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Reproductive dysfunction induced by naphthalene in an estuarine crab *Scylla serrata* with reference to vitellogenesis

K. Vijayavel[b,*], M.P. Balasubramanian[a]

[a]*Department of Pharmacology and Environmental Toxicology, Dr. ALM - Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani, Chennai-600 113, Tamil Nadu, India*
[b]*Water Resources Research Center, University of Hawaii at Manoa, 2540 Dole Street, Holmes Hall 283, Honolulu, HI 96822, USA*

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Abstract

Biomarkers are useful tools for understanding complex interactions that elicit organisms response to environmental pollutants and their sublethal effects on organisms health. Effect of naphthalene on vitellogenin (VTG) and vitellin (VTN) were assessed in hepatopancreas, haemolymph and ovary of an estuarine crab *Scylla serrata* with reference to vitellogenic phases. In addition, the gonadosomatic index (GSI) was also assessed. Significant reductions in VTG and VTN contents were observed in hepatopancreas, haemolymph (VTG) and ovary (VTN). The GSI exhibited a decreasing trend in crabs exposed to naphthalene irrespective of the vitellogenic phases. We attempted to use the alterations in vitellogenic proteins and GSI as biomarkers of reproductive disturbances occurred in the crab due to naphthalene stress.

Keywords: *Scylla serrata*; Naphthalene; Vitellogenesis; Vitellogenin; Vitellin; Gonodsomatic index; Biomarker

1. Introduction

It is well known that reproduction is one of the fundamental activities of all living beings involving cellular changes, hormonal regulations, nutritional factors and enzyme systems. The success of every organism depends on how efficiently it conserves available energy and also the ability to transform conserved energy into reproductive activities. Vitellogenesis in crustaceans is an important energy demanding physiological process in reproduction (Meusy, 1980; Meusy and Payen, 1988). It is a crucial event, where the process of yolk deposition takes place during female gametogenesis (Ezhilarasi, 1982). This is characterized by the co-expression of two major proteins, viz. vitellogenin (VTG) and vitellin (VTN). In *Scylla serrata*, VTG, the precursor of the major yolk protein, is first synthesized in hepatopancreas and thereafter secreted into haemolymph. Subsequently, VTG is transported into ovary and processed into VTN by the process of proteolytic cleavage. VTN thus serves as an important source of nutrients during the process of ovarian maturation (Rani, 1998). In *S. serrata*, vitellogenesis occurs in two major phases: (i) primary vitellogenesis (Vitellogenic Phase I), which extends for several months and results in slow increase in the size of oocytes; and (ii) secondary vitellogenesis (Vitellogenic Phase II) which results in rapid increase in oocyte size leading to oviposition (Rani and Subramoniam, 1997).

Polyaromatic hydrocarbons (PAHs) are found to be toxic and impair the process of vitellogenesis in aquatic animals (Nicolas, 1999). Naphthalene is a common semivolatile PAH persistent in the environment and resistant to photooxidation. It has a lower molecular weight (128.16) and extremely toxic to exposed aquatic animals (Vijayavel et al., 2004; Vijayavel and Balasubramanian, 2006b). In crustaceans, pollutants also accumulate in the lipid rich yolk (Molven and Goksoyr, 1993), the contents of which are mobilized from the extra ovarian synthesis sites like hepatopancreas and utilized for the

*Corresponding author. Fax: +1 808 956 5044.
E-mail address: viji222@rediffmail.com (K. Vijayavel).

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oocyte development (Ezhilarasi, 1982). Reproductively active aquatic animals exposed to pollutant stress undergo alterations in their biochemical and physiological functions leading to the disturbed reproductive process. Johnson et al. (1989) opine that measurement of the biochemical constituents involved in vitellogenesis are used for assessing the impact of pollutants on the reproductive physiology of aquatic animals.

The aquatic animals when exposed to xenobiotics during their active vitellogenic stages undergo dysfunction in their reproductive process (Lee et al., 1996). Several laboratory studies have documented PAH associated disturbances in the biochemical constituents and reproductive hormones during the vitellogenesis process of aquatic animals (Truscott et al., 1983, 1992; Thomas and Budiantara, 1995; Nicolas, 1999; Elumalai and Balasubramanian, 1999; Vijayavel et al., 2004; Tintos et al., 2006). In the present study, toxic effect of naphthalene on *S. serrata* was evaluated with reference to variations in the levels of VTG, VTN and gonadosomatic index (GSI) during the active vitellogenic phases.

2. Materials and methods

2.1. Experimental setup

Female crabs (120 ± 15 g in weight) were collected from the brackish water regions of Muttukadu near Chennai, Tamil Nadu, India and transported to the laboratory. The crabs were maintained in large glass aquaria of seawater and fed with fresh prawn meat. The water temperature and pH were maintained at 28 ± 1 °C and 7.9, respectively, and the crabs were acclimatized for 15 days and divided into two groups of 10 specimens each. Group I was used as the control and group II was exposed to a sublethal concentration of 10 mg naphthalene l^{-1}. This concentration was chosen on the basis of a preliminary acute toxicity study carried out in our laboratory (Vijayavel and Balasubramanian, 2006a). The lethal (LC_{100}), median lethal (LC_{50}) and sublethal (LC_0) levels of naphthalene to *S. serrata* for 96 h exposure were 26, 18 and 10 mg l^{-1}, respectively. Therefore, the crabs were exposed to 10 mg l^{-1} of naphthalene and the exposure by static renewal method was continued for 30 days during which sufficient aeration and feed was provided.

