Vitellogenin gene expression in the intertidal blenny *Lipophrys pholis*: A new sentinel species for estrogenic chemical pollution monitoring in the European Atlantic coast?

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A B S T R A C T

The presence of estrogenic chemicals (ECs) in the aquatic environment is a growing problem. While most attention was initially given to fresh water and estuarine ecosystems, it is now evident that coastal marine areas are also vulnerable to these pollutants. The use of vitellogenin induction in male fish, a specific biomarker of EC exposure, has been the most widely applied methodology. However, in some occasions, the high mobility and migratory behaviour of common sentinel fish species makes data interpretation difficult. Hence, there is the need to validate new sentinel marine fish species which should display, among other features, a strong homing behaviour. The shanny, *Lipophrys pholis*, is an intertidal fish that combines many of the required characteristics for a sentinel species: abundance and easy of catch, wide geographical distribution and restricted home range. Thus, in order to evaluate, in the field, the species sensitivity to ECs, *L. pholis* males were collected at two sites reflecting different degrees of anthropogenic contamination. The vitellogenin II gene (VTGII) was isolated and its liver expression evaluated by RT-PCR in the field samples. A significant induction of gene expression was observed in the specimens collected in the urban area, if compared to the reference site, which suggests exposure to ECs. Moreover, a 21-days laboratory exposure to environmentally relevant concentrations of ethinylestradiol (EE2) was also performed. A significant induction of *L. pholis* VTGII gene in EE2 exposed males was observed suggesting similar sensitivity to that of other marine/estuarine fishes. Even though further validation is currently in progress, the available data indicates that *L. pholis* is responsive to ECs, thus favouring its future integration in monitoring programmes designed to evaluate the presence of ECs in European marine ecosystems.

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1. Introduction

Worldwide concerns have recently increased due to the presence of natural and synthetic chemicals that can interfere with the endocrine system of several wildlife species, such as birds, reptiles, fish, amphibians, molluscs and mammals (Sumpter, 2005). The reported effects include alterations in development, growth, fertility, as well as female masculinization and male feminization (Howell et al., 1980; Cody and Bartone, 1997; Jobling et al., 1998; Larsson et al., 2000; Parks et al., 2001; Van Aerle et al., 2001; Santos et al., 2002; Sole et al., 2003; Jobling et al., 2004; Rodrigues et al., 2006; Holbech et al., 2006). Aquatic species seem to be particularly vulnerable to endocrine disrupting chemicals (EDCs), which is not surprising as the aquatic ecosystems receives a wide variety of pollutants. Although several mechanisms of action may be involved in endocrine disruption, most of the field studies dealing with aquatic vertebrates have shown that estrogenic effects such as fish feminization are predominant. Estrogenic chemicals (ECs) such as some organochloride pesticides, polychlorinated biphenyls, phthalates, alkylphenolic compounds and natural and synthetic estrogens can interact with the estrogen receptor or alter estrogen metabolism, therefore mimicking the action of the natural steroid 17-β-estradiol (E2) (Jobling et al., 2004). The first reports of fish feminization came from observations in some UK rivers displaying high levels of ECs (Purdom et al., 1994; Jobling et al., 1998). Notably, a high proportion of male fish were found to show the presence of oocytes within the male gonad tissue and elevated levels of vitellogenin (VTG). Additional laboratory and field studies have validated the use of VTG (the precursor of egg yolk...
protein) as a specific and highly sensitive biomarker of estrogenic exposure (Sumpter, 2005). VTG is usually produced by mature females in response to elevated levels of 17β-estradiol. Although males normally show no or low levels of VTG in plasma, their VTG genes are highly inducible after estrogen exposure (Purdom et al., 1994; Harries et al., 1997; Arukwe et al., 1998; Kime et al., 1999). More recently, the expression of VTG genes in the liver of males was shown to be a valid approach to monitor estrogenic exposure since VTG mRNA levels quickly rise after an exposure to Ecs (Bowman et al., 2000; Craft et al., 2004).

