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Estrogen receptor genes in gastropods: phylogenetic divergence and gene expression responses to a synthetic estrogen

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ABSTRACT

Endocrine disrupting chemicals (EDCs) have the potential to affect development and reproduction in gastropods. However, one is today lacking basic understanding of the Molluscan endocrine system and one can therefore not fully explain these EDC-induced affects. Furthermore, only a few genes that potentially may be connected to the endocrine system have been sequenced in gastropods. An example is the estrogen receptor gene (*er*) that have been identified in a restricted number of freshwater and marine gastropods. Here, we have identified a new partial coding sequence of an estrogen receptor gene (*er*) in the European common heterobranch *Radix balthica*. The following phylogenetic analysis divided the *ers* of heterobranchs and ceanogastropods in two branches. Furthermore, exposure to the synthetic estrogen 17α-ethinylestradiol (EE2) showed that exposure could significantly affect *er* expression level in the heterobranch *R. balthica*. This paper is the first that phylogenetically compares gastropods' *er*, basal *er* expression profiles, and transcriptional estrogenic responses in gastropods from two different evolutionary groups.

1. Introduction

Gastropods are essential parts of marine and freshwater ecosystems. They also represent one of the most diverse and species rich phyla in the world (Zapata et al. 2014). Available information about the Molluscan endocrine system, including gastropods, is today scarce (Kaur et al. 2015; Matthiessen 2008; Zheng et al. 2014). More information is consequently needed to evaluate if it is the presence of this system that makes gastropods susceptible to endocrine disrupting chemicals (Stange et al. 2012; Sun et al. 2014).

In mammals, estrogen binds a transcriptionally active estrogen receptor (ERα/NR3A1/ESR1). The ER are involved in basic biological functions, such as homeostasis, reproduction and immunology (Gronemeyer et al. 2004). Laboratory studies have determined that gastropods were susceptible to the synthetic estrogen 17α-ethinylestradiol (EE2) (Borysko and Ross 2014; Hallgren et al. 2012; Luna et al. 2013; Stange et al. 2011). However, information about the basic mechanisms that could explain these effects is still lacking, since binding-assays indicated that the Molluscan estrogen receptor could not bind estrogen (Kajiwara et al. 2006; Thornton et al. 2003).

Opposing studies suggest that estrogens play a role in the endocrinology, physiology and reproduction of some mollusk species (Ciocan et al. 2010; Giusti and Joaquim-Justo 2013; Lei et al. 2015; Matthiessen 2008; Nagasawa et al. 2015). There is evidence of vertebrate-like steroids being present (e.g. estradiol, testosterone, progesterone) (Giusti and Joaquim-Justo 2013; Janer and Porte 2007; Kajiwara et al. 2006; Matsumoto et al. 1997) and metabolized in mollusks (Giusti and Joaquim-Justo 2013). Furthermore, the metabolic levels of these steroids correlate with sexual maturation and reproduction in a marine snail (*Thais clavigera*) and two mussel species (*Mytilus sp.*) (Kajiwara et al. 2006; Nagasawa et al. 2015). Moreover, the *er* in octopus were responsive to estrogenic compounds (De Lisa et al. 2012). This opens up for the possibility of a transcriptionally active estrogen receptor (*er*) gene in gastropods (Hultin et al. 2014; Nagasawa et al. 2015; Stange and Oehlmann 2012; Stange et al. 2012). There was also evidence of estradiol-induced changes in the phosphorylation state of transcription factors in mollusks (Canesi et al. 2004).

Gastropods were divided in different evolutionary groups: Vetigastropoda, Neritimorpha, Ceanogastropods and Heterobranchia (Zapata et al. 2014). The *er*s have so far only been identified in a limited number of these groups. Examples of available *er* sequences in gastropods were: *Bithynia tentaculata, T. clavigera* and *Potamopyrgus antipodarum* (Hultin et al. 2014; Kajiwara et al. 2006; Nagasawa et al. 2015; Stange et al. 2012).

The objective of this study was to compare the *er* sequence and gene expression-level in two evolutionary groups of gastropods: heterobranchs and ceanogastropods. We here tested the hypothesis that these two groups of gastropods would respond differently at mRNA-level after short-term exposure to a synthetic estrogen (EE2).

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To answer the hypothesis, natural gastropods were collected from a lake in Sweden. After acclimatization, gastropods were exposed to EE2 during 72 hours. After exposure, their hole-body homogenates (without the shell) were used for molecular analysis. Gastropods were here exposed to EE2 because this pharmaceutical has been frequently detected in sewage treatment plant effluents worldwide (Sun et al. 2014). Moreover, EE2 has been added to EU's "watch list" under the Water Framework Directive (Sumpter 2014).

2. Materials and methods

2.1. Test species

The collected species *B. tentaculata* (Caenogastropoda) and *Radix balthica* (Heterobranchia) have different reproduction and feeding patterns. *B. tentaculata* has separate-sex and feeds both by grazing periphyton and through filter feeding. *R. balthica* is a hermaphroditic gastropod that only feeds through grazing periphyton (Hallgren et al. 2012; Lagadic et al. 2007). The two test species are essential parts of freshwater ecosystems in Europe (Hallgren et al. 2012).

Both of our test species were collected from natural populations in Lake Sövde (South of Sweden, 55°34′33″N··· 13°39′53″E) during one week of March in 2012. The snails were fed daily and kept for a 1–2 week period of acclimation in tap water before starting experiments. For detailed description of conditions we refer to our previous publications (Hallgren et al. 2012; Hultin et al. 2014).

2.2. EE2 exposure

During the entire exposure period, snails from each species were kept in 2 L tanks, filled up with 1.5 L test media at 18 ± 2 °C under a natural photoperiod (approx. 11 h darkness:13 h light) at the Department of Biology, Lund University. Both *B. tentaculata* and *R. balthica* have previously been reported as responsive to EE2 under highly similar conditions and setup (Hallgren et al. 2012). The half-life of EE2 has been determined to be approximately 54 h under highly similar setup and conditions (Hallgren et al. 2012). For detailed descriptions of chemicals, parameters, experimental setup, feed, and water analyses see our previous publications (Hallgren et al. 2012; Hultin et al. 2014).

Twelve individual snails per tank were exposed simultaneously to nominal concentrations of EE2. The tested concentrations were 10 ng/L and 100 ng/L (prepared by a dilution series from the stock solution). To be able to compare results from a previous publication we used 100 ng/L of EE2 as the highest concentration (Stange et al. 2012). In addition to three replicate tanks per concentration, the experiment also included three solvent tanks and three water control tanks. All exposure groups (except water control) had the same final concentration of DMSO (0.001% of total water volume per test vessel). After 72 h of exposure 18 snails from each species were randomly taken from each treatment group (6 snails per tank). The gene expression levels of individual snails were analyzed after 72 h because the previous analysis showed no significant transcriptional effects in *B. tentaculata* after 24 h of EE2 exposure (Hultin et al. 2014).

R. balthica shells were removed and individuals were directly snap-frozen in −80 °C. The *B. tentaculata* snails were dissected to determine sex before snap-frozen in −80 °C. *B. tentaculata* shells were carefully removed and their sex was determined based on the presence of a penis using a stereo microscope (Gloeer and Yildirim 2006). Except for a penis (under the shelf), no additional external anatomical differences were visible between the sexes (Gloeer and Yildirim 2006) and therefore the number of males varies between the different exposure groups (it was not possible to assign which snails were males and females pre-exposure). For the methods and results of *B. tentaculata* females see Hultin et al. (2014).

2.3. RNA extraction and cDNA amplification

Total RNA was extracted from individual snails (a hole-body homogenate) using TRIzol® (Life Technologies), following the manufacturer's instructions. Total RNA concentrations was measured using a NanoDrop 2000 spectrophotometer (NanoDrop Products, Thermo Fisher Scientific). Prior to cDNA synthesis, all RNA samples were treated with DNase I (Life Technologies) to remove traces of genomic DNA. Two mg total RNA was reversely transcribed into single-stranded complementary DNA (cDNA) using 200 U SuperScript™ II Reverse Transcriptase (Life Technologies) and 100 ng Random hexamers (both from Life Technologies). The reverse transcription reaction was carried out for 10 min at 25 °C; 45 min at 42 °C; 15 min at 50 °C and 10 min at 70 °C. The cDNA was diluted in 20 μL ddH_2O and stored at −20 °C.

2.4. Gene cloning

Degenerated RACE primers (Table 1) for identification of the *er* in *R. balthica* were manually designed using the conserved gene regions of the *er* for *Aplysia californica* (NCBI accession no. AAQ95045), *Thais clavigera* (NCBI accession no. BAC66480) and *Nucella lapillus* (NCBI accession no. ABQ96884). All sequences were imported from the National Centre for Biotechnology Information (NCBI) server.

Partial coding sequence of *R. balthicas*'s *er* gene was successfully isolated using a SMART-RACE kit from Life Technologies according to the protocol provided by the manufacturer. All isolated PCR products from RACE PCR were cloned into a TOPO TA vector using the manufacturer's protocol (Life Technologies) and sequenced using the M13 primer at the SweGene sequencing platform at the Department of Biology, Lund University, Sweden.

2.5. Homology comparison

To determine the degree of homology of the *er's* DNA binding domain (DBD) among selected mollusk and vertebrate species, the aligned sequences were compared according to the BLOSUM62 aa substitution matrix using the BioEdit program (www.mbio.ncsu.edu/BioEdit) (Table 2). The sequence identity of the ligand-binding domain (LBD) was not analyzed as the amino acid sequence obtained for *R. balthica* only contained a small part of the LBD.

2.6. Phylogenetic analysis

Gene sequences from *R. baltica* were assembled and translated into deduced amino acid sequence in the BioEdit alignment editor program version 7.0.5.3. BioEdit was also used for CLUSTAL W alignment of the sequences. Phylogenetic analysis of CLUSTAL W aligned deduced amino acid sequences were conducted by the Molecular Evolutionary Genetics Analysis (MEGA) program (Tamura et al. 2007), version 6, under maximum likelihood (ML) with a bootstrap approach (Jones–Taylor–Thornton (JTT) model). The estrogen-related receptor gene of *Drosophila melanogaster* (NCBI accession no. AF359420) was used as the out-group.

Table 1
Primers used for RACE-PCR and Real-time qPCR of the estrogen receptor (*er*) gene in *R. balthica*.

Primer	Primer sequence (5′-3′)	Annealing °C
RACE-reverse	TTTTGCCCTTCAGGCCACTTGT	67
RACE-forward 1	ACATGCCTGACCCTGAGTGACCAG	67
RACE-forward 2	AACACGTGCCAGGTTACACATGCC	67
qPCR-forward	TTGTAGGTTACGGCGGTGCT	63
qPCR-reverse	CGACCTTTTGGCTGCTTTTG	63