

Review

Endocrine disruptors in marine organisms: Approaches and perspectives

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Received 8 September 2005; received in revised form 14 March 2006; accepted 15 March 2006

Available online 22 March 2006

Abstract

Organic pollutants exhibiting endocrine disrupting activity (Endocrine Disruptors—EDs) are prevalent over a wide range in the aquatic ecosystems; most EDs are resistant to environmental degradation and are considered ubiquitous contaminants. The actual potency of EDs is low compared to that of natural hormones, but environmental concentrations may still be sufficiently high to produce detrimental biological effects. Most information on the biological effects and mechanisms of action of EDs has been focused on vertebrates. Here we summarize recent progress in studies on selected aspects of endocrine disruption in marine organisms that are still poorly understood and that certainly deserve further research in the near future. This review, divided in four sections, focuses mainly on invertebrates (effects of EDs and mechanisms of action) and presents data on top predators (large pelagic fish and cetaceans), a group of vertebrates that are particularly at risk due to their position in the food chain. The first section deals with basic pathways of steroid biosynthesis and metabolism as a target for endocrine disruption in invertebrates. In the second section, data on the effects and alternative mechanisms of action of estrogenic compounds in mussel immunocytes are presented, addressing to the importance of investigating full range responses to estrogenic chemicals in ecologically relevant invertebrate species. In the third section we review the potential use of vitellogenin (Vtg)-like proteins as a biomarker of endocrine disruption in marine bivalve molluscs, used worldwide as sentinels in marine biomonitoring programmes. Finally, we summarize the results of a recent survey on ED accumulation and effects on marine fish and mammals, utilizing both classical biomarkers of endocrine disruption in vertebrates and non-lethal techniques, such as non-destructive biomarkers, indicating the toxicological risk for top predator species in the Mediterranean. Overall, the reviewed data underline the potential to identify specific types of responses to specific groups of chemicals such as EDs in order to develop suitable biomarkers that could be useful as diagnostic tools for endocrine disruption in marine invertebrates and vertebrates.

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Keywords: Endocrine disruptors; Marine invertebrates; Top predators; Steroid metabolism; Cell signalling; Biomarkers; Vitellogenin; CYP1A1

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1. Introduction

Endocrine disruptors (EDs) are a structurally diverse group of compounds that may adversely affect the health of humans, wildlife and fisheries, or their progenies, by interaction with the endocrine system (Colborn et al., 1993; Colborn, 1998; EEA Report, 2002; Gillesby and Zacharewski, 1998). They include organic chemicals used heavily in the past, specifically in industry and agriculture, such as polychlorinated biphenyls and organochlorine pesticides. EDs also include chemicals currently used, such as plasticizers and surfactants. Many known EDs are estrogenic, affecting particularly reproductive functions. Because of the lipophilic and persistent nature of most xenobiotic estrogens and their metabolites, many bioaccumulate and biomagnify in different environmental compartments, including marine biota (Colborn, 1998; Arukwe et al., 1996; Matthiessen, 2003; Langston et al., 2005; Lye, 2000).

Most data on the biological effects and mechanisms of action of EDs on marine organisms come from studies on vertebrates. A review of the wide and complex field of endocrine disruption in marine vertebrates, including the vast literature on fish, as well as the recent data emerging from the application of the -omics approach is outside the scope of this work, and the reader is referred to a number of excellent papers on these subjects (Goksøyr et al., 2003; Matthiessen, 2003; Langston et al., 2005).

In this work selected aspects of endocrine disruption on which limited information is available will be reviewed, in particular those concerning invertebrates. These aspects emerged during the 22nd Conference of the European Society of Comparative Physiology and Biochemistry—ESCPB, held in Alessandria, Italy, in 2003, where contributions to the section “Biological effects of organic pollutants—endocrine and signalling disruption”, underlined the potential for development of studies on endocrine disruption by organic chemicals in marine invertebrates, such as molluscs, and vertebrate top predators (pelagic fish and cetaceans).

The importance of developing invertebrate studies is largely due to the limited knowledge on the endocrine physiology of many invertebrate groups that represent important components of marine ecosystems; moreover, little is known on the molecular targets for the action of EDs in these organisms and only in few marine invertebrate species the genomics and proteomics approach is being developed. With regards to marine top predators, that play a key role in food chains, the role of endocrine disruption among the factors contributing to the general decline of predatory fish communities as well as cetaceans, is still largely unexplored.

2. Steroid synthesis and metabolism in invertebrates: potential targets for endocrine disruptors

It is now generally accepted that endocrine-disrupting chemicals (EDCs) or endocrine disruptors (EDs) are at least partially res-

ponsible for disruption of reproduction and development in wildlife populations (Vos et al., 2000), and that both vertebrates and invertebrates are susceptible to the action of EDs. In fact, the development of imposex in gastropods exposed to tributyltin (TBT) is considered one of the clearest examples of chemically induced endocrine disruption. However, progress on understanding endocrine disruption and the mechanisms of action of EDs in invertebrates has been hampered by the lack of detailed knowledge on their endocrinology.

Although the role of steroid hormones in invertebrates is still under debate, key steps of steroidogenesis leading to androgens or estrogens have been described in species from different phyla (Fig. 1). Thus, in some invertebrate species the metabolism of cholesterol to pregnenolone by cytochrome P450 side chain cleavage is followed by conversion of pregnenolone to progesterone by $3\beta/\Delta^5$ - Δ^4 -hydroxysteroid dehydrogenase (3β -HSD- Δ^5 - Δ^4 isomerase). This yields an active vertebrate-type steroid, which is also the precursor of other sex steroids, and is metabolized by various cytochrome P450s to cortisol, but also to 17α -hydroxyprogesterone and androstenedione, a precursor of active androgens and estrogens in vertebrates. In molluscs and echinoderms, androstenedione is also formed from dehydroepiandrosterone (DHEA), a Δ^5 steroid; and it is aromatized to estrone by cytochrome P450 aromatase. 17β -Hydroxysteroid dehydrogenases (17β -HSDs) catalyze the formation of testosterone from androstenedione and estradiol from estrone. Thus, similarly to vertebrates, a combination of cytochrome P450s and steroid dehydrogenases catalyze the conversion of cholesterol to sex steroids in echinoderm and molluscan species (Fig. 1). Some of those steroidogenic pathways have not been demonstrated in crustaceans, where ecdysteroids (the molting hormones) play a key role in the control of reproduction and embryogenesis (Lafont, 2000).