The first reports of elevated male VTG levels and ova-testis development were reported in freshwater and estuarine ecosystems, which is not surprising taking into consideration that, in some cases, over 25% of river flow is constituted by effluents (Sumpter, 2005). More recently, the finding of intersex and elevated VTG levels in the Mediterranean swordfish (Xiphias gladius L.) (Fossi et al., 2001; De Metrio et al., 2003) demonstrated that Ecs are not circumscribed to freshwater and estuarine ecosystems, and thus coastal ecosystems may also be at risk. Hence, there is the need to validate the use of new fish species that can be incorporated as sentinel organisms for Ecs monitoring in coastal seas. Several fish species have been increasingly used as sentinels in marine ecosystems biomonitoring programmes. However, some problems arise from the use of several of these species: i) the number of used sentinel species is limited and the obtained results (e.g. sensitivity to a particular chemical) may not be comparable; ii) the selected species represent only a restricted group of natural habitats; iii) in some periods, the migratory behaviour makes data interpretation difficult. Thus, there seems to be place for the use of new species whose ecological and behavioural characteristics, such as restricted home range, might increase the confidence in the biomarker’s responses.

Rocky shores are amongst the best studied habitats, due to its intrinsic richness and accessibility (Lewis, 1964). Many fish species have settled in this particular habitat, amid which benthioiods (one of the most abundant fish groups in tropical and warm temperature habitats) have received considerable interest (Monteiro et al., 2005). The shanny, Lipophrys pholis (L.), is a common inhabitant of the north-eastern Atlantic rocky intertidal, from Mauritania to Norway (Zander, 1986), usually found in rock pools from where it emerges at high tide to feed (Monteiro et al., 2005). Apart from a wide geographical distribution, abundance and easy sampling, L. pholis reunites other valuable characteristics that could encourage its use as a sentinel species in monitoring programmes, namely a well described life cycle and defined homing behaviour (the life cycle takes place almost entirely in the intertidal area). Thus, in order to validate the use of L. pholis as a sensitive sentinel species of Ecs contamination, an analysis of VTG gene expression was conducted in male specimens collected from two populations inhabiting sites that differed in the degree of anthropogenic contamination. In parallel, a laboratory exposure to environmentally relevant concentration of the model xenosterogen ethinylestradiol (EE2) was also performed. The reported findings suggest that L. pholis has the potential to be used as a sentinel species in the assessment of Ecs contamination in coastal seas.

2. Material and methods

2.1. Study area and sampling

Animals were collected at two locations in the Portuguese coast displaying different degrees of anthropogenic contamination: Cabo do Mundo (N 41, 22401; W 008, 71667), located in one of the most densely populated areas in the north of Portugal (the Porto coast). Cabo do Mundo and the surrounding beaches receive high loads of treated and untreated effluents from the cities of Matosinhos and Porto. High pollution levels have been reported in the past, including microbiological contamination (Bordalo, 2003; Cairrao et al., 2004; Cunha et al., 2005; Lima et al., 2008; www.inag.pt); Castelejo (N 37, 10241; W 008, 94521), located at the Natural Park of Sudoeste Alentejano and Costa Vicentina, a protected area of about 74788 ha and a submarine coastline of 2 km width. Neither large population aggregates nor industrial areas exist close to the coast which explains why this area was shown to be one of the least contaminated along the Portuguese coast (Santos et al., 2002; Castro et al., 2004; Lima et al., 2008). Additionally, no microbiological contamination has been observed in the last decade at Castelejo (www.inag.pt). Other biomarker responses of L. pholis collected in Cabo do Mundo and Castelejo in November 2005 and 2006 have been evaluated by two parallel studies (Lima et al., 2008; Solé et al., 2008). Hence, L. pholis collected at Cabo do Mundo displayed a significant induction of EROD and PAH bile metabolites and a decrease activity of acethylcholines- terases in comparison with specimens collected at Castelejo. This further validates the selection of the study locations.

