*Interdisciplinary Studies on Environmental Chemistry — Biological Responses to Contaminants*, Eds., N. Hamamura, S. Suzuki, S. Mendo, C. M. Barroso, H. Iwata and S. Tanabe, pp. 121–126.


# Endocrine Disruption, Reproductive Cycle and Pollutants in Blue Mussel *Mytilus edulis*

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(Received 2 February 2010; accepted 17 February 2010)

**Abstract**—Adult *Mytilus edulis* were exposed to environmentally relevant concentrations of 17$\beta$-estradiol in seawater for 10 days. Exposure was performed to assess effects on *vitellogenin* mRNA expression during early gametogenesis stages. The results show an increase in *VTG* expression, in contrast with previous experiments where no statistically significant change in the expression of *VTG* in ripe mussels was recorded.

Keywords: endocrine disruption, mussel, *vitellogenin*

## INTRODUCTION

Endocrine disruptors are a very diverse group of compounds, including organic chemicals used heavily in the past (in industry and agriculture) but also chemicals currently used in the pharmaceutical industry. Because of their lipophilic and persistent nature they can bioaccumulate and biomagnify in different environmental compartments, including marine invertebrates (Langston *et al*., 2005). The occurrence of vertebrate-like steroids in the marine bivalve *Mytilus edulis* was documented by early studies that also indicate the presence of key enzymes in their biosynthesis (De Longcamp *et al*., 1974). The identity of the sterols was determined by GC-MS studies that indicate the presence of testosterone, estradiol, estrone and other steroids in the tissues of *M. edulis* (Reis-Henriques *et al*., 1990).

The levels of estradiol in bivalves have been shown to vary along the year; the profile is synchronised with variations of oocyte diameter and gonad index. Subsequently, estradiol is considered to exhibit a seasonal change associated with the reproductive cycle and to be involved in the regulation of several reproductive processes via the estrogen receptor in bivalves such as vitellogenesis (Matsumoto *et al*., 1997; Osada *et al*., 2004). Nonetheless, the role and metabolism

Table 1. Overview of exposure experiments.

| Time of exposure | Exposure regime (abbreviation on graph axis) | Nominal concentration of contaminant (ng/l) | Average concentration measured in water (ng/l) | Number of mussels analysed | Developmental stage of mussels |
|---|---|---|---|---|---|
| February 2008 | Control (C) | — | — | 20 | Early gametogenesis |
| | E2 (E5) | 5 | 3.6 ± 1.2 | 20 | Early gametogenesis |
| | E2 (E50) | 50 | 30.7 ± 8.1 | 20 | Early gametogenesis |
| | E2 (E200) | 200 | — | 20 | Early gametogenesis |
| | EE2 (EE5) | 5 | 4.2 ± 1.9 | 20 | Early gametogenesis |
| | EE2 (EE50) | 50 | 33.8 ± 8.7 | 20 | Early gametogenesis |
| | EB (EB) | 200 | — | 20 | Early gametogenesis |

Table 2. Real time PCR primer sequences.

| Primer name | GenBank accession number | Primer forward (5′-3′) | Primer reverse (3′-5′) |
|---|---|---|---|
| VTQ | AY679116 | GGA CCT CCA CCA GTG CTA ATC C | ATC TCA GCG GTT CCG ACT GC |
| 18S | L78854 | GTG CTC TTG ACT GAG TGT CTC G | CGA GGT CCT ATT CCA TTA TTC C |

of estradiol in bivalves is far from being elucidated. Recent findings suggest that *VTG* protein is heterosynthetically produced in the follicle cells in contact with the vitellogenic oocytes, and directly provided to the oocyte, not transported through blood flow. Indeed, *vitellogenin* protein content in the hemolymph was quite low and showed no variation during scallop oogenesis (Osada *et al*., 2003). It may simply be for this reason that the existence of *vitellogenin*-like protein in the hemolymph of bivalves has not been detected to date.

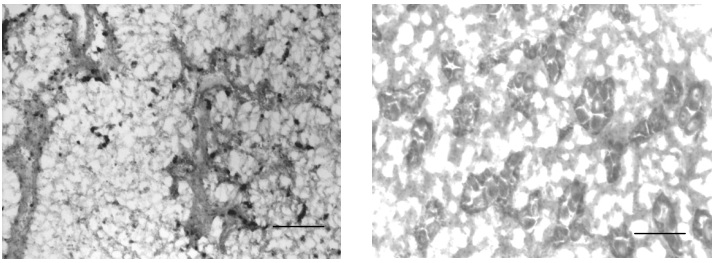
## MATERIALS AND METHODS

### *Exposure experiment*

Mussels were collected from concrete groins on Brighton beach (U.K.) (50°49′ longitude and 0°8′ latitude) at low tide, in February 2008, brought to the laboratory, and placed in artificial seawater (Sera Premium, Heisenberg, France). Following acclimatization (4 d), mussels (4.43 ± 0.34 cm) were exposed to estrogen compounds or were part of a control group (summarised in Table 1). E2 and EE2 (Sigma-Aldrich Company Ltd., Gillingham, U.K.) dissolved in methanol were added to two glass flasks and the methanol evaporated under a stream of nitrogen. The estrogen compounds were resuspended in 1 l deionised water using a magnetic stirrer and added to the aquaria seawater to give a nominal concentration of 5 ng/l, 50 ng/l, 200 ng/l of E2, and 5 ng/l, 50 ng/l EE2, respectively. The required amount of EB (as 17$\beta$-Estradiol 3-benzoate >98%, Sigma-Aldrich Company Ltd., Gillingham, U.K.) dissolved directly in artificial seawater, was added to one aquarium, giving a nominal concentration of 200 ng/l. The control group of mussels was maintained in clean artificial seawater. The water was changed every day (allowing 1 l of water per mussel) and re-spiked with the test compounds. 250 ml water samples were taken from each tank prior to and following the addition of fresh spiked water to measure the concentration of estrogens during the exposure period. The exposure duration was 10 days for all experiments, then terminated and the mussels dissected immediately. The sex and stage of gonadal development was characterized by examining a gonadal smear under a light microscope and by employing histological techniques.