Despite the existence of common steroidogenic pathways in the different invertebrate species studied so far, an in-depth study of those catalytic activities evidences significant differences between phyla. For instance, androstenedione is actively oxidized to testosterone in crustaceans and echinoderms, similarly to vertebrates, but reduced to 5α -androstenedione in molluscs (Janer et al., submitted for publication). Also, the activity of cytosolic sulfotransferases using testosterone as a substrate was found to be much higher in echinoderm species than in molluscs or crustaceans, which suggest the existence of different steroid-inactivating enzymes in different invertebrate groups (Janer et al., 2005).

Within this context, several studies have assessed the ability of endocrine disruptors to alter steroid hormone metabolism in invertebrates. In vitro studies have demonstrated the ability of TBT to inhibit P450-aromatase activity in *Crassostrea gigas* and *Ruditapes decussata* (Morcillo et al., 1998; Le Curieux-Belfond et al., 2001), and testosterone sulfotransferase and palmitoyl-CoA-

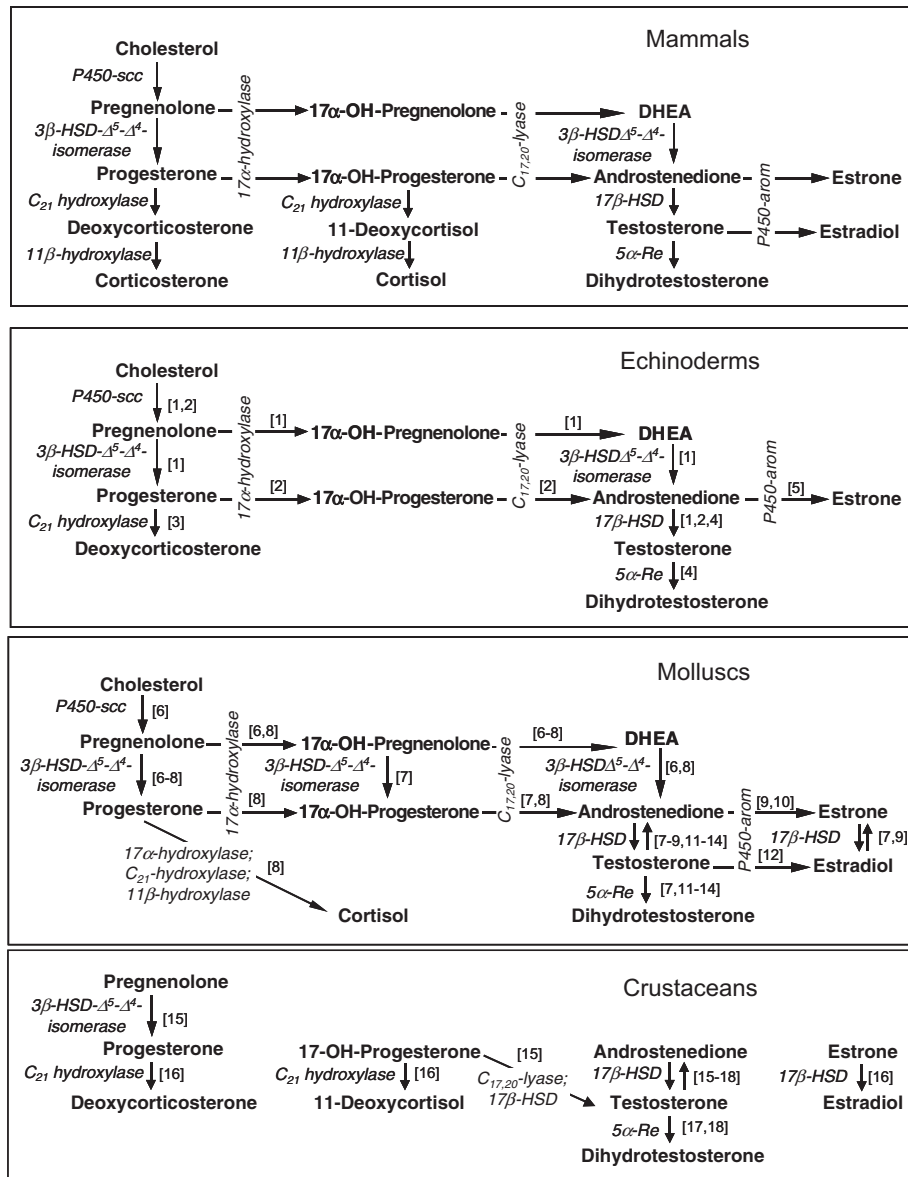


Fig. 1. Principal steroidogenic pathways for echinoderms, molluscs and crustaceans, and comparison with those described in humans—top figure adapted from Normand and Litwack, 1998. [1] Voogt et al., 1990; [2] Schoenmakers, 1979; [3] Wasson and Watts, 2000; [4] Wasson et al., 1998; [5] our group, unpublished results; [6] Gottfried and Dorfman, 1970; [7] De Longcamp et al., 1974; [8] Lupo di Prisco and Dessi' Fulgheri, 1975; [9] Le Curieux-Belfond et al., 2001; [10] Morcillo et al., 1999; [11] Morcillo et al., 1998; [12] Le Guellec et al., 1987; [13] Ronis and Mason, 1996; [14] Oberdörster et al., 1998b; [15] Swevers et al., 1991; [16] Blanchet et al., 1972; [17] Verslycke et al., 2002; [18] Baldwin and LeBlanc, 1994b.

testosterone transferase in *Paracentrotus lividus* (Janer et al., 2005). The data obtained indicates that 5 α -reductases and HSDs are probably not specific targets for organotin compounds in invertebrates, whereas they are strongly inhibited in subcellular fractions isolated from human or rat tissues (Doering et al., 2002; Loo et al., 2003; McVey and Cooke, 2003). Also the imidazole-like fungicide fenarimol did not alter the metabolism of testosterone in the mollusc *Marisa cornuarietis* and the crustacean *Hyalella azteca*, but strongly enhanced the synthesis of DHT and 5 α -androstenediol in *P. lividus* gonad microsomes (Janer et al., submitted for publication). This might have relevant physiological effects, if, similarly to mammalian species, DHT behaves as a potent androgenic steroid in echinoderms.

