EFFECTS OF 17β-ESTRADIOL EXPOSURE IN THE MUSSEL *MYTILUS GALLOPROVINCIALIS*

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Abstract

Mussels *Mytilus galloprovincialis* were exposed to different concentrations of estradiol (20, 200, and 2000 ng/l) in a semi-static regime (1-day dosing intervals) for up to 7 days in an attempt to see how mussels dealt with exogenous estrogenic compounds. Sex hormone levels were determined in whole tissue. Free-estradiol was only significantly elevated at the highest exposure dose (up to 10-fold). Most of the estradiol was in the tissues as fatty acid esters (>78%), which sharply increased in a dose-dependent manner (from 4 ng/g in controls to 258 ng/g at the high exposure group). In contrast, neither free nor esterified testosterone levels showed significant differences between control and exposure groups. The results suggest the existence of mechanisms that allow mussels to maintain their hormonal status, and the important role that fatty acid esterification may play within those mechanisms. Synthesis and conjugation rates of estradiol were further investigated by measuring the activity of P450 aromatase, and palmitoyl-CoA:estradiol acyltransferase, in digestive gland microsomal fractions. Overall, the study contributes to the better knowledge of molluscan endocrinology, and defines new mechanisms of regulation of free steroid-levels in mussels.

Keywords: estradiol, *Mytilus galloprovincialis*, P450-aromatase, esterification.

There is evidence that some pollutants in the environment interfere with the hormonal function in wildlife. The presence of endocrine disrupting substances has been well-documented in inland waters, and recent work has also reported endocrine disruption on estuaries, and the open sea (Vos et al., 2000). Similarly to vertebrate wildlife species, invertebrates may be adversely affected by EDCs (SETAC, 1999).

However, information on the effect of xenoestrogens in invertebrates is rather limited. Thus, this study was designed to characterize the response of the mussel *Mytilus galloprovincialis* to estradiol (E2) as a model estrogenic compound, and aimed to facilitate future assessments of the effects of xenoestrogens in molluscs.

Mussels, collected from a relatively unpolluted site, were placed in 50 l glass aquaria (50 randomly selected individuals per tank) fitted with constant air bubbling and maintained at 15ºC. Mussels were exposed for 7 days (daily dosing) to different concentrations of E2: 20 (L), 200 (M), and 2000 ng/l (H), or to the solvent, i.e. 0.004 % TEG (C). L & M doses were of environmental relevance. Levels of E2 right after dosing were close to nominal concentrations (20, 217, and 2988 ng/l in tanks L, M, and H, respectively). However, 24 hours after, E2 levels in water decreased to 17, 13, and 129 ng/l in tanks L, M and H. The control tank had measurable levels of E2 (8-12 ng/l), the source of E2 is uncertain, but it might be excreted by the mussels due to handling stress (Halm et al., 2002).

Sex steroids were determined in whole tissue of mussels as described in Morcillo et al. (1999) using commercial RIA kits (Diagnostic System Laboratories Inc.). No significant differences were observed among treatments, except for the H group that exhibited a significant 10-fold increase in tissue E2 titres (Fig. 1A). However, when tissue extracts were saponified (Gooding et al. 2003), in order to release esterified estradiol, we observed that total E2 levels (free + esterified) increased in a dose-dependent manner, from 4 ng/g in C to 258 ng/g in H (Fig. 1A). Testosterone levels, either free or esterified, did not significantly differ between control and exposure groups. The results suggest that homeostatic mechanisms may exist that help mussels to maintain endogenous levels of estradiol stable, and the potential role that esterification may play within those mechanisms. Esterified steroids do not bind steroid receptors, but

they have been considered as long-acting steroids, since they can be hydrolized by esterases (Hochberg 1998). To our knowledge, the physiological function of steroid-esters in molluscs is unknown, and future efforts should be addressed to reveal their role.

Synthesis and conjugation rates of estradiol were further investigated in control and exposed organisms by measuring the activity of both, P450 aromatase and acyl-CoA:estradiol acyltransferase, in microsomal fractions isolated from digestive gland. P450 aromatase was measured by the tritiated-water release method as indicated in Morcillo et al. (1999), and palmitoyl-CoA:estradiol acyltransferase as indicated in Janer et al. (2003).

P450 aromatase was significantly reduced at the low E2 dose, but the trend was reversed at the high dose, and organisms had significantly higher P-450 aromatase than controls (Fig. 1B). This is well in agreement with vertebrate studies, which indicate that E2 can both, up-regulate and down-regulate, aromatase activity (Halm et al. 2002).

Palmitoyl-CoA:estradiol acyltransferase activity was significantly reduced at the low exposure group (L: 1.4 ± 0.3; C: 2.4 ± 0.3 pmol/min/mg protein), and increased from low- to high-exposure group in a dose-dependent manner. However, despite of the high levels of esterified estradiol detected in mussels from the H group (Fig. 1A), no significant induction of palmitoyl-CoA:estradiol acyltransferase was recorded (H: 2.3 ± 0.2 pmol/min/mg protein). Thus, further work is needed to clarify if constitutive levels of the enzyme are enough to esterify the excess of estradiol, or other tissues and fatty acids are involved in E2-ester formation.

Additionally, gonads were examined to assess whether changes in gamete maturation had occurred as a consequence of the exposure. To this end, gonads were dissected, fixed in buffered formaldehyde, sections (7 µM) stained with

Haematoxylin/Eosin, and scored as briefly indicated in Figure 2. The gonads in most of the individuals (8 out of 10) in tank C were stage 0. However, in group L, there were significantly fewer individuals with stage 0 gonads, and correspondingly higher number of individuals exhibiting mature (stage 3 & 5) gonads (Fig. 2). These findings are in accordance with earlier studies that described accelerated sexual maturation in female oysters exposed to E2 (Mori et al, 1969).

Overall, these results demonstrate that estradiol, at environmentally relevant concentrations, may affect metabolism and gamete maturation in mussels. The highest sensitivity observed in the low-exposure group for the different endpoints assessed (P450 aromatase, palmitoyl-CoA:estradiol acyltransferase, gametogenesis) suggests that E2 at low concentrations behave as an endogenous steroid regulating physiological functions, while at higher concentrations behaves as a xenobiotic.

AKNOWLEDGEMENT

G. Janer, PhD student at Universitat Autònoma de Barcelona, acknowledges a predoctoral fellowship from the Spanish Government and partial support from EU Project BEEP (ENVK3-CT-2000-00025). Dr. Antonio Figueras (IIM-CSIC, Vigo) is gratefully acknowledged for his comments and help on gonad development classification.

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Figure 1. (A) Whole tissue levels of free and total estradiol, and (B) P450-aromatase activity in microsomal fractions isolated from digestive gland, of control and estradiol exposed specimens. Data expressed as mean ± SEM (n = 5-6). Significant differences with respect to controls indicated by $*p < 0.05$ (one way ANOVA, Dunnet's test).

Figure 2. Distribution of gonad developmental stages in the experimental groups. Gonads were classified in 6 groups: 0: not developed; 1: some follicles, many reserve cells; 2: follicles and spermatogonia/oogonia; 3: espermatogonia/oogonia and gametes; 4: gametes and few reserve cells; 5: mature, almost no reserve cells.