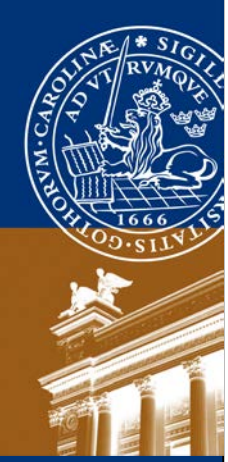

The estrogen receptor in the freshwater pulmonate gastropod Radix balthica:

Investigating changing activity levels from exposure to 17α -ethinylestradiol

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The estrogen receptor in the freshwater pulmonate gastropod Radix balthica:

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Abstract

Endocrine disruption from synthetic human estrogens in molluscs has not been investigated as closely as in vertebrates. This has led to a missing piece in the important puzzle investigating the differences in effects between diverse animal groups after exposure to endocrine disrupting chemicals, EDCs. The *Mollusca* is one of the largest phyla in the world and the different species can be found in many aquatic habitats. Investigations of their sensitivity to synthetic substances discharged by humans is therefore of potentially high interest. *17α-ethinylestradiol* (EE2) is the active compound in human contraceptives and has been found in the effluents from wastewater treatment plants in concentrations that affect fish and now recently also molluscs. This study examined the estrogen receptor (ER) gene of the freshwater gastropod (snail) mollusc *Radix balthica*, to see if the gene is transcribed differently when exposed to EE2. The proposed hypothesis is that the ER gene is activated by EE2 and increases ER gene transcription levels in exposed snails compared with unexposed snails. The results from the qRT-PCR analysis show no significant effect on the receptor gene transcription levels after 24 hours exposure.

1. Introduction

Synthetic female hormones are today widely used by women as contraceptives. These substances are constantly released into rivers and estuaries through waste water treatment plants. Although waste water treatment plants can remove a lot of the estrogenic activity (Kirk et al. 2002) studies have shown that the human contraceptive *17α-ethinylestradiol* (EE2), at levels as low as 1-10 ng/L still can have substantial effects on fish species (Jobling et al. 2003, Lange et al. 2008, Purdom et al. 1994). Recently it has been suggested that fish and molluscs may show similar reactions to exposure (Jobling et al. 2003).

Even if Mollusca is one of the most species rich phylum in the animal kingdom (Gruner 1993) studies have in the past primarily been focused on vertebrates (Castro et al. 2007). It was first in 1970 that the first case of imposex (development of male sex organs in females) was reported. After that more and more cases of imposex in molluscs have been found causing an urge to understand the underlying reasons for the cause (Matthiessen, Gibbs 1998, Oehlmann et al. 2007). The most famous example is the one about the dogwhelk (*Nucella lapillus*) that showed to be affected by tributyltin (TBT) from marine anti fouling paints (Matthiessen, Gibbs 1998). The "poorly understood endocrine pathways" (Stange et al. 2012) in molluscs have recently ended up in several studies trying to differentiate the mode of action of sex steroids (Stange et al. 2012, Benstead et al. 2011, Bannister et al. 2007, Castro et al. 2007, Kajiwara et al. 2006, Keay et al. 2006). Following the knowledge that sewage effluents are estrogenic and that intersex fish found in English rivers probably are feminized males (Tyler, Jobling 2008), has led to the focus of studies on estrogens in particular.

Sex steroids such as *17β- estradiol* (E2), testosterone and progesterone have been found in molluscs and there is evidence that they constitute an endocrine function and also possibly a role in reproduction (Keay et al. 2006, Kajiwara et al. 2006). Jobling et al. (2003) found elevated embryo production in the prosobranch mollusc *Potamopyrgus antipodarum* when exposing it to the synthetic estrogen EE2. The estrogenic effluents from waste water treatment plants (Jobling et al. 2003) which have been reported to contain *estrone* (E1), E2, EE2 and alkylphenolic chemicals as the major estrogens (Desbrow et al. 1998, Rodgers-Gray et al. 2000, 2001) have also been shown to effect molluscs (Jobling et al. 2003, Castro et al. 2007).