Exposure experiments and even field studies have often associated exposure to TBT with an inhibition of P450-aromatase activity in several molluscan species (Morcillo et al., 1999; Santos et al., 2002), and with induction of 17 β -HSD in the crustacean *Neomysis integer* (Verslycke et al., 2003). In addition, exposure to TBT resulted in a decrease in testosterone sulfation in *Littorina littorea* (Ronis and Mason, 1996), and a decrease in testosterone esterification in *Ilyanassa obsoleta* (Gooding et al., 2003). Metabolic androgenization, defined as an increase in the ratio between reduced and dehydrogenated metabolites of testosterone—preferentially retained in the organism—to hydroxylated and conjugated metabolites—preferentially eliminated—was reported in *N. integer* and *Daphnia*

magna exposed to TBT (Verslycke et al., 2003; Oberdörster et al., 1998a). Other compounds have also been shown to interfere with the metabolism of testosterone in invertebrates. Namely, the organophosphorous pesticide malathion caused a decrease in testosterone hydroxylases in *D. magna* (Baldwin and LeBlanc, 1994a), and nonylphenol polyethoxylates and pentachlorophenol led to a decrease in polar testosterone conjugates (glucosides and sulfates) (Baldwin et al., 1998; LeBlanc and Bain, 1997).

Some studies have additionally assessed whether xenobiotic exposure can affect endogenous steroid levels, which may in turn be an indication of altered steroid synthesis and/or metabolism. Thus, increased tissue testosterone levels have been observed after exposure of clams to TBT (Morcillo et al., 1998). Exposure to cadmium or PCBs (Clophen A50) led to a decrease in testosterone and progesterone levels in the pyloric caeca, but not in gonads of *Asterias rubens* (den Besten et al., 1991).

Exposure to model steroids, such as estradiol, may help to better characterize the response of invertebrates to endocrine disrupting chemicals (estrogenic, androgenic), and to identify key biochemical pathways that can be altered by exposure, as well as the physiological consequences for the organism. In a recent work, exposure of mussels to different concentrations of estradiol evidenced the existence of mechanisms that allow mussels to maintain their hormonal levels stable, and the important role that fatty acid esterification may play within those mechanisms (Janer et al., 2004).

Finally, it should be mentioned that the endocrine system has complex feedback signalling pathways that act as homeostatic mechanisms, therefore, an alteration observed in a specific endpoint might not be due to a direct interaction of the xenobiotic on that level, but the result of indirect regulatory mechanisms. Nonetheless, several lines of evidence suggest that steroidogenic enzymes, steroid-inactivating enzymes, and even steroid recep-

tors (Thornton et al., 2003) may have an important functional role in invertebrates, and that they are modulated by endogenous compounds, are potential targets for some xenobiotics, and certainly deserve further research.

3. Effects of natural and environmental estrogens on kinase-mediated cell signalling in *Mytilus* immunocytes

EDs include a variety of natural and synthetic steroid estrogens, as well as of estrogen-mimicking chemicals. These compounds can bind mammalian intracellular estrogen receptors (ERs); ERs, after estrogen binding and nuclear translocation, directly act as ligand-inducible transcription factors specifically regulating the expression of target genes; this is generally referred to as genomic or 'classical' pathway of estrogen action (Fig. 2). In vertebrates, the activity of most EDs is weak, due to their interactions with the ER; moreover, ERs from different species exhibit different ligand preferences and relative binding affinities for estrogenic compounds (McLachlan, 2001; Witorsch, 2002; Rotchelle and Ostrander, 2003). However, ER binding per se can have limited influence on endocrine disruption, and the nature (estrogenic or antiestrogenic) and magnitude of the response can be a function of other factors (Witorsch, 2002). In fact, estrogens can also act through 'alternative' pathways (Fig. 2), involving either membrane ERs or receptor-independent mechanisms that can result in both direct local effects (such as modulation of ion fluxes) and regulation of gene transcription secondary to modulation of kinase cascades (Nadal et al., 2000; Driggers and Segars, 2002; Segars and Driggers, 2002; Lösel et al., 2003). In this light, the mechanisms of action of EDs can include complex cross-talk between ER and other signalling pathways and alternative modes of estrogen action, involving both kinase- and Ca^{2+} -mediated signalling (Nadal et al., 2000; Witorsch, 2002).

