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Influence of Salinity in Hemolymph Vitellogenin of the Shore Crab *Carcinus maenas*, to be Used as a Biomarker of Contamination

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Estuaries have strong gradients in many physical and chemical variables, including salinity, pH, dissolved oxygen, temperature, nutrients and the amount and composition of particles. Unlike freshwaters, where pH is the controlling factor, in estuaries salinity is the controlling factor for the partitioning of contaminants between sediments and overlying or interstitial waters; both are key variables in controlling the bioavailability, and hence the toxicity, of heavy metals bound to sediments (Riba et al. 2004).

In the toxicological assessment of contamination episodes in estuarine systems, physicochemical factors can induce structural and functional modifications in living organisms. Consequently these factors affect the assessment of toxicity. Once the suitable sentinel organism and biomarker are chosen for toxicity assessment, the influence of environmental conditions such as salinity should be analyzed as well as their effects on the organism and on the biomarker. When this influence is established, the responses of the biomarker to the toxicity can be assessed.

The green crab *Carcinus maenas,* (whose basic physiology is well known) is capable of withstanding wide variations in water salinity (Trutchot 1986), due to its adaptive responses to salinity changes (Péqueux 1994). *Carcinus maenas* is a native species in estuarine environments, and considerable research related to the effect of several contaminants in this species has been carried out, including work to monitor the effects of the Aznalcóllar mining spill in the Guadalquivir estuary (Martin-Díaz 2002).

The inhibition and/or stimulation of vitellogenin/vitellin (VTG) levels in the hemolymph of this species could provide an useful indicator of the direct repercussions of contaminants on the reproductive capacity of the female crabs (Fingerman et al. 1996). Nevertheless, its potential use as a contamination biomarker in the environment requires the study of the influence of variations in the physicochemical controlling factors, particularly salinity, which are characteristic of some environments. Such variations could produce misleading biomarker responses to contamination. The purpose of the present study was to determine the influence of variations in salinity concentrations on the induction

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or inhibition of VTG (vitellogenin/vitellin) in the hemolymph of female green crabs, so as to assess the potential use of VTG levels in hemolymph as a biomarker in toxicity tests.

MATERIALS AND METHODS

Female specimens of the shore crab *Carcinus maenas* (4-5 cm width of carapace) at the intermoult stage of their cycle were obtained from fishermen in Cadiz Bay (SW, Spain) and were acclimatized in the laboratory for 21 days in a 500L tank with a continuous flow of aerated, filtered sea water with a salinity of 35.

The bioassay consisted of a battery of eight aquaria, each containing eight specimens in 20L of filtered sea water. Specimens were subjected to eight different treatments (one treatment per aquarium) and each treatment was duplicated. Treatments lasted 49 days and consisted of eight different regimens of weekly salinity variation (including one of no variation) from an initial value of 35 in all treatments, in steps of 5, to a minimum of 0 (Figure 1).

The water in the aquaria was continuously aerated to maintain oxygen saturation and was replaced every three days. The replacement was performed after feeding of the crabs with frozen mussels (0.5 cm length), in order to avoid debris accumulation. Aquaria were kept in an incubator with a temperature maintained at 13±1°C and the photoperiod set at 12L:12D. During the first week all aquaria were kept at the same salinity, 35. The second week, the first aquarium was kept at a salinity of 35 and all the others were reduced to 30. The third week, aquarium 1 was kept at 35, aquarium 2 at 30, and all the other aquaria were reduced to 25. The procedure was followed in successive weeks to achieve the differing final salinity values required in each tank, ranging from 35 to 0 (Fig. 1). Salinity was changed by adding different amounts of distilled water (Milli-Ro) to filtered sea water (S= 35).

Hemolymph samples from each crab were taken on days 0, 14, 28, 42 and 49 from the base of the walking leg (100 µL) and immediately placed in liquid nitrogen, prior to storage at –80°C. Vitellogenin in the hemolymph of the intermoult female crabs was measured using a direct enzyme-linked immunosorbent assay (ELISA). Purified *C. maenas* VTG was used to prepare standard solutions (0, 2, 10, 20, 50, 75 and 100 ng· 100 μL^{-1}). The method used 96-well microtiter plates which were coated with the standard solutions and hemolymph samples from each crab (200 µL). Vitellogenin concentration was identified by a polyclonal antibody raised in rabbits against *C. maenas* VTG. Photometric data were read through a microtriter plate reader at (405nm). Vitellogenin standards were fitted to a linear regression. The standard curve was linear. No non-specificity of the antibody to hemolymph was found to demonstrate this absence; diluted male hemolymph was used as an antigen. The concentrations of vitellogenin in the hemolymph of *Carcinus maenas* obtained for each treatment were normalized by means of a transformation in order to obtain the Percentage of Inhibition/Activation (% I/A) [1], which indicates the amount of