### *Quantification of VTG expression*

Real-time PCR reactions were performed in duplicate, in a final volume of 25 $\mu$l containing 12.5 $\mu$l of qPCR Fast Start SYBR Green Master Rox (Roche Applied Science, U.K.), 5 $\mu$l of diluted cDNA (1/60) and 3.75 $\mu$M primers (Table 2). A control lacking cDNA template was included in qPCR analysis to determine the specificity of target cDNA amplification. Amplification was detected with a Mx3005P real time PCR system (Stratagene, U.K.) and with the following cycling parameters: 50°C for 2 min, 95°C 10 min, 40 cycles of 30 s at 95°C, 1 min at 60°C and 30 s at 72°C. Melting curves were determined following the instrument instructions to identify the presence of primer dimers and analyse the specificity of the reaction. The amplification efficiency of each primer pair was



A B

Fig. 1. Sections of gonads at different stages of mussel gametogenic cycle: A, resting stage; B, female in early gametogenesis stage. Scale bars: 100 $\mu$m.

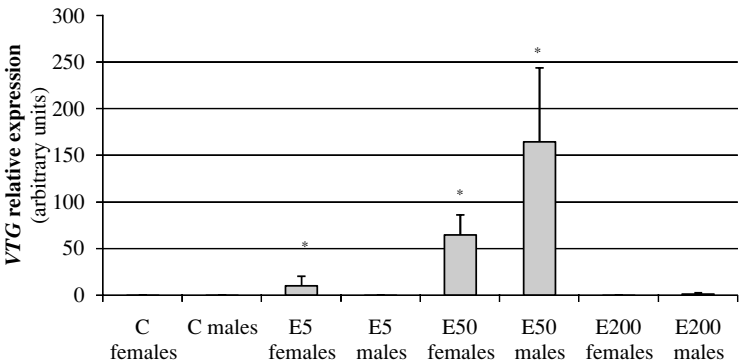



Fig. 2. *Vitellogenin* relative expression in *M. edulis* gonads. C = control mussels; E5 = mussels exposed to 5 ng/l 17$\beta$ estradiol; E50 = mussels exposed to 50 ng/l 17$\beta$ estradiol; E200 = mussels exposed to 200 ng/l 17$\beta$ estradiol. Pair wise comparisons (between females treated and control females; males treated and control males) were done using the Tukey-Kramer method and the accepted level of significance was $p < 0.05$. Treatments marked with * are significantly different from their corresponding control.

calculated using a dilution series of cDNA. Mussel *18S rRNA* was used as the internal reference. All statistical analyses were carried out using Sigmastat (version 2.0; SPSS Inc. Chicago, IL, U.S.A.). Statistical significance was accepted at $p < 0.05$ for all comparisons.

## RESULTS AND DISCUSSION

The endocrine disrupting effect of two concentrations of 17$\beta$ estradiol was investigated in mussel, and the timing of the exposure (February 2008) varied to coincide with the onset of early gametogenesis in these organisms. Mussels in the English Channel begin the gametogenesis process in mid December–January

(Fig. 1). This stage is predominant until the end of April when spermatozoids develop and become mobile, and developed ovocytes mark the onset of the first spawning (Puinean, 2006). It was hypothesised that this stage of gonad development represented the period of the life cycle that was potentially the most susceptible to endocrine disruption.

Exposure of *M. edulis*, during their early gametogenesis stage of development, to E2 (at certain doses) significantly increased the *VTG* gene expression levels in gonad tissues (Fig. 2). The relative expression of *VTG* (normalised against *18S*) increased significantly in samples from the 50 ng/l exposure group, with males being more responsive than females. Females exposed to 5 ng/l E2 also showed a significant increase compared to controls. Exposure to the higher dose of 200 ng/l of E2 did not produce any significant change in *VTG* mRNA expression.

An interesting finding was the significant increase in *VTG* mRNA expression at all the exposure doses, except for the higher E2 dose (of 200 ng/l). One possible explanation may be the biomarker behavior—a very high level of contaminant can induce a concentration-dependent maximum level of response, which is followed by an apparent reduction in activity when the concentration of inducer is further increased (Petrulis and Bunce, 1999).

Also, *VTG* mRNA expression is slightly elevated in males compared with females, with the exception of the lowest E2 dose (5 ng/l). Similar results have been reported in other bivalves: manila clam, *Tapes philippinarum* exposed to nonylphenol, (Matozzo and Marin, 2005), male zebra mussels *Driessena polymorpha* exposed to sewage treatment work effluents (Quinn *et al*., 2004) and even males of *M. galloprovincialis* collected at three sites in the Venice historic centre showed significantly high *vitellogenin*-like protein levels in haemolymph (Pampanin *et al*., 2005).

Mussels are considered resilient animals since prolonged exposure to various contaminants, including EDCs has little effect on mortality. Still, the increased response in *VTG* mRNA expression in exposed males indicates a possible change in fecundity, which could have an effect at the population level.

*Acknowledgments*—This work was funded by an INTERREG IIIA European Regional Development Fund grant (project number 162/025/266). This work was also supported by Grant-in-Aid for Scientific Research (17580153) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and Joint Project of Japan-UK Research Cooperative Program from Japan Society for the Promotion of Science (to M.O.).

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