The DNA binding domain, DBD, in vertebrates and molluscs are very similar; according to Kajiwara et al. (2006) the marine snail *Thais clavigera* have an identity of more than 80 % with the DBD in the vertebrate estrogen receptor (ER). Further, studies on the marine snails *Aplysia californica* (Thornton et al. 2003) and *Nucella lapillus (*Castro et al. 2007) as well as the freshwater snails *Marisa cornuarietis* (Bannister et al. 2007) and *P.antipodarum* (Stange et al. 2012*)* has lead to the discovery of ERs in these species as well and also to that the receptors appear to be orthologues. The presence of sex steroids and ERs in molluscs might point to that they have a connection. Earlier studies have not been able to show the binding to the receptor (Bannister et al. 2007, Kajiwara et al. 2006, Keay et al. 2006, Thornton et al. 2003) and the function of the ER is therefore yet to be discovered.

There seems to be some differences between species of molluscs in their reaction to estrogens (Castro et al. 2007). Therefore more studies on species of molluscs needs to be carried out in order to understand the similarities and differences and to be able to understand the function of the ER.

Stange et al. (2012) examined the prosobranch snail *P.antipodarum* that is a promising freshwater OECD-test organism and found an up regulation of the ER transcriptional level after 24 hours exposure to EE2 (100 ng/L) but no significant change was observed after 7 days when normalized with solvent control.

In this study the transcriptional activity of the ER in the freshwater snail *Radix balthica* has been examined after exposure to EE2. The isolation of the ER gene has previously been done in our laboratory by C.Hultin (unpublished data). The hypothesis for this study is that the ER gene will respond to the EE2 through an up regulation of the ER-mRNA production. The aim is to see if there are any similarities and

differences between the freshwater species (prosobranchs and pulmonates) and to see whether perhaps *R.balthica* could also be suitable as an OECD test organism since its relative, *Lymnaea stagnalis*, already is recommended by OECD (2009). The test species that are recommended today might not meet the requirements after some years testing, therefore more candidates of molluscs needs to be examined.

Sensitivity to exposure of endocrine disrupting chemicals, EDCs, is an important feature for a species to be chosen as an OECD standard test organism (OECD, 2009). Therefore emphasis will be put on analyzing this endpoint.

2. Materials and methods

Animals

Radix balthica belongs to the gastropods within the family *Lymnaeidae* signifying a pulmonate (with lungs) freshwater species. The species is a simultaneous hermaphrodite (Dillon et al. 2000) with sexual mating and egg laying normally starting in March (Welter Schultes 2012). It can also self fertilize but this normally to a fitness cost (Dillon et al. 2000). As other pulmonates it is highly adaptable (Strong et al. 2008) and the family is widely distributed in the world both as native and non native species (Strong et al. 2008, Kipp et al. 2012).

Experimental setup and sampling

R.balthica was collected in a small pond in the vicinity of Lund (55°47'21" N, 13° 10'29" E) Sweden, in the middle of April 2012. Before the experiment started the snails were acclimatized to aquaria for 1-2 weeks with similar water and feeding conditions as in the setup later on. The setup constituted of 2 L aquaria filled up with 1,5 L test media, 12 snails were randomly added to each aquaria with three replicates per treatment.

The nominal concentrations of EE2 were 10 and 100 ng/L. The concentrations were chosen because of the earlier study on the fresh water snail, *P.antipodarum*, by Stange et al. (2012) which showed an up regulation of the ER-mRNA level at 100 ng/L. The concentration 10 ng/L was then put as an environmentally relevant value. Depending on the country, EE2 in effluents from waste water treatment plants can range between 0.1 and 15 ng/L with a mean approximately around 1-4 ng/L in Europe (Kuch and Ballschmiter 2001 (Germany), Ternes et al. 1999 (Germany), Larsson et al. 1998 (Sweden), Cargouet et al. 2004 (France), Belfroid et al. 1999 (Netherlands), Desbrow et al. 1998 (U.K.).