Sampling was performed in November 2005, within L. pholis breeding season (Monteiro et al., 2005). During the reproductive season, males can be identified by the presence of a club gland on the tip of each dorsal fin ray whilst parental males display a uniform dark coloration that contrast with white lips (Northcott and Bullock, 1991). Males of L. pholis were collected with hand-nets in rocky pools and channels during ebb tides. In order to avoid using animals at different maturation stages, only adults were selected for the present study (measuring between 10 and 12 cm). After being collected, animals were transported alive to the laboratory in a refrigerated and aerated recipient. In the laboratory, fish were anaesthetized in saline water and ice, and body length and weight were determined. After dissection, the liver of five males per site was collected to RNAlater (Sigma) for downstream applications in molecular biology.

2.2. Histological procedures

To determine the male gonad maturation stage and to test for the presence of abnormalities such as testi-ova in field collected animals, histological preparations of the sampled specimens were performed. Gonad tissues were collected from all specimens and preserved on a Bouin buffer 1% with ethanol, embedded in paraffin, and sectioned to 5–7 μm thicknesses, stained with haematoxylin-eosin for histological identification of the gonadal developmental stage. Male maturation stages were evaluated based on the scheme of Weltzie (2002). All male gonads were found to be at stage IV (lumen of seminiferous tubules filled with spermatozooa) (Fig. 1B,C).

2.3. Laboratory exposure

Adult L. pholis were collected in November 2007 at Praia do Mindelo, an area of low contamination in the north of Portugal (Cunha et al., 2005). The animals were allowed to acclimate in the laboratory for approximately 2 month prior to the onset of the experiment. After this period, 3 animals per replicate were assigned to 70 L aquaria (2 replicates per treatment) filled with 42 L of artificial sea water (salinity 35‰) and maintained at 14.5±1 °C in an acclimatized room under natural photoperiod. Sera premium salt and carbon activated filtered tap water were used to prepared artificial sea water in the day before use (pH=8.3, conductivity=48 ms/cm, redox potential=– 76 mv). Water was changed daily, and animals were feed immature mussels from their origin site every other day. Stock solutions of Ethinylestra- diol (EE2) (98% purity) was obtained from Sigma and prepared in acetone. The following experimental treatments were established: solvent control; 5 ng/L EE2; 15 ng/L EE2. The concentration of acetone in all treatments was 0.000056%. After a 21-day exposure, animals were anaesthetized in saline water and ice, and body length and weight were determined. After dissection, the liver of males was collected to RNAlater (Sigma) for downstream applications in molecular biology.
designed in sequence conserved regions detected after aligning several described sequences in fish. Primer sequence as follows: VTGF: 5'-TGAATTCATGGTTGTGGGGAATG-3' and VTGR 5'-TTCTCAGCCTG-CACAGATT-C-3'. PCR conditions were: initial denaturation 94 °C 5 min, 93 °C 30 s, 52 °C 45 s and 68 °C 30 s for 40 cycles. A single DNA band was retrieved with the expected size, purified with the QIAquick gel extraction kit (Qiagen), and cloned using the pGEM-T Easy Vector System (Promega, USA). Clones were digested with the appropriate restriction enzyme (Promega) and separated on an agarose gel. A clone with the expected size was sequenced on both strands using M13 forward and M13 reverse primers (Stab Vida).

### 2.5. Phylogenetic analysis

The amino-acid sequence obtained in this work for the vitellogenin of *L. pholis* was aligned with other fish VTG sequences using ClustalX 1.83 (Thompson et al., 1997). The neighbour-joining tree was visualised with the software TreeView version 1.6.6 (Page, 1996).