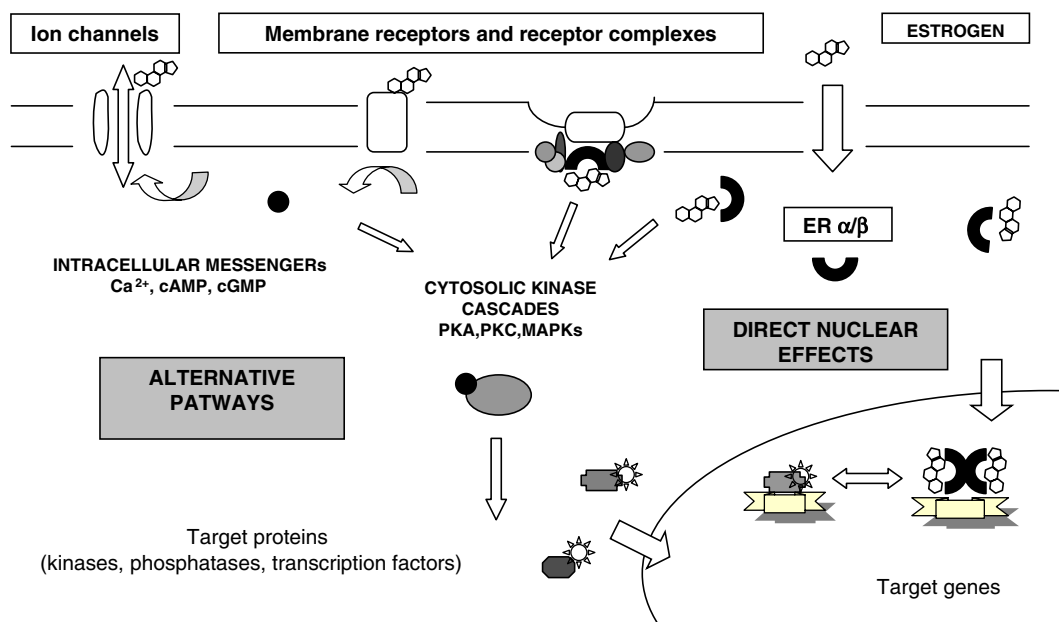


Fig. 2. Possible signalling pathways involved in the cellular action of natural and environmental estrogens. ER=estrogen receptor; PKA=Protein kinase A; PKC=protein kinase C; MAPKs=mitogen-activated protein kinases.

In comparison to vertebrate systems, a limited number of studies have examined the effects of EDs in invertebrates (Depledge and Billingham, 1999; Rotchelle and Ostrander, 2003). This is largely due to lack of knowledge on basic endocrine physiology of most invertebrate groups, the presence of estrogen and steroid receptors, and the mechanisms of action of natural hormones. In different molluscan species ER-like proteins have been described (Di Cosmo et al., 2002; Stefano et al., 2003; Osada et al., 2003; Canesi et al., 2004a). Sequences highly homologous to human ERs are present in *Aplysia*; however, the transcriptional activity of this ER seems to be constitutive, and independent of estrogen activation (Thornton et al., 2003). In analogy with mammalian cells, the effects of estrogenic compounds in invertebrate cells may be also independent of intracellular receptors, and due to both activation of membrane ER forms and cross-talk with other signalling pathways.

We have recently investigated the possible effects and the mechanisms of action of the natural estrogen 17 β -estradiol (E₂) in the hemocytes of *Mytilus galloprovincialis* (Canesi et al., 2004a). The results showed that nanomolar concentrations of E₂ rapidly induced cell shape changes, lysosomal destabilization and hydrolytic enzyme release, and stimulation of bactericidal activity; these effects were related to transient activation of MAPK- (Mitogen Activated Protein Kinases) and STAT- (Signal Transducers and Activators of Transcription) like proteins that are involved in the immune response (Canesi et al., 2002, 2003a). In the same cells, the immunotoxic effects of certain PCBs congeners were also associated to increased phosphorylation of MAPKs and STATs (Canesi et al., 2003b). In *Mytilus edulis* ganglia, E₂ induced Ca²⁺-dependent NO production through activation of a signalling pathway at the cell surface (Stefano et al., 2003). In both hemocytes and ganglia, most of the effects of E₂ were prevented by the classical anti-estrogens tamoxifen and ICI 172780, indicating a role for ER-like proteins (Stefano et al., 2003; Canesi et al., 2004a). Overall, these data are apparently consistent with the hypothesis that estrogen mechanisms of action involving alternative pathways are also present in invertebrate cells and that they may also modulate functions other than reproduction. In particular, the results obtained with mussel hemocytes indicate that, like in mammalian immunocytes (Ansar Ahmed, 2000), estrogenic compounds can also affect immune function.

Different synthetic estrogens and other estrogenic chemicals were subsequently shown to rapidly affect mussel hemocyte lysosomal function in vitro through modulation of kinase-mediated cascades (Canesi et al., 2004b). Like for PCBs, the effects of DES (diethylstilbestrol), BPA (bisphenol A) and NP (nonylphenol) were observed at concentrations about 1000 times higher than those of E₂ (Table 1). Each compound showed a distinct effect on the phosphorylation state of MAPK and STATs members. Interestingly, both BPA and NP induced a persistent decrease in the level of p-p38 MAPK and of p-STAT members (Canesi et al., 2004b). MAPKs exert their effect directly, by phosphorylating substrates as transcription factors, or indirectly, by activating downstream kinases which in turn phosphorylate their own substrates. P38 MAPK plays multiple roles in an array of cellular responses to environmental stress in different organisms (Cowan and Storey, 2003). STAT proteins are activated by a variety of extracellular stimuli (cy-

Table 1

Changes in the phosphorylation state of MAPK- and STAT-like members in mussel hemocytes incubated in vitro with 17 β -estradiol and estrogenic chemicals for different periods of time

% Change vs. controls				
Treatment	5'	15'	30'	60'
<i>E2</i> (17 β -estradiol) (25 nM)				
p-ERK ₂ MAPK	+100*	+80*	+50*	–
p-p38 MAPK	+400*	+250*	+100*	+100*
p-STAT3	+100*	+300*	+80*	–
p-STAT5	+300*	+200*	+100*	+20
<i>PCB 47</i> (2,2'-4,4'-tetrachlorobiphenyl) 3 μ M				
p-ERK ₂ MAPK	–	–	–	–
p-p38 MAPK	–	+50*	+100*	+260*
p-STAT3	–	–	–	–
p-STAT5	–	–	+40*	+30*
<i>DES</i> (diethylstilbestrol) 25 μ M				
p-ERK ₂ MAPK	–20	+40*	+20	–65*
p-p38 MAPK	+300*	+580*	+610*	+260*
p-STAT3	–20	–40	–60*	–80*
p-STAT5	+100*	+120*	+80*	–40*
<i>BPA</i> (bisphenol A) 25 μ M				
p-ERK ₂ MAPK	+100*	+50*	–10	–
p-p38 MAPK	–	–50*	–50*	–50*
p-STAT3	+50*	+210*	+210*	–
p-STAT5	–15	–25	–55*	–80*
<i>NP</i> (nonylphenol) 25 μ M				
p-ERK ₂ MAPK	+40	+60*	+10	–
p-p38 MAPK	–40*	0	–15	–50*
p-STAT3	–60*	–60*	–90*	–50*
p-STAT5	–40*	–60*	–55*	–40*

