first order kinetic approach. Results showed a good correlation among experimental values and estimated ones. Metal exposure resulted in an increase in vitellogenin concentration in hemolymph, especially for cadmium.

Keywords: Heavy metals; Biomarker; Kinetic model; Aznalcóllar mining spill; Toxicity; Vitellogenin; *Carcinus maenas*

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The Aznalcóllar mining spill poured into the nearby Guadiamar river that flows the Guadalquivir estuary. The mining accident produced almost 6 Hm^3 of mud and acidic waters, with high concentrations of metals in solution including Cd, Cu, Mn, Pb and Zn, and metalloids as As. As a consequence, sediment and water quality from the river and the estuary was negatively affected (Riba, DelValls, Forja, & Gómez-Parra, 2002).

The hormonally-regulated processes can serve as indicators of the health of the environment, some more readily than others (Fingerman, Jackson, & Nagabhushanam, 1998). Inhibition or stimulation of vitellogenin (VTG) levels in hemolymph could provide an useful indicator of direct repercussions on the reproductive capacity in the female crabs.

The present study was carried out to investigate the effect of the mining spill in the Guadalquivir estuary, using a bioindicator species, *Carcinus maenas* and vitellogenin levels variation along time in hemolymph. Concentrations of cadmium, copper and zinc analysed in the Guadalquivir estuary after the accident were simulated in the laboratory to assess the impact in the biological community.

Heavy metals selected for the present study were Cd (3 $\mu g\,l^{-1}$), Cu (15 $\mu g\,l^{-1}$) and Zn (700 $\mu g\,l^{-1}$) using concentrations previously determined from April to September, 1998 in the estuary (Gómez-Parra, Forja, DelValls, Sáenz, & Riba, 2000). Eight intermoult crabs were housed, after two weeks of acclimatization, in glass 20 l aquariums, aerated and exposed to different treatments of heavy metals (Cd, Cu and Zn) and control for 21 days in an static renovation. Every three days, crabs were fed with frozen mussels. After feeding the animals water was siphoned, waste food, faeces, and any other debris were carefully cleaned. Then a volume of the stock solutions previously prepared ($CdCl_2$, $ZnCl_2$, $CuCl_2$, SIGMA) in distilled water was added to each 20 l aquarium in order to maintain the concentration of heavy metals required. The parameters monitored routinely within the test tanks throughout the bioassays were dissolved oxygen (6.8 ± 0.4 $mg\,l^{-1}$), pH (7.5 ± 0.2), temperature (15.1 ± 0.6 °C) and salinity (34.7 ± 0.2). The photoperiod was maintained at 12 h light:12 h dark. Hemolymph samples were collected the days 0, 7, 14 and 21 to determine the concentration of VTG along the time. Samples were taken from the base of the walking leg (100 μl), and introduced in liquid nitrogen, prior to store at −80 °C.

Vitellogenin was measured in the hemolymph of intermoult female crabs, *C. maenas* using a direct enzyme-linked inmonosorbent assay (ELISA). The 96-well microtiter plates were coated with the standard solutions, purified VTG (0, 2, 10, 20, 50, 75 and 100 ng 100 μl) and hemolymph samples from each crab (200 μl). A polyclonal antibody raised in rabbits against *C. maenas* VTG identified vitellogenin concentration. Photometric data were read through microtiter plates reader at (405 nm). VTG standards were fit to a linear regression (correlation coefficient $(r) = 0.98$; slope $(b) = 0.38$. The sensitivity of the assay (the lowest concentration of VTG giving an optic density significantly greater than background) was 60–64 $ng\,ml^{-1}$. No non-specificity of the antibody to hemolymph was found. A toxicokinetic approach was fitted with VTG results. The differential equation that describes the variation of VTG is shown in the next expression:

$$\frac{d[VTG]}{dt} = K[VTG],$$

where [VTG] is the vitellogenin concentration in hemolymph, and K is the velocity constant. The resolution of this differential equation using the initial and final conditions ($t = 0$, $t = t[VTG] = C_0$, [VTG] = [VTG]) produces the next 1st kinetic equation:

$$\mathrm{Ln}[VTG] - \mathrm{Ln}\, C_0 = Kt \quad \text{or} \quad \mathrm{Ln}[VTG\, t] = \mathrm{Ln}\, C_0 + Kt, \tag{a}$$

where C_0 is the initial VTG and [VTG] is that experimentally measured.

The error associated with the model prediction is calculated using the expressions:

$$\%\ \text{error} = \frac{C_0(\exp) - C_0^*}{C_0(\exp)} * 100,$$

where C_0(exp) is the initial concentration of VTG and experimentally determined by means of ELISA and C_0^* the theoretical concentration given by the 1st kinetic equation.