The EE2 stock solution (0,1 g/L) was prepared by dissolving EE2 (Sigma-Aldrich, ≥98%) in dimethyl sulfoxide, DMSO, and stored in darkness at 4° C. It was then diluted in series to obtain the nominal concentrations of 100 and 10 ng/L. Two negative controls were used, one solvent control and one water control (without solvent). The number of replicates was three also for the controls. All the treatments, including the solvent control, had the same concentration of DMSO (0,001 %, 10 µL/L as recommended by OECD (2009)).

Before the experiment all the snails were measured (mean shell height 9,2 +/- 0,20 mm) and checked for mortality. The snails were then placed in the aquaria together with the newly added test media and feed with green salad (equal amount per aquaria). The water in the aquaria was constantly aerated and the test media was not changed during the exposure period (96 h, EE2 half life approximately 54 h (Hallgren et al. 2012)). The water temperature during the exposure was 17-20°C and in the surrounding air it was on a constant 20°C, pH on average 7,35. The light/dark period consisted of 13 h light and 11 h dark which was the natural light/dark hours at the time of exposure.

Six snails were collected from each test vessel after 24 hours and the last six after 96 hours. The exposure was planned for seven days in order to compare with the study by Stange et al. (2012), but the temperature had gone up too high at an uncertain time range between day one and day three so the results might not have been trustworthy. Therefore snails from the 96 hours exposure were not analyzed.

After the collection of snails the shells were gently cracked and the snail tissues carefully poled off their shells before stored into the freezer (-80°C). The mortality in each aquarium was notated for each exposure period.

RNA extraction and cDNA synthesis

The whole tissue of the snail was used for the RNA-extraction. The individual tissues were homogenized using 1 mL TRIzol and a pestle and the total RNA was purified through several steps before stored in the freezer (-80°C). To measure the RNA concentrations a spectrometer, NanoDrop 2000 (NanoDrop Products Thermo Fisher), was used that works through UV-absorption. For the cDNA synthesis, total RNA (1 µg) was subsequently converted into complementary DNA using SuperScript™ II (Invitrogen). The cDNA reaction was conducted through the following steps: at 25° C for 10 min, at 42°C for 45 min, at 50°C for 15 min and at 70°C for 10 min. 20 µl ddH_20 was added for diluting the cDNA and it was frozen to -20°C.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The transcriptional activity of the ER was measured using quantitative real-time polymerase chain reaction (qRT-PCR) analysis on a Stratagene Mx 3000P (MxPRO software). Relative mRNA transcription was determined using absolute quantities, based on threshold cycle values, calculated from standard curves. A standard (a cloned snail ER-fragment in a 100 ng/µl) had been prepared previously by C.Hultin and was used to set up a series of x10 dilution standards against which the ER-mRNA level in the different samples were to be compared.

In a 96-well plate each sample was distributed as duplicates, consisting of 2 µl cDNA template, 0,5 µl of each primer (ER-F and ER-R, 18S-F and 18S-R), 12,5 µl SYBR Green PCR I Dye, 0,25 µl ROX and 10,25 µl ddH_20. Reactions were measured twice for each sample. The same amount of standard and sample was used for the qRT-PCR, where standard curves were set as six 10-fold dilution series. Two non- template control treatments were added to each plate. The reaction for the qRT-PCR started at 50° C for two minutes, then two min at 95°C, 40 cycles for 15 s at 95° C, thereafter at 64° C for 30 s and the last step constituted of a melting cycle to assure that one single product was present in the reactions.

Data analysis

The results from the qRT-PCR were analyzed with the accurate real time standard curve (recommended by the manufacturer). The housekeeping gene, 18S, was used as a reference when normalizing the ER gene levels for each sample: specifically, the absolute value of the ER gene was divided with the absolute value of the standard gene. The relative gene transcription level for the individual snails could then be compared.

3. Results

The mortality rate after 24 hours was 0 % in all the treatments and 5,6 % in the water control (calculated on all 12 snails per aquaria). Analyzes were performed on three of the six snails exposed for 24 hours. Since some samples did not give enough RNA, qRT-PCR could not be performed on all nine snails included in the treatments. The transcriptional levels of the ER gene are presented as mean values +/- standard deviation (SD) for all the treatments.