Data, obtained by SDS-PAGE and Western blotting of hemocyte protein extracts with specific anti-phospho-antibodies, represent the results of densitometric analyses (mean of three independent experiments). Relative band optical densities (arbitrary units) were normalised for the control band in each series and results are expressed as mean % change vs. controls; * $P \leq 0.05$.

tokines, growth factors, hormones) and they are involved in many physiological processes, including apoptosis, proliferation and immunity; they take part in tumorigenesis by deregulating the signal transduction pathways in which they are implicated (Calò et al., 2003). Our data indicate that both MAPK and STAT members, that play a key role in activation of the immune response of mussel hemocytes (Canesi et al., 2002, 2003a), represent a target for the action of both natural and environmental estrogens in vitro. Overall, the results demonstrate that different EDs may act through disruption of kinase mediated signalling pathways and suggest that, like in mammalian cells, also in mussel cells both natural and environmental estrogens may affect the phosphorylation state of transcription factors; this, in turn, possibly leads to changes in gene expression secondary to modulation of cytosolic kinase cascades.

The possibility that EDs may affect hemocyte function and kinase-mediated pathways has been further investigated in vivo in mussels injected with more realistic environmental concentrations of NP or BPA. In particular, BPA in vivo was shown to induce a dramatic decrease in the phosphorylation level of the stress-activated p38 MAPK and of a CREB-like (cAMP-responsive element binding protein) transcription factor (Canesi et al., 2005).

The results confirm the *in vitro* data (Canesi et al., 2003c, 2005); moreover, they are consistent with the observation that exposure to BPA induces changes in protein tyrosine phosphorylation in mussel cells (Berti et al., 2003). This suggests that determination of changes in the phosphorylation state of critical signalling components (both cytosolic kinases and transcription factors) in mussel tissues might be utilized in evaluating the effects of environmental exposure to EDs.

Modulation of kinase-mediated pathways does not represent the only possible effect of EDs on cell signalling in mussels. Both E₂ and EDs have also been shown to affect Ca²⁺ homeostasis in different cells and tissues, although at higher concentrations (Burlando et al., 2002; Canesi et al., 2004a, c; Canesi et al., *in press*). Further studies are needed in order to recognize the distinct mechanisms of cellular signalling by endogenous steroids and estrogenic chemicals in invertebrates. Our data address to the importance of investigating full range responses to estrogenic chemicals and may help understanding their basic mechanisms of action in ecologically relevant invertebrate species.

4. Biomarkers of exposure to endocrine disruptors in marine bivalve molluscs and their use in marine pollution assessment

Biomarkers could be defined as measurements of body fluids, cells or tissues that indicate in biochemical or cellular terms the presence of contaminants or the magnitude of the host response (Livingstone et al., 2000). Biomarkers indicating exposure to pollutants and their effects are increasingly applied to assess the health of estuarine and marine ecosystems. However, there is a limited number of well-established “core” biomarkers used routinely in marine environmental programmes (Cajaraville et al., 2000) and still a need exists to develop new biomarkers that could be useful as diagnostic tools for specific groups of chemicals or specific types of responses. A wide spectrum of potential biomarkers could be applied to the study of endocrine disruption in the aquatic environment. In fish, these include changes in hormone titres (steroid hormones, thyroid hormones), abnormal gonad development, low gamete viability, alterations in some enzyme activities (i.e., aromatases) and protein levels (i.e., vitellogenin, zona radiata proteins, spiggin) (WHO/IPCS, 2002; Matthiessen, 2003; Kleinkauf et al., 2004; see also other sections in this review). Induction of vitellogenin (Vtg), the precursor molecule of yolk proteins, in oviparous males or juveniles is a well known effect of xenoestrogenic contaminants in fish, and has been extensively used as biomarker both in laboratory and field studies (Matthiessen and Sumpter, 1998; WHO/IPCS, 2002; Arukwe and Goksøyr, 2003; Goksøyr et al., 2003; Ortiz-Zarragoitia and Cajaraville, 2005a).

Several methodologies have been developed for determination of Vtg: immunotechniques based on the use of specific antibodies such as radioimmunoassays, enzyme-linked immunosorbent assays (ELISAs), western blot and immunohistochemistry, molecular tools such as RNA protection assays and transcript analysis by Northern blotting or various variants of polymerase chain reaction (PCR), and protein expression studies by proteomic approaches (Denslow et al., 1999; Arukwe and Goksøyr, 2003;

Marin and Matozzo, 2004). Most of these methods have been used in vertebrate aquatic organisms such as fish, but little is known in aquatic invertebrate species, such as bivalve molluscs used worldwide in biomonitoring programmes (Cajaraville et al., 2000). There are few specific antibodies developed against bivalve Vtg or Vtg-like molecules, and these antibodies usually show low cross-reactivity across species (Li et al., 1998; Blaise et al., 1999; Kang et al., 2003; Osada et al., 2003; Park and Choi, 2004). Therefore, indirect methods could be used to study alterations provoked by EDs, such as increase in RNA contents, lipid deposition, glycogen depletion, increase in protein levels, calcium, magnesium and phosphoproteins contents (Verslycke et al., 2002; Arukwe and Goksøyr, 2003; Marin and Matozzo, 2004). Among these methods, the measurement of phosphoproteins by the alkali-labile phosphate (ALP) method has been widely used in different aquatic organisms such as fish and bivalve molluscs (Kramer et al., 1998; Blaise et al., 1999; Verslycke et al., 2002; Marin and Matozzo, 2004). In fish, ALP levels have been shown to associate with Vtg levels measured using specific immunotechniques and gene expression tools (Versonnen et al., 2004; Robinson et al., 2004). In a recent international intercalibration study using adult male zebrafish exposed to 17-β-estradiol for 2–9 days (Porcher, 2003), we found a significant positive correlation between Vtg levels measured by specific ELISA and ALP methods (Spearman’s correlation index 0.925, $p < 0.05$), although the ELISA technique was more sensitive than ALP (Ortiz-Zarragoitia, 2005). As far as we know, specific antibodies for Vtg-like proteins are not available in marine mussels *M. edulis* and *M. galloprovincialis*. Thus, the ALP method could have potential as a simple cost-effective biomarker of endocrine disruption in mussels and other widely used molluscan sentinel species. Seasonality studies in different bivalve molluscs have shown that ALP levels follow the same trend of the gametogenic cycle in females (Blaise et al., 1999, 2002; Ortiz-Zarragoitia, 2005). ALP levels in female *M. galloprovincialis* increased during active gametogenesis reaching maximum levels at gonad maturation.