Summarized results VTG concentration in the hemolymph exposed to Cu (15 $\mu g\, l^{-1}$), Cd (3 $\mu g\, l^{-1}$) and Zn (700 $\mu g\, l^{-1}$) along time is shown in Fig. 1. It is observed an increase of the protein concentration along the time, for zinc, copper and cadmium. The behaviour of the concentration of VTG in the crabs for each heavy metal treatment was fitted to a 1st order equation. The fitted parameters, the expression of the kinetic model and the error associated the approximated values of protein for each day are shown in Table 1. The fitted results show a good correlation between the experimental data and the predicted by expression (a). It confirms a good approximation of VTG induction to a 1st order kinetic model. The best estimation is obtained for the Cu exposure, it showed the lowest correlation error 1.8, and the best correlation coefficient ($r^2 = 0.980$). The estimation made for Cd and Zn treatments also showed a good correlation coefficient ($r^2 = 0.956$; $r^2 = 0.967$) and low error percentages, 8.2 and 8.7, respectively.

Zinc is the heavy metal showing the highest K value, followed Cd and finally Cu. In other studies developed with female squirrelfish (Thompson, Mayer, Walsh, & Hogstrand, 2002) and in *Xenupus laevis* (Falchuk, Montorzi, & Vallee, 1995), it was observed that changes in zinc concentrations in bloodstream followed the course vitellogenin transport from the liver. Although no studies have been done related to crustaceans, this affirmation could explain zinc behaviour. Discrepancies between experimental and predicted VTG concentration at day 21 have been shown in all the treatment and especially in Cu and Zn treatment. Copper and zinc are essential metals and regulated by the organisms, specially by metallothioneins, serving as storage forms for these metals and playing a regulator role, specially as a metals donor for apohemocyanin (Cu) and carbonic anhydrase (Zn) (Engel, 1987). After a constant exposure of these metals, part of the metal concentrations could not be regulated, then basal levels in the individuals could be surpassed, and vitellogenin induction is out of the predicted values output by the model.

The biological significance of the VTG system on the reproduction of these organisms allows to predict potential effects in future generations. This study has

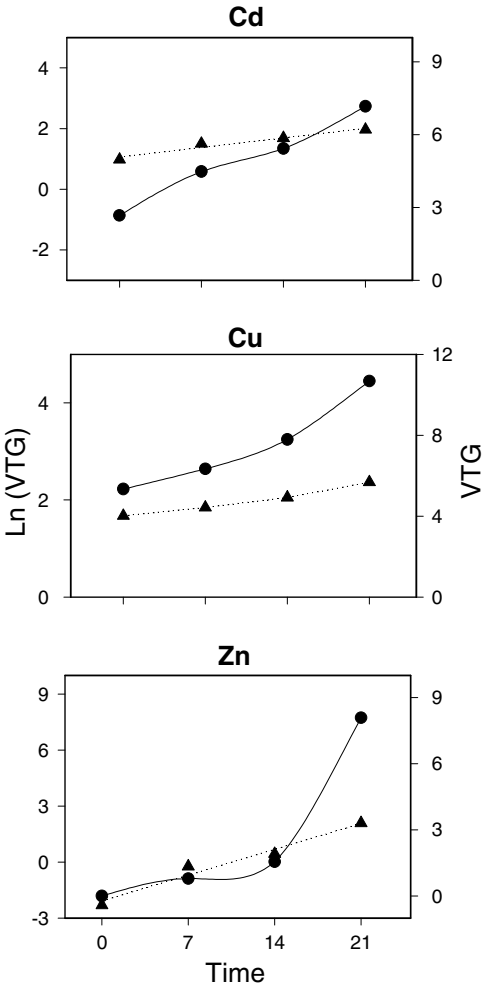

Fig. 1. Vitellogenin (ng ml^{-1} 100) concentration in hemolymph of *C. maenas* along the 21 days of exposure (0, 7, 14, 21). In the graph are shown the experimental data (●) and the predicted values by the 1st order kinetic approach (▲).

Table 1
Fitted parameters resulting from the 1st kinetic order model for each heavy metal treatment, Cd (3 μg l^{-1}), Cu (15 μg l^{-1}) and Zn (700 μg l^{-1})

Metal	r^2	K	Ln C_0^*	Equation	Ln C_0(exp)	% Error
Cd	0.956	4.510^{-2}	1.06	$v = 0.045$[VTG]	0.98	8.2
Cu	0.980	3.310^{-2}	1.64	$v = 0.033$[VTG]	1.67	1.8
Zn	0.967	2.010^{-1}	−2.1	$v = 0.200$[VTG]	−2.30	8.7

Results of correlation coefficient, r^2, velocity constant, K, % error and Ln C_0^* (Ln of VTG concentration the day 0) are shown.

demonstrated that heavy metal exposure affects the VTG production in *C. maenas*, showing differences between heavy metal treatments. The development of a first kinetic order model fits the experimental results of the induction of VTG in *C. maenas*, after exposure to environmental concentrations of Cd, Cu and Zn after an accidental spill and provides a useful tool to establish the biological effects associated with the mining spill.

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