There is a small increasing trend from the lowest to the highest concentration, the increase between the water control and 10 ng/L EE2 is greater than between the 10 and 100 ng/L EE2 treatments (Fig.1). Although both unexposed snails and snails from the solvent control had higher levels.

The high standard deviation between the individuals in all aquaria gives a quite big uncertainty to the results, although some diversity is expected as it is snails collected from a wild population. The normal distribution in the treatments was tested with a Shapiro Wilk test. The test showed that all the treatments were normally distributed except for the water control and the 100 ng/L EE2 treatment. Therefore no statistic significance could be tested between the treatments with a one way ANOVA test.

Both controls had an increase in ER gene transcription levels after the 24 hours, especially the solvent control that had the highest level of all treatments tested. This puts a question mark to weather the solvent is a cofactor to the results. A notation can also be made on that even the unexposed snails have a high ER gene transcription level.

There are two samples that differ considerably in the ER-mRNA level compared to the other individuals in the same group (Fig. 2), one in the water control (ER-mRNA 3,01) and one in the 100 ng/L treatment (ER-mRNA 5,07). When testing for the outcome without these two values the populations in the different treatments all get a normal distribution. The difference between the treatments was tested in a one way ANOVA test but did not show any significant result.

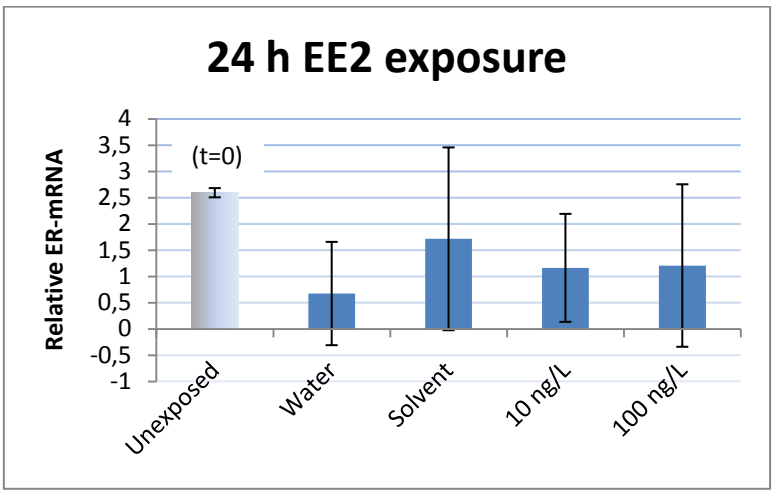

Figure 1. ER-mRNA level in *R.balthica* when treated with the nominal concentrations of 10 and 100 ng/L EE2 and the negative controls with water and solvent (mean +- SD). qRT-PCR was used to measure the mRNA and the values were normalized with the housekeeping gene 18S. ER specific primers for *R.balthica* was used for the transcription. Total number, n_t, of snails in the treatments were; unexposed n_t=2, water n_t=8, solvent n_t =6, 10 ng/L n_t =9, 100 ng/L n_t =9.