### 2.6. Semi-quantitative RT-PCR analysis of VTGII expression

The strategy entailed first the isolation of total RNA and first strand cDNA synthesis from the liver of each specimen as previously described. Quantification specific primers were designed from the original sequence of *L. pholis* VTGII to originate a fragment of 334 base pairs. Primer sequence as follows: VTGF 5'AGCAACCAACAGAAT-GAGG3' and VTGR 5'GTGCCTGTCATGCTTTGT3'. A potential source of error when determining variation on gene expression by PCR comes from genomic DNA contamination carried over from RNA extraction to cDNA synthesis. Although DNaseI treatment should prevent such a problem, the design of quantification primers outflanking intron(s) further helps to determine if genomic contamination is present. We have used the quantification primers to determine intron presence, location and size in the VTGII sequence from *L. pholis*. Genomic DNA was extracted with the gDNA Mini Tissue Kit (Invitrogen). PCR conditions were those described for the semi-quantification analysis (see below). A single DNA band was retrieved with approximately 800 bp and directly sequenced with the flanking primers.

For the semi-quantitative PCR, 1 μL of single strand cDNA was amplified using 2.5 U of Taq DNA polymerase (Eppendorf) in a total volume of 20 μL containing 1X PCR buffer, 200 μM dNTP's and 200 nM of specific primers. PCR reactions were performed in a Biometra T-Personal Thermal cycler. Each cycle consisted of 30 s of denaturation at 93 °C, 30 s of annealing at 58 °C/65 °C for VTG and β-actin, respectively, and 25 s of extension at 68 °C. The number of cycles used was optimised for each target, when setting up the PCR reactions; master-mixes were always used to assure a minimum of variation in the reaction conditions. Trial runs were performed to determine the cycle number so that the reaction did not reach the plateau, i.e. they were in the exponential phase of amplification (not shown). The validation of the method as well as its applicability as a semi-quantitative approach to evaluate gene expression has been discussed in detail by Freeman et al. (1999) and Marone et al. (2001).

The β-actin primers were originally designed for *Sparus aurata* (forward 5'GGCCGCGCACTACGACTAC3' and reverse 5'ACCGAG-GAAGATGGCTTGAAA3'), and produce a DNA band of 250 base pairs (Santos et al., 1997).

The generated PCR products were run alongside with 1 Kb DNA marker (Invitrogen), separated on 2% agarose gel stained with ethidium bromide. For quantitative determination of the RT-PCR products, gels were run in duplicate and photographed by a digital camera (Kodak EDAS 290 Digital) under UV illumination with an image analysis system (Kodak ID 3.5). The fluorescence intensity of the band, which is related to the concentration of DNA in the gel, was determined using Molecular Analyst /PC Software Kodak ID 3.5. The β-actin expression was used to normalize the VTGII expression.

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**Fig. 1.** A photograph of the intertidal rocky blennioid *Lipophrys pholis* (A) and examples of the histological sections of the testis from males sampled in “Castelejo” (B) and “Cabo do Mundo” (C). The arrows indicate the presence of spermatozoa in the lumen of the seminiferous tubules.
Data from field samples were tested for significance using the Mann–Whitney Rank Sum test. Differences in VTGII normalized band intensity among EE2 exposed groups and control were evaluated through a one-way analysis of variance (ANOVA) followed by a multiple comparison test (Student–Newman–Keuls). All analyses were conducted in Statistica 7.0. All probabilities are two-tailed and a significant level of 0.05 was used.

3. Results

3.1. VTGII isolation

The isolated VTG fragment (460 nucleotides, see Fig. 2 A) was converted into an amino acid sequence (152 amino acids, Fig. 2 B). The alignment of L. pholis amino acid sequence with other available fish VTG sequences, namely VTGI and VTGII (Fig. 2 B), showed that the obtained sequence was VTGII-like. This was further confirmed by the phylogenetic analysis which places L. pholis sequence within the type II group (Fig. 2 B).

3.2. Field study

Since the sampling was performed within L. pholis breeding season, the microscopic analysis of the testes showed that males from “Castelejo” and “Cabo do Mundo” were mature and at similar stages of maturation (Fig. 1 B,C). No testis-ova were observed in the conducted histological samples. These observations validated all further comparisons on VTG expression levels in the two selected populations.