Synthesis of Vtg-like proteins in bivalve molluscs occurs in gonads (Li et al., 1998; Matsumoto et al., 2003) and its regulation is not well understood even if estrogenic steroids seem to play an important role (Matsumoto et al., 2003; Osada et al., 2003, 2004). Injection of soft shell clams (*Mya arenaria*) and freshwater mussel *Elliptio complanata* with estradiol elevated Vtg-like protein levels measured as ALP levels (Blaise et al., 1999; Gagné et al., 2001a,b, 2002b, 2005). Furthermore, *E. complanata* injected with estradiol showed increased expression of Vtg coding gene and ALP levels in gonads (Gagné et al., 2005). Pacific oyster (*C. gigas*) injected with estradiol also showed high vitellin levels in gonads (Li et al., 1998). These results suggest that estradiol is an important hormone in the regulation of vitellogenesis in bivalve molluscs. However, Riffeser and Hock (2002) did not observe any change in ALP levels of blue mussels (*M. edulis*) and freshwater mussel *Anodonta cygnea* exposed to and injected with estradiol. Therefore, more detailed studies are needed to elucidate the function of estrogenic hormones in bivalve reproduction, gonad development and vitellogenesis. The possible role of other steroid hormones has to be also considered.

Injection of the androgenic hormone testosterone provoked elevated Vtg-like protein levels in *E. complanata* (Gagné et al., 2001a).

In comparison with the extensive literature about effects of EDs on Vtg levels in fish, studies in bivalve molluscs are scarce. Only effects of few potential xenoestrogens, such as alkylphenols, polybrominated compounds, phthalates and bisphenol A, on Vtg-like protein levels have been evaluated in molluscs. Female soft shell clams injected with nonylphenol and pentachlorophenol showed elevated ALP levels (Blaise et al., 1999; Gagné et al., 2001b, 2002b). Exposure of Manila clam (*Tapes philippinarum*) to nonylphenol provoked elevated Vtg-like protein levels both in females and males, with estrogenic effects more evident in male clams (Matozzo and Marin, 2005) and endocrine disrupting effects were also noted in zebra mussel (*Dreissena polymorpha*) (Quinn et al., 2006). Accordingly, exposure of blue mussels to a combination of North Sea oil, alkylphenols and polycyclic aromatic hydrocarbons (PAHs) simulating produced water, increased significantly Vtg-like protein levels in male mussels but not in female mussels (Ortiz-Zarragoitia and Cajaraville, 2005b). In the same experiment, female mussels showed a high percentage of atretic oocytes. In the marine prosobranch *Nucella lapillus* exposed to octylphenol, enlargement of accessory pallial sex glands and massive stimulation of oocyte production in females and reduced length of penis and prostate gland in males were described (Oehlmann et al., 2000). Similarly, alterations in the accessory pallial sex glands of female *N. lapillus* and in the length of penis and prostate gland of males were described after exposure to bisphenol A (Oehlmann et al., 2000). Exposure of blue mussels to bisphenol A, tetrabromodiphenyl ether congener 47 (TBDE-47) and diallyl phthalate did not cause changes in Vtg-like protein contents both in male and females but gamete resorption occurred upon exposure to bisphenol A (Ortiz-Zarragoitia and Cajaraville, 2005b). The lack of changes in Vtg-like proteins in mussels exposed to these potential xenoestrogens could be attributed to the relatively low doses used or, most probably, to the fact that exposure took place when gamete maturation was complete.

Estrogenic effects have also been described in bivalve wild populations. Male mussel *M. galloprovincialis* from Venice Lagoon, an area with high urban and industrial contamination, showed high Vtg-like protein levels (Pampanin et al., 2005). Soft shell clams inhabiting downstream of a domestic sewage effluent showed elevated Vtg-like proteins in gonads (Blaise et al., 2002; Gagné et al., 2002b). Estrogenicity of sewage and domestic effluents in bivalve molluscs were confirmed after transplant experiments (Blaise et al., 2003; Gagné et al., 2001b, 2002a; Quinn et al., 2004) and in vitro experiments (Gagné et al., 1999, 2001b). These effects could be due to the presence of hormone mimicking compounds (Gagné et al., 2001a) or modulators of neuropeptides such as serotonin and dopamine (Gagné and Blaise, 2003; Gagné et al., 2004). Furthermore, changes in biochemical composition of Vtg-like proteins have been described in bivalve molluscs from polluted environments (Gagné et al., 2002b) indicating the importance of characterizing Vtg-like proteins in bivalves.

Anti-estrogenic effects have been reported in soft shell clams inhabiting PAH contaminated sites, which showed low Vtg-like protein levels (Gagné et al., 2002b). Similarly, female blue

mussels exposed to North Sea oil showed significantly lower Vtg-like protein levels and gamete development than controls (Ortiz-Zarragoitia and Cajaraville, 2005b). These females also showed a high percentage of atretic oocytes in comparison with control females, in agreement with earlier studies with mussels exposed to diesel oil and PAH derivatives (Lowe and Pipe, 1987; Lowe, 1988). No effects were found in male mussels. These results agree with the idea of a possible anti-estrogenic effect of PAHs described in other aquatic organisms such as fish (Johnson et al., 1997; Gagné et al., 1999; WHO/IPCS, 2002; Matthiessen, 2003). The anti-estrogenic effect of PAHs in fish appears to be mediated by activation of the Ah receptor (Navas and Segner, 2000). Mussels *M. galloprovincialis* exposed to three different oils showed accelerated spawning at high doses, but at low and medium doses retarded gonad development and severe haemocytic infiltration in the gonads were observed in both sexes (Cajaraville et al., 1992). Soft shell clams collected from a harbour polluted by PAHs and heavy metals also showed delayed gametogenesis (Gauthier-Clerc et al., 2002).