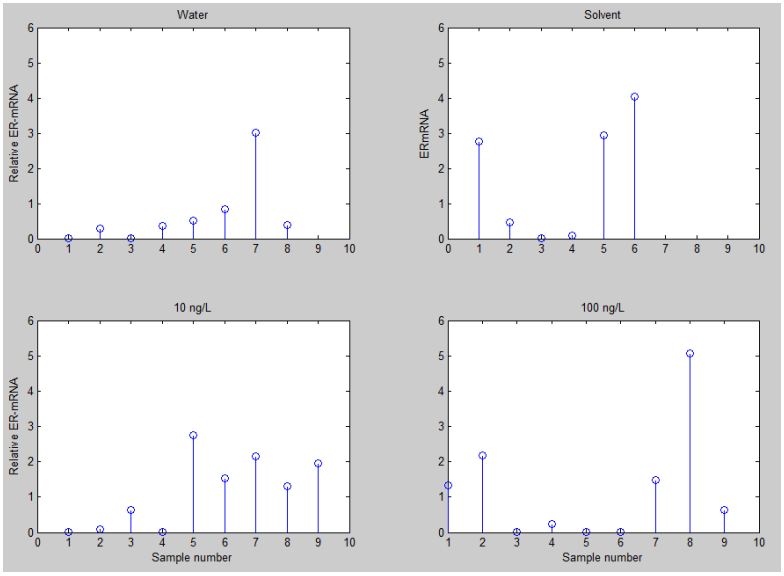

Figure 2. The ER-mRNA level in *R.balthica* showed for every individual snail in all the different treatments, the nominal concentrations of 10 and 100 ng/L EE2 and the negative controls with water and solvent. Relative ER-mRNA level presented on the y-axis and the number, n, of the snail sample on the x-axis. Total number, n_t, of snails in the treatments were; water n_t=8, solvent n_t =6, 10 ng/L n_t =9, 100 ng/L n_t =9

4. Discussion

In this study the ER-mRNA transcription level of the freshwater snail *R.balthica* was analyzed after exposing it for 24 hours to two different concentrations of EE2 -10 and 100 ng/L. The results show no significant difference in the transcriptional activity for the concentrations tested which falsifies the hypothesis that EE2 will alter the transcription level of the ER gene in *R.balthica*. Even though the transcription level is rising with increasing concentrations, the standard deviation is very high and therefore no conclusions can be drawn from that statement.

The individuals in the solvent control show the highest levels of the ER-mRNA. Stange et al. (2012) could also see this effect on the ER gene by DMSO in the *P.antipodarum* after seven days exposure. Both studies applied concentrations set up by OECD (2009). The conclusion from this is that the effects from this solvent can alter the results of the ER gene transcription levels. Other solvents might have to be considered to be used instead. Methanol and ethanol have shown effects in previous studies (Stange et al. 2012) so the ultimate solution would of course be to not use any solvent at all. Regular stirring would perhaps be a good alternative.

The no significant result is in agreement with the result from a previous study in our laboratory where P.Hallgren and his coworkers (2012) tested the effect of EE2 on several reproductive endpoints (days to and size at first reproduction, egg production and hatching success) and could not see any significant effect on *R.balthica*.

The result from our study is not in agreement with the work presented by Stange et al. (2012) and the freshwater species *P.antipodarum* where after 24 hours a significant up regulation was shown after exposure to EE2. The reason for the different outcomes between the two snails can only be speculated. The insignificant effect and high standard deviations can be due to the limited test data, few individuals were exposed (18 per treatment) and analyzed (nine per treatment), and since the snails are collected from a wild population the genetic variations between individuals will be greater compared to a laboratory breed population (Garant et al. 2004). The collection of snails from a wild population also leads to the question about their difference in age and sexual maturity. As stated in Jobling et al. (2003) it is today a well known fact that ERs change their numbers and affinity for estrogens seasonally. For example in mussels it has been shown that the ER gene has its maximum expression in March and April during the spring month (Ciocan et al. 2010) and in the mollusc *P.antipodarum* a seasonal variation of estradiol and testestorone has been found where E2 levels are significantly higher during the reproductive period (Gust et al. 2011). Since *R.balthica* starts reproduction in March (Welter Schultes 2012), comparison of responses by individuals at different stages of sexual development can be complicated (Jobling et al. 2003). As can be seen in the results, even the unexposed snails have a high ER-mRNA level.

A possible explanation for the insignificant results can also be that *R.balthica* is less sensitive to EE2 compared to the *P.antipodarum* because of the difference in endocrine systems of this hermaphroditic pulmonate (Dillon et al. 2000) mollusc and the parthenogenetically reproducing (Wallace, 1979) prosobranch respectively. In the prosobranch molluscs steroids have been found to have a functional role where as in the pulmonates they have not (Oetken et al. 2004). Effects on embryo production by EDCs like EE2, BPA and OPs on prosobranchs have for example been shown by Jobling et al. (2003), Duft et al. (2003) and Oehlmann et al. (2000).