After 30 days, the experiment was terminated and the ovary and hepatopancreas were dissected from control and test crabs and staged by vitellogenic phases according to the identification methods described by Ezhilarasi and Subramoniam (1984). The colour change in the hepatopancreas, haemolymph and ovary during vitellogenesis are important indicators in identifying vitellogenic stages in crustaceans. In *S. serrata,* Vitellogenic Phase I (early vitellogenesis) was identified by the presence of pale yellow coloured hepatopancreas, brownish orange tinged haemolymph and deep yellow coloured ovary. Vitellogenic Phase II (peak vitellogenesis) was identified with yellowish brown coloured hepatopancreas, bluish grey haemolymph and bright orange coloured ovary.

2.2. Sample preparation

The whole haemolymph including haemocytes was collected by cutting the propodus of an appendage after prechilling the animal for 5 min as this procedure prevents coagulation of haemolymph and clumping of haemocytes. Haemolymph (50 µl) was collected in a vial containing 1 ml of ice-cold Tris-HCl buffer (pH 7.8, 40% sucrose). Hepatopancreas and ovary were dissected out and rinsed with distilled water in order to remove the adhering haemolymph. Hepatopancreas (50 mg) and ovary (50 mg) were weighed and homogenized with 1 ml of homogenization buffer (100 mM NaCl, 50 mM Tris, 1 mM EDTA (pH 7.8). The haemolymph and tissue homogenates were centrifuged separately for 30 min at 20,000*g* at 4 °C in a refrigerated centrifuge to remove the cellular debris. After centrifugation, the clear supernatant was collected and used to quantify VTG, VTN, and free amino acid (FAA) content.

2.3. Polyacrylamide gel electrophoresis (PAGE) analysis

Electrophoretic extraction and estimation of VTG and VTN were carried out according to the procedures of Rani and Subramoniam (1997). The samples (hepatopancreas, haemolymph and ovary) of control and naphthalene exposed crabs (Vitellogenic Phases I and II) were subjected to PAGE analysis as described by Davis (1964) using a mini-tube gel system. The resolving gel (7%) was prepared by mixing 3.5 ml of monomer solution, 3.75 ml of Tris-HCl buffer (pH 8.8; 1.5 M), 7.75 ml of distilled water, 0.5 ml of 10% ammonium persulfate solution and 0.01 ml of *N*,*N*,*N*-Tetramethyl ethylene diamine (TEMED). The stacking gel was prepared by mixing 0.65 ml of distilled water, 1.25 ml of ammonium persulfate solution and 0.005 ml of TEMED. The lower tank of the electrophoretic apparatus was filled with the electrophoresis buffer (Tris-glycine buffer, pH 8.3). One drop of glycerol was mixed with 30 µl of each sample and placed in the sample wells. Subsequently, the sample wells were overlaid with the marker dye bromophenol blue (0.05%). The upper tank of the electrophoretic apparatus was also filled with the electrophoresis buffer (pH 8.3). A current of 24 mA was maintained until the end of electrophoretic run. As the marker dye reached the bottom of the gel, the power supply was disconnected and the gel was carefully removed from the glass tubes and subjected to Coomassie brilliant blue stain in order to detect the protein fractions (Fig. 1).

2.4. Identification and quantification of VTG and VTN

The major yolk protein VTN was identified in the ovary extract lanes by an orange coloured band in the unstained gels. Using this orange coloured band as a reference point, the corresponding band of VTG in the hepatopancreas and haemolymph was sliced out from the gel. The gel fragments containing these protein bands were separately minced into small bits and were then homogenized in Tris-HCl buffer (pH 8.8; 1.5 M) and centrifuged at 5000*g* for 30 min in a refrigerated centrifuge at 4 °C. The clear supernatants were used for quantifying the VTG and VTN content according to the method of Lowry et al. (1951) as described by Rani and Subramoniam (1997). The relative proportion of TCA (trichloro acetic acid) precipitable fraction of the VTG and VTN from eluted PAGE bands and total TCA precipitated protein of the respective samples were expressed in terms of µg/mg protein. To confirm the recovery of the yolk proteins, known quantities of protein standards like myosin, phosphorylase, ovalbumin and bovine serum albumin were also subjected to PAGE analysis and the mean recovery of the four standards were 91.5% (Fig. 2).

2.5. Assessment of FAA and GSI

The FAA content was analysed according to the method of Helland et al. (2000) using a fluorometer (Hitachi–F250). The GSI was obtained by finding the ratio between the total weight of the ovarian tissue and body weight of the control and naphthalene exposed crabs as described by Rani (1998).

2.6. Statistical analysis

The statistical significance of differences between the two groups was determined using Student's '*t*' test according to the method of Bailey (1959). The data was presented as Mean ± SD of 10 animals per group and the significance was tested.