In summary, evidences are slowly growing which indicate that gamete development and vitellogenesis of marine bivalve molluscs are targets of EDs. Nevertheless, further studies are needed before changes in Vtg-like proteins measured by ALP could be used as a biomarker of endocrine disruption in bivalve molluscs. First, a great research effort should be devoted to gain more knowledge on the functioning of molluscan endocrine system and its role in controlling reproduction. Second, additional laboratory studies are needed to test potential EDs at a range of environmentally realistic concentrations. Most importantly, field studies should be conducted to determine basal levels of Vtg-like proteins in indigenous male and female bivalve molluscs and their seasonal variation. Measurements of Vtg-like proteins should be combined with histological examination of gonad (mantle) sections in order to monitor possible alterations in gamete development. Finally, innovative proteomic approaches that could identify specific protein expression signatures (Mi et al., 2005) and genomic approaches, such as the use of DNA arrays (Dondero et al., in press), could give important clues for deciphering mechanisms of action of EDs and for developing novel biomarkers of endocrine disruption in bivalve molluscs. Recently, genes encoding Vtg-like proteins have been cloned in *M. edulis* (GenBank AY679116), scallop *Patinopecten yessoensis* (GenBank AB055960, Osada et al., 2004), Pacific oyster *C. gigas* (GenBank AB084783, Matsumoto et al., 2003) and Eastern oyster *Crassostrea virginica* (GenBank CD647526), providing the opportunity of investigating regulation of vitellogenesis and interactions of EDs with this process.

5. Toxicological hazard due to endocrine disruptors in marine top predators

Recent alarm regarding rapid worldwide depletion of predatory fish communities raised serious concern about the ecological effects of industrialized fishing. Myers and Worm (2003) estimate that large predatory fish biomass (including swordfish and tuna) today is only about 10% of pre-industrial levels. In this context, serious concern about Mediterranean

pelagic longlines has been recently expressed by both the public and the scientific community. Pelagic longlines catch a wide range of species in a systematic way and over a vast spatial scale. The Mediterranean swordfish population is particularly affected by this industrialized fishing technique. However, there is another unexplored factor that could drastically interfere with the stability of populations of Mediterranean top predators, including large pelagic fish: the toxicological effects of EDs.

Man-made EDs range across all continents and oceans; some geographic areas, such as the Mediterranean Sea, are potentially more threatened than others. This basin has limited exchange of water with the Atlantic Ocean, and is surrounded by some of the most heavily populated and industrialized countries in the world. Levels of some xenobiotics are therefore much higher here than in other seas and oceans (Aguilar et al., 2002). Mediterranean marine fauna could therefore be a target for EDCs. In this peculiar environment, top predators, such as large pelagic fish and marine mammals, tend to accumulate large quantities of organochlorine contaminants (OCs) and toxic metals (Corsolini et al., 1995; Marsili, 2000). The levels of OCs in a top predator of the Mediterranean, the striped dolphin (*Stenella coeruleoalba*), are 1–2 orders of magnitude higher than in Atlantic and Pacific dolphins of the same species (Marsili, 2000). This suggests the hypothesis that Mediterranean top predator species are potentially “at risk” due to EDs contamination.

Here we summarize the final results of a project in which the potential estrogenic effects of polyhalogenated aromatic hydrocarbons on Mediterranean top predators is investigated using sensitive biomarkers such as vitellogenin (Vtg), zona radiata proteins (Zrp) and CYP1A activities in order to evaluate the toxicological hazard in swordfish (*Xiphias gladius*) and bluefin tuna (*Thunnus thynnus thynnus*). Non-lethal techniques, such as non-lethal biomarkers (BPMO (CYP1A) activities in skin biopsy) are also used in order to carry out hazard assessment of threatened species exposed to EDs, such as cetaceans (*S. coeruleoalba*, *Tursiops truncatus*, *Delphinus delphis* and *Balaenoptera physalus*).

The first warning about toxicological risk to large Mediterranean pelagic fish due to EDs was pinpointed by the results of Fossi et al. (2001) in swordfish and Fossi et al. (2002) in bluefin tuna. Dramatic induction of typically female proteins (Vtg and Zrp) was detected by ELISA and western blot in adult males of the two species. In a 4-year survey on the Mediterranean population of swordfish (Fossi et al., 2004), the potential toxicological effects of some PHAHs (organochlorines) and trace elements (Hg, Cd, Pb) on 192 specimens of swordfish, caught in the spawning seasons from 1999 to 2002 in the Straits of Messina, Sicily (Italy), were investigated using Vtg, Zrp (Goksoyr, 1991), and cytochrome P4501A (CYP1A) activities (Kurelec et al., 1977; Lubet et al., 1985) (EROD, BPMO). These present results confirmed the finding of dramatic induction in adult male swordfish of Vtg and Zrp. It is interesting to see that several Mediterranean male specimens show values of Vtg and Zrp (Fig. 3A–B) that are higher than average male values and/or in the same range as those of reproductive females, which suggests that this species is exposed to xenoestrogen in the Mediterranean Sea. A role of organochlorines (PCBs in liver ranged 128–22847 ppb d.w.) in this induction phenomenon is suggested by the statistically significant correlations between Zrp

levels in plasma and PCB concentrations in muscle (Kendal’s Tau $b=0.312$; $p<0.032$) and Vtg levels in plasma and PCB concentrations in liver (Kendal’s Tau $b=0.618$, $p<0.034$) of male specimens. Organochlorine levels (PCBs in liver) were also correlated with total length of male specimens (Kendal’s Tau $b=0.377$, $p<0.021$). These results confirm that induction of Vtg and Zrp can be used as a diagnostic and prognostic tool for exposure assessment of Mediterranean swordfish stocks exposed to OCs with ED capacity (Fossi et al., 2004). These data, and those recently published by De Metrio et al. (2003) demonstrating a high percentage of intersex in Mediterranean swordfish, sound a warning about potential reproductive alterations in large pelagic fish and suggest the need for continuous monitoring to avoid reductions in their populations.