The semi-quantitative RT-PCR on L. pholis VTGII expression showed no significant differences between sampled populations in the expression of β-actin (Mann–Whitney U Test, N1=5, N2=5, U=6, Z=−1.36, P=0.22), thus validating its use as a reference gene. A visual inspection of the obtained agarose gel showed that all “Cabo do Mundo” males were expressing VTGII, while four out of the five analysed males in “Castelejo” also presented background levels of VTGII gene expression. However, males from the most contaminated site were expressing VTGII at increased levels when compared to males from the control sampling location. These results were confirmed when the gel bands fluorescence intensity was analyzed (Fig. 2 C, E). The mRNAVTGII/mRNAb-actin ratio showed significant differences between the selected populations (Mann–Whitney U Test, N1=5, N2=5, U=1, Z=2.40, P=0.05) with a mean 4.18 fold increase in VTGII normalized band intensity in males from “Cabo do Mundo”, if compared with those from the reference site.

3.3. Laboratory exposure

Fig. 2 (D and F) displays the results of the laboratory exposure. A dose-dependent increase in VTGII normalized band intensity was observed in the 21-days EE2 exposed males in comparison with control. A trend towards an increase in VTGII normalized band intensity was already evident at 5 ng/L EE2 while the highest dose (EE2 at 15 ng/L) induced a 13-fold increase in VTGII normalized band intensity (P<0.001).

4. Discussion

The use of fish in environmental monitoring programmes is a well established approach. However, one of the main drawbacks associated with its use as sentinels in environmental monitoring programs is the high mobility and migratory behaviour displayed by several species. In some cases, the unique ecological features of a particular species obscures data interpretation and may explain the lack of biomarker induction in some contaminated sites, and the observation of highly induced animals collected in apparently clean environments. Hence, in monitoring programs, the use of sentinel organisms which are sessile or display a strong homing behaviour, is highly recommended.

In European marine coastal ecosystems, the eelpout (Zoarces viviparus) has been the most widely used marine species to monitor chemical pollution, including ECs (Schladt et al., 1997; Gercken and Sordyl, 2002; Frenzilli et al., 2004; Lyons et al., 2004; Gercken et al., 2006; Napierska and Podolska, 2006; Vuorinen et al., 2006). While the eelpout shares many advantageous characteristics with L. pholis, such as restricted home range, the eelpout’s southern distribution limit lies, nowadays, on the English Channel, being absent in southern European marine habitats. It seems important to stress that, with the ongoing climatic change, the southern limit of distribution of some boreal species is currently receding (Monteiro et al., 2006). Thus, for these regions, it is important to validate the use of other coastal marine fish species, with contrasting or at least partially non-overlapping geographical distributions, which are responsive to pollution. Thus, L. pholis seems to be particularly suitable for environmental monitoring program; not only has a restricted home range and a wide geographical distribution, as it is extremely abundant and easy to capture in the intertidal rocky shores of the northeastern Atlantic. Furthermore, the results obtained in the present study seem to indicate that L. pholis is responsive to ECs pollution, and therefore are in favour of its use to screen the presence of ECs in the marine environment.