Few studies have been made on endocrine disruption in pulmonate molluscs (Lagadic et al. 2007) so the possibility for a function of steroids cannot be excluded nevertheless, since increased embryo production also has been found when exposing the freshwater snail *L.stagnalis* to EDCs (Segner et al. 2003). The increased embryo production at relatively high concentrations (500 ng/L) of EE2 in the pulmonate mollusc *L.stagnalis* (Segner et al. 2003, OECD 2009) compared to the prosobranch *P.antipodarum* (1 ng/L) (Jobling et al. 2003) might point to a difference in sensitivity between the two species and possibly even between the two gastropod subclasses. A supporting example for this is TBT, the chemical inducing imposex in female dogwhelks (Matthiessen, Gibbs 1998), that has been shown to effect a lot of prosobranchs

(Oehlmann et al. 2007) but not pulmonates (Hallgren et al. 2012). Prosobranchs have been affected at very low concentrations, 1-3 ng/L (Matthiessen, Gibbs 1998) compared to the pulmonate *L.stagnalis* that has been affected at relatively high, Segner et al. (2003) showed effects on the prostate gland in the range of 230-3200 ng/L.

A contradictive finding to this hypothetic theory is that in the study by Hallgren et al. (2012) no difference in sensitivity to EE2 could be found between the prosobranch *B.tentaculata* and the pulmonate *R.balthica* (endpoints; days and size to first reproduction, egg production and hatching success). The only endpoint with significant effect on the two species was somatic growth rate with *R.balthica* showing an increase and *B.tentaculata* a decrease with rising concentrations.

So although steroids have been found in molluscs (Kajiwara et al. 2006, Keay et al. 2006) and reproductive effects from estrogens have been seen (Jobling et al. 2003, Segner et al. 2003) the biological mechanism behind it is still unclear (Stange et al. 2012). According to the previous literature studied (Bannister et al. 2007, Kajiwara et al. 2007, Keay et al. 2007, Thornton et al. 2003) none has been able to show or prove the binding of E2 to the ER. The explanations for this are many; one is that the ER is not the receptor mediating the observed effects (Keay et al. 2006) and that perhaps non-genomic functions are involved in the signaling instead (Kajiwara et al. 2006). Another is that the active sex hormone might not be E2 itself but a "derivate" (Stange et al. 2012). According to Bannister et al. (2007) the function of the ER can be that it has a key role in cell growth which in the cause of ER mediating EE2 signaling would support the findings of the study by Hallgren et al. (2012).

P.antipodarum is according to OECD (2009) a suitable test species because of its many advantages in the lab environment e.g. easy to breed, rapid generation time and known sensitivity to EDCs (OECD 2009). *R.balthica* does not according to these results seem like a suitable test species for OECD guidelines. It was not sensitive to the test substance EE2 and it appears to be a host for parasites, similar to other pulmonate molluscs (Lagadic et al. 2007) which according to OECD (2009) needs to be eliminated in order to avoid cofactors. The OECD recommendations for DMSO concentrations should according to this and the study by Stange et al. (2012) be reconsidered.

A future study where exposure and examination of more wild snails is carried out would clear out if the result from this study is repetitive. This would clarify whether the finding of these results is because *R.balthica* really is less sensitive to EE2. Furthermore, a study comparing juveniles to adults would give a better understanding to which age-groups that are more vulnerable for EDCs in the environment.

Even though the first case of imposex in dogwhelk molluscs where reported already in the 1970s, there is still a scientific debate about the exact mechanism behind the endocrine disruption of TBT and other EDCs (Oehlmann et al. 2007). The different reactions to estrogens by diverse species of molluscs (Castro et al. 2007) make the investigations to find it complicated. Gastropod molluscs morphological and developmental differences have lead to a suggestion of a new classification system of this taxon (Ponder, Lindberg 1997). More research needs to be done on the basic endocrine systems of molluscs and with this a better understanding of the biochemical pathway of the steroids within molluscs can be established.

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