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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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ABSTRACT

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 environmental 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.

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

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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 blennioids (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 xenoestrogen 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 acethylcholinesterases 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 Weltzien et al. (2002). All male gonads were found to be at stage IV (lumen of seminiferous tubules filled with spermatozoa) (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 acclimatize 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 filtrated 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 Ethinylestradiol (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.