The most common methodological approach to monitor the presence of ECs in the aquatic environment has been the quantification of VTG or zona radiata protein (ZRP) induction in male fish (Sumpter, 2005). More recently, the evaluation of VTG and ZRP gene expression has also been applied with the same purpose, with the advantage of a more agile response after EC insult, if compared with protein synthesis (Garcia-Reyero et al., 2004). Furthermore, in some cases, as no suitable VTG or ZRP antibody is available for the species of interest, it is less laborious and time consuming to isolate the gene of interest than develop and validate the specific antibody. Conversely, the decay rate of VTG protein in fish plasma after ECs insult is longer than that of the VTG mRNA, and this may be disadvantageous in some monitoring programs (Craft et al., 2004). In the present study, we have isolated a VTGII partial sequence of L. pholis. In order to further validate the applicability of this methodology in the field, and the use of L. pholis as a sentinel species for EC monitoring, we have compared the VTGII gene expression in male L. pholis from an urban and industrialized area (Cabo do Mundo) with males collected in a reference site (Castelejo). Our data clearly showed a significant induction of the VTGII gene in the males collected in Cabo do Mundo when compared with those from Castelejo, thus suggesting exposure to ECs. In parallel, we have also screened for the presence of testis-ova in the analysed males, but no signs of feminization were observed. However, the number of scored male gonads is too limited to reach a definitive conclusion, and therefore further assessment is in progress. Yet, our observations are in line with several previous studies that demonstrated elevated VTG levels in males collected in rivers and estuaries receiving high loads of industrial and domestic effluents (Sumpter, 2005). To date only a few studies have evaluated the presence of fish feminization in coastal and open-sea ecosystems. Despite the higher chemical dilution that can be anticipated for marine ecosystems, when compared to rivers and estuaries, most of these previous studies have been able to detect the presence of VTG induction and/or testis-ova development in male fish collected in the vicinity of urban areas (Fossi et al., 2001; Gercken and Sordyl, 2002; De Metrio et al., 2003; Fossi et al., 2004; Desantis et al., 2005; Stentiford and Feist, 2005; Barucca et al., 2006; Martin-Skliton et al., 2006; Scott et al., 2006). Thus, our results further demonstrate that the negative impact of ECs is not circumscribed to fresh water and estuarine ecosystems, and therefore coastal marine species may also be exposed to high enough levels of ECs capable of eliciting deleterious effects.
Over the past five years, the station of Cabo do Mundo has displayed, in several analyses, a degree of microbiological water contamination (total and faecal coliforms) above the standards for recreational uses (www.inag.pt), most likely due to the release of treated and untreated domestic sewage. The presence of natural and synthetic estrogens, known to act as xenoestrogens in fish, has been previously associated with this kind of effluents (Sumpter, 2005; Rodrigues et al., 2006). Furthermore, Cabo do Mundo has several other potential sources of ECs in the vicinity. An important oil refinery is located 2 km to the south, thus explaining the accumulation of high levels of PAHs metabolites in the bile of Lipophrys pholis specimens collected at Cabo do Mundo (Lima et al., 2008).
activities have been reported to display high levels of alkylphenols which act as estrogen receptor agonists (Tollefsen et al., 2007). The estuary of the Leça river is located only 4 km to the south. This river has been shown to be highly contaminated with ECs both from urban and industrial origin. The most common xenoestrogens are nonylphenol, octylphenol, phthalates, 2-hydroxybenzothiazole, with levels up to the μg/L range (Azevedo et al., 2001; Petrovic et al., 2002; Céspodes et al., 2004). The recombinant yeast assay found an estrogen equivalent in one location of the Leça river (Ponte Moreira) of 3 ng/L (Céspodes et al., 2004), and effluents from one of the sewage treatment plants discharging in the Leça river are estrogenic and anti-androgenic to the gastropod Nucella lapillus (Castro et al., 2007; Santos et al., 2008). Moreover, a previous study performed in the Douro estuary, located at approximately 10 km south of Cabo do Mundo, showed that 21% of male mullets (Mugil cephalus) analysed displayed testis-ova (Ferreira et al., 2004). Tissue analyses from those animals revealed the presence of several PCBs and DDE, known to act as xenoestrogens in fish. Similar to Cabo do Mundo, the microbiological water quality of the Douro estuary and surrounding beaches has often presented faecal contamination above the standards for recreational uses, an indication of domestic sewage contamination (Bordalo, 2003), and the presence of ECs has been recently demonstrated in water and sediments (Almeida et al., 2007; Ribeiro et al., in press). Because of the predominant coastal sea currents toward north during autumn and winter raining periods, water from the Douro and the Leça River as well as effluents from the oil refinery reaches Cabo do Mundo and surrounding beaches (Da Silva et al., 2008), and thus may be one of the sources of ECs in the study area. Although the identification of the ECs that may be present in water at Cabo do Mundo was not one of the aims of this study, the significant induction of VTGII in male fish from that location indicates that future more in-depth studies are needed to characterize the chemicals and the sources of the observed estrogenicity. In addition, with the exception of the study performed by Jobling et al. (2002) with Aulius rutilus from UK rivers where a significant decrease in sperm quality and egg fertilization success were observed for intersex males, the population-level impact of ECs in fish have not been addressed in detail in wild populations. Hence, we plan to proceed with more thorough studies to investigate if the observed induction of male _L. pholis_ VTGII gene has any implication on the reproductive capability of that population.

In order to determine _L. pholis_ VTGII gene sensitivity to ECs, a laboratory experiment was conducted in which male _L. pholis_ were exposed to aqueous EE2 at concentrations of 5 and 15 ng/L. The threshold for VTGII induction observed in the present study between 5 and 15 ng/L after 21-days exposure is similar to that reported for other marine fish species (Kirby et al., 2003); in the sand goby Pomatoschistus minutus, and the flounder, Platichthys flesus, a threshold for VTG gene induction after 21-days EE2 exposure of between 1 and 10 ng/L was observed. For the fresh water teleost zebrafish, Danio rerio, both VTGII and III gene induction was found to start at 2.5 ng/L EE2 in a 21-days trial (Islinger et al., 2003), thus rendering the response to EE2 slightly more sensitive than _L. pholis_ VTGII induction.

Fish have been shown to have multiple VTG genes. In zebrafish, VTGII displays the highest expression levels in the liver (Wang et al., 2005). In male plaice, Pleuronectes platessa, exposure to EE2 at 20 ng/L for 21-days lead to a significant induction of both VTGII and VTGIII genes, with the VTGII gene being slightly more inducible (25%) than VTGIII (Brown et al., 2004). In zebrafish, exposure to estradiol (5 μg/L for 3 days) led to a 160% induction of VTGII gene and 80% induction of VTGII in the liver of females (Wang et al., 2005). Hence, available data seems to indicate that VTGII is more inducible by estrogens than VTGIII. Therefore, the isolation of _L. pholis_ VTGII should be a target in future studies, as it may reveal to be even more sensitive to low levels of ECs than VTGII.

Several European countries have developed national programs to provide evidence for endocrine disruption in fresh water, estuarine and coastal ecosystems, with particular emphasis on ECs (Sumpter, 2005). To date, no similar program has been developed for Portuguese ecosystems, although the Portuguese Ministry of Environment set out in 1999 a monitoring programme aimed at evaluating the levels of ECs in water and sediments in fresh water and estuarine areas, and to identify potential “hot spots” (Reis-Henriques et al., 2006). Generally, samples collected in industrialised areas near Porto and Lisbon presented high estrogenic loads (Petrovic et al., 2002; Céspodes et al., 2004). Alkylypholic compounds and 4-nonylphenol isomers, in particular, seemed to be the main source of estrogenicity in those samples (Petrovic et al., 2002; Céspodes et al., 2004). However, almost no information is available on the effects of ECs in Portuguese aquatic ecosystems. Besides the observation of testis-ova in male _Mugil cephalus_ from the Douro estuary (Ferreira et al., 2004), we have recently observed feminization of male sand goby (Pomatoschistus minutus) from two Portuguese estuaries (Rodrigues et al., 2006). Hence, to the best of our knowledge, the results of the present study are the first evidence of the negative impact of ECs in fish sampled along the Portuguese marine coastal ecosystems, thus adding additional concern to the limited set of data available for Portuguese estuaries. These preliminary results (Ferreira et al., 2004; Rodrigues et al., 2006; present study) clearly demonstrate that feminization of male fish is occurring in Portuguese estuaries and marine coastal areas, thus showing the need for the establishment of a national program on this topic.

In conclusion, the significant VTGII induction observed in _L. pholis_ collected in an urban and industrialized area, together with the massive VTGII gene induction observed in the laboratory exposure to EE2 indicates that this species seems to be EC responsive under field and laboratory conditions. If the species particular ecology and behaviour are also taken into consideration, then the available evidence encourages the species future integration in monitoring programmes designed to evaluate the presence of ECs in European marine ecosystems.

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