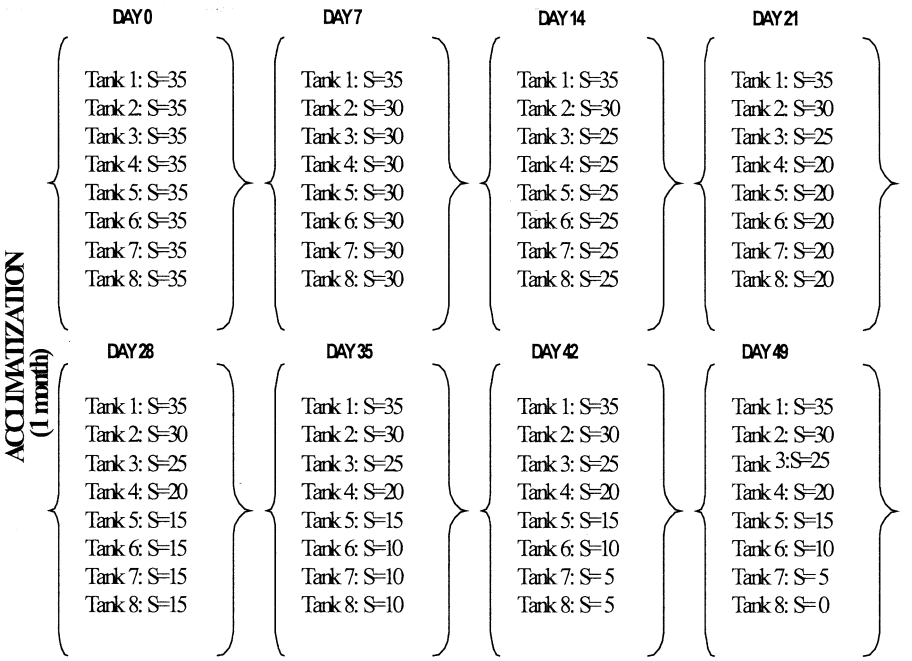

Figure 1. Schematic description of the temporal evolution of the salinity values in each aquarium (tank) during the bioassay using *Carcinus maenas*.

protein concentration that fluctuates from the first day to the end of the experiment, in the same aquarium. The expression used was:

[1]

$$\frac{\text{VTG Concentration (49 day)} - \text{VTG Concentration (0 day)}}{\text{VTG Concentration (49 day)}} \times 100 = \%\ \text{I/A}$$

Vitellogenin concentration was analysed statistically using a Student t-test for independent samples in order to determine differences in: a) VTG concentration at different days between tank 1 (constant salinity value: 35) and the other tanks (variable salinity values over time); and b)VTG fluctuation over 49 days of exposure (%I/A) in different tanks.

RESULTS AND DISCUSSION

Vitellogenin concentrations in female crabs are shown in Figure 2 for the days 0, 14, 28, 42 and 49. All the vitellogenin concentrations correspond to female *C. maenas* in the intermoult period. The sensitivity of the assay [the lowest concentration of VTG giving an Optic Density (O. D.) significantly greater than background] was 20 ng· mL^{-1}. No significant differences were observed between VTG concentrations of replicates. In general, the results from tank 1, where the salinity was kept constant during the bioassay, showed a considerable decrease in

VTG concentration in hemolymph on day 14 and then a progressive increase over time. In the other seven tanks there was a similar decrease in VTG concentration recorded on day 14, except in tank 5 in which there was an increase (not statistically significant) of VTG concentration at day 14 (Fig. 2). The pattern of VTG variation observed in tank 1 can be explained as reflecting the normal variation of the requirements for this protein during the intermoult period of these female crabs. In *Carcinus maenas*, the degree of vitellogenesis in the culture of fragments taken from the hepatopancreas has been related to the stage of ovarian maturation (Lee and Chang 1997).

Vitellogenin concentrations of crabs from tank 1 (constant salinity values) were statistically compared with that of crabs belonging to the other tanks (variable salinity values) at days 0, 14, 28, 42 and 49.

Results obtained from the statistical analysis showed that the protein concentration in crabs from tank 1 were significantly different ($p<0.05$) from that found in tank 4 on day 28 (Fig. 2).

Significant differences in VTG concentrations between crabs from tanks 1 and 2 ($p<0.01$) at the end of the experiment (day 49) were also observed (Fig. 2).

Results from the statistical analysis comparing the percentage of inhibition/activation between crabs exposed to a constant salinity value (tank 1) and crabs exposed to a variable salinity value (tank 2, 3, 4, 5, 6, 7, 8) are shown in Fig. 3. From these results it can be seen that, in general, the % I/A shows an increase (activation) for all the treatments except for tank 4 (almost 0), tank 7 ($p<0.01$), and especially tank 2 ($p<0.01$) which shows high negative values. These negative values are associated with an inhibition of VTG concentration in hemolymph in tank 7 (salinity 5 on day 49), in tank 4 (salinity 20 on day 49), and in tank 2 (salinity 30 on day 49). The inhibition found in tanks 2 and 4 compared with the activation determined in tank 1 is consistent with statistically significant lower values of VTG concentration in hemolymph that were recorded between crabs from tank 1 and tanks 2 (on day 28) and 4 (on day 49).

The present study represents a first approach to measuring the effect of the change in a broad range of salinity values and has demonstrated that VTG production, an aspect of the reproductive biology of the shore crab *Carcinus maenas,* might have to be modified at least at salinities 20 and 30 in tanks 2 and 4, where the behaviour was statistically different from the control.

Previous results reported by McKenney (1996) demonstrate that salinity values have a considerable influence on *Mysidopsis bahia*, in which a decrease in reproductive capacity was found at physicochemical conditions where salinity varied from 31 to 3. Relatively few studies have been conducted on the effects of salinity in the reproduction of crustaceans (eg. Sastry 1983), although such studies have been performed using amphipod species (eg. Steele and Steele 1991). In these studies no references were made to the influence of salinity in vitellogenin

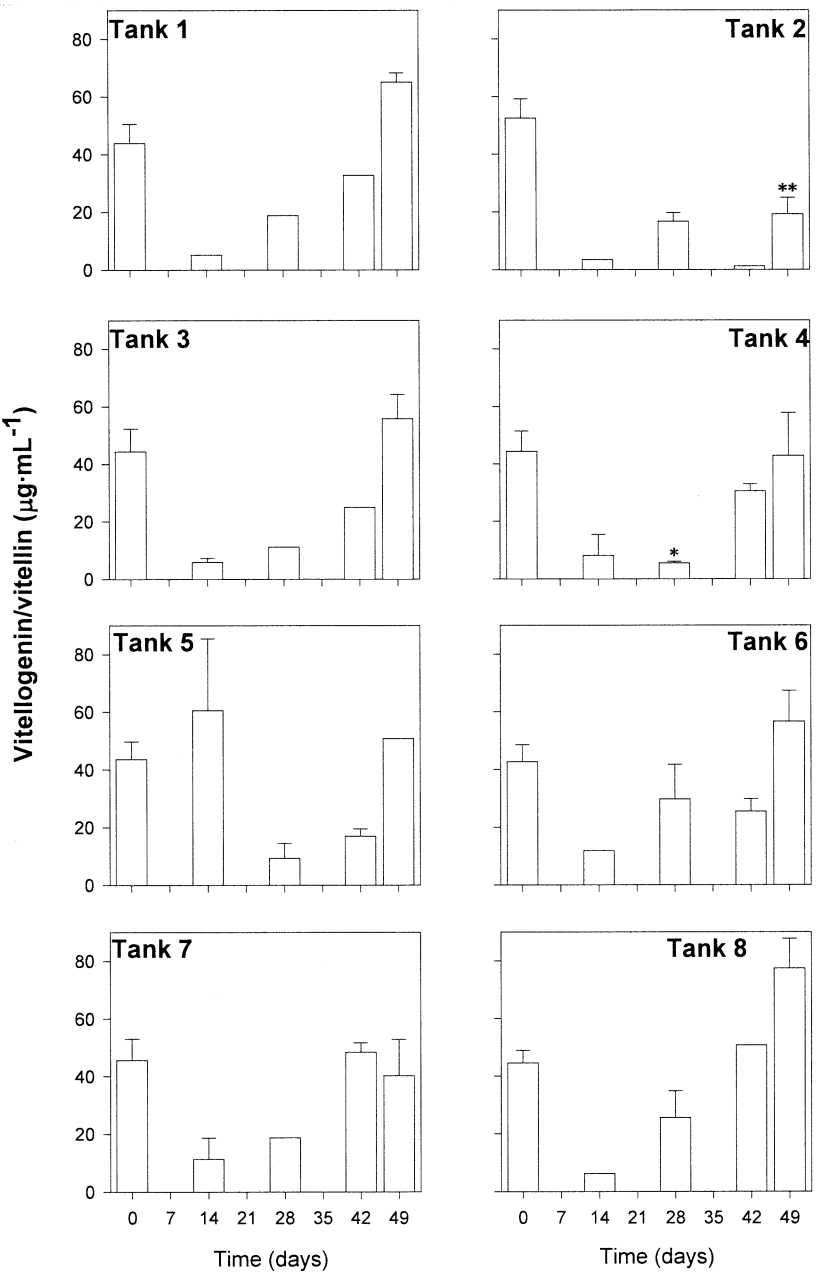

Figure 2.. Vitellogenin/vitellin concentration in *Carcinus maenas* hemolymph in the different tanks over time. Asterisks indicate significant differences resulting from the comparison of VTG (Vitellogenin/vitellin) concentrations of crabs from tank 1 (constant salinity values) with those of crabs exposed to varying reductions in salinity values, at days 0, 14, 28, 42 and 49 (**$p<0.01$), (*$p<0.05$).

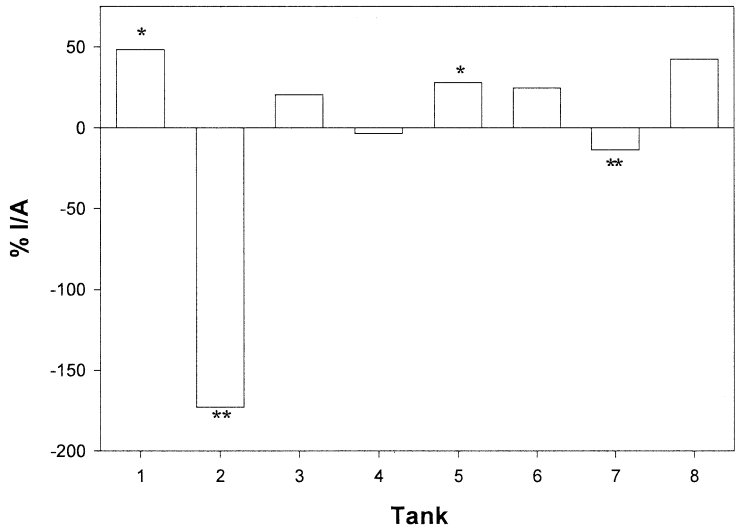

Figure 3. Percentage of inhibition (values lower than zero) or activation (values higher than zero) of VTG in hemolymph of *Carcinus maenas* in different tanks. Asterisks indicate significant differences obtained from the comparison of VTG concentration on day 0 and on day 49 in the same tank (**$p<0.01$), (*$p<0.05$).

concentration in the hemolymph of crustaceans.

Fluctuating environmental conditions, concurrent with other exogenous and various endogenous factors, influence the amount of energy consumed by individuals for gamete production (Sastry 1983). The change in respiration rates as a consequence of salinity fluctuations in crustaceans suggests that more energy is required due to osmotic stress (Kutty et al. 1971; Simmons and Knight 1975), for example the hyperosmoregulating crab *Carcinus maenas* responds to a decrease in salinity with an increase in activity, which may serve as an initial escape response (McGaw et al. 1999).

This increase in activity leads to an increase in energy consumption and a decrease in lipid reserves. The success of an organism requires the allocation of a certain amount of the assimilated energy to the gonads, in competition with the interrelated processes of maintenance and somatic growth (Calow 1979).

During the intermoult period, the hepatopancreas synthesizes and releases serum VTG, which is immunologically identical to lipovitellin, the major high density lipoprotein in the fully-developed ovary (Paulus and Laufer 1987). Reduction of lipid reserves could provoke a reduction in VTG synthesis and, as a consequence, a decrease in its concentration in crab hemolymph. However, it is considered that the reduction of VTG concentration in tanks 2 and 4 observed in the present study could also be explained by mechanisms produced in the hormonal-regulating system as a consequence of reducing salinity values. Hypo-osmotic stress elevates

methyl farnesoate (MF) levels in the hemolymph (Verzi et al. 1998), and stress in general stimulates the release of crustacean hypoglycemic hormone (CHH) (Webster 1996) and moult inhibiting hormone (MHI) (Mattson and Spaziani 1986).

Future studies of the interaction of salinity changes in VTG production and consequently in reproduction should include lipid determination in the gonads and hepatopancreas of the crab, as well as osmolarity in the hemolymph. It could also be interesting to study the effect of changes in salinity values on the hormonal system of the crustacean and its influence in VTG production. Osmotic stress could influence the energy reserves and requirements, and in the hormonal system provoke a higher sensitivity of crabs to contaminants.

These determinations, together with further toxicity tests proposed, in which crabs will be exposed to different salinity values and concentrations of contaminants, will provide a better understanding of interaction of salinity in VTG production. Thus the present study should be considered as a first approach. The thinking underlying this line of research is that the natural fluctuations in the two main variables being considered, salinity and VTG production in this species, requires a toxicokinetic approach instead of a chemical toxicology assessment of VTG production.

With the object of developing a useful toxicity test based on VTG as a biomarker in the female shore crab *Carcinus maenas*, using environmental samples from sites where salinity is a fluctuating variable and a controlling factor in contaminants bioavailability such as in estuaries, it can be concluded from the results obtained in this study that the following tests are required next: i) the use of female shore crabs at the intermoult period and at the same ovarian stage, for the comparison of the determinations observed in the different toxic treatments and ii) the use of a control test with individuals exposed to the same physicochemical conditions (especially salinity values) as those used in the toxicity treatments.

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