Surprisingly, if we compare levels of OCs in swordfish with levels found in free-ranging striped dolphins, we find levels 10 to 20 times higher in cetaceans. Four types of organochlorine endocrine disruptors (Adami et al., 1995; Hilscherova et al., 2000; Fossi and Marsili, 2003) are commonly found in Mediterranean cetaceans (Aguilar et al., 2002; Marsili, 2000; Fossi et al., 2003): 1) environmental estrogens, 2) environmental androgens, 3) anti-estrogens and 4) anti-androgens. The relative estrogenic power of

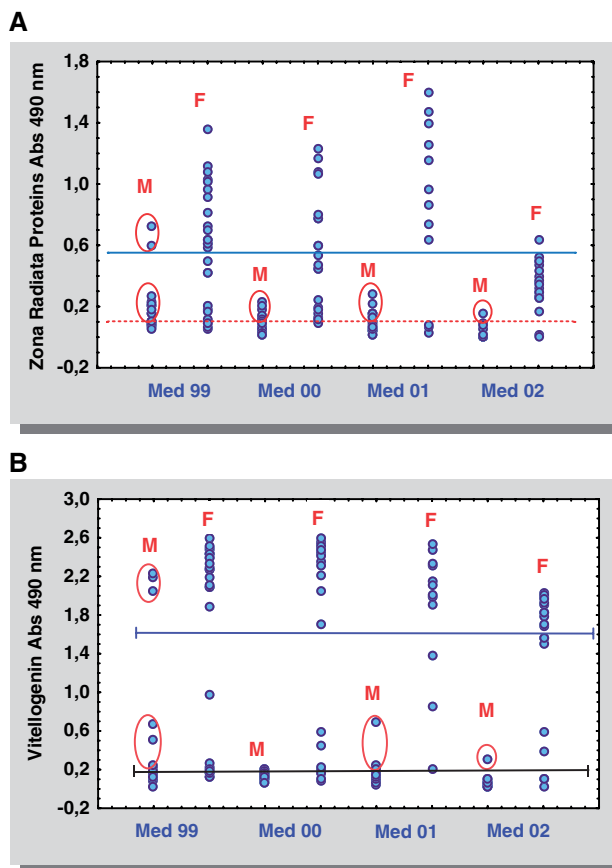


Fig. 3. (A) *Zona radiata* proteins (Zrp) and (B) vitellogenin (Vtg) of male and female swordfish (*Xiphias gladius*) captured in the Mediterranean Sea (Straits of Messina, Sicily, Italy) in summer 1999, 2000, 2001, 2002, during the spawning period. Circles indicate male specimens showing values higher than average male values (line) and/or in the same range as those of reproductive females (Fossi et al., 2004, modified).

Table 2

Pearson produced-moment correlations between OCs with endocrine disruptors capacity and BPMO (CYP1A1) activity in the different cetacean species (Fossi et al., 2003, modified)

	BPMO											
	<i>Balaenoptera physalus</i>			<i>Stenella coeruleoalba</i>			<i>Tursiops truncatus</i>			<i>Delphinus delphis</i>		
	n	r	p	n	r	p	n	R	p	n	r	p
DDTs	27	-0.2875	0.146	7	0.6685	0.101	7	-0.3741	0.408	11	0.8040	0.003*
pp'DDT	27	-0.2700	0.173	7	0.6147	0.142	7	-0.2175	0.639	11	-0.0411	0.905
op'DDT	27	-0.2777	0.161	7	0.8210	0.024*	7	-0.6360	0.125	11	0.7610	0.007*
op'DDE	27	-0.2110	0.291	7	0.1408	0.763	7	0.1434	0.759	11	0.3248	0.330
pp'DDE	27	-0.2746	0.166	7	0.6648	0.103	7	-0.3681	0.417	11	0.8371	0.001*
PCBs	27	-0.2115	0.290	7	0.6766	0.095	7	-0.3784	0.403	11	0.6529	0.029*
95	27	-0.0968	0.631	7	0.0168	0.971	7	-0.2125	0.647	11	0.3506	0.290
99	27	-0.2432	0.222	7	0.6891	0.087	7	0.0539	0.909	11	0.4223	0.196
101	27	-0.1717	0.392	7	-0.0770	0.870	7	-0.2331	0.615	11	-0.0013	0.997
153	27	-0.1642	0.413	7	0.8665	0.012*	7	-0.5592	0.192	11	0.7239	0.012*

these chemicals, identified by in vitro and in vivo screening methods (Safe, 1995, 2000) is rather weak (10^{-3} or less) compared with the reference of 17-estradiol or DES (Miyamoto and Klein, 1998). However, the high levels of organochlorine compounds detected in marine mammals, particularly in pinnipeds and odontocetes, and consequently, the high levels of organochlorines with ED capacity, cannot be ignored.

Some general considerations on potential hazard to these Mediterranean species can be drawn from comparison of the data commonly detected in Mediterranean cetaceans and that of other cetacean species with known reproductive impairment (Fossi and Marsili, 2003). Several examples suggest that exposure to OC insecticides and PCBs has affected endocrine function and reproduction in marine mammals. Here, it is worth noting that levels of PCBs found in Mediterranean free ranging odontocetes (Fossi et al., 2003) are similar to those detected in the population of beluga whales of the St. Lawrence estuary where a hermaphrodite specimen was detected (PCBs mean value=78900 ng/g lipid mass (l.w.)) (Muir et al., 1996); levels of PCBs detected in Mediterranean free ranging fin whales in the same period (mean value=7331 ng/g l.w.) (Fossi et al., 2003) are approximately 10 times higher than those found in the population of bowhead whales (*Balaena mysticetus*) where pseudohermaphroditism and other reproductive dysfunctions have been detected (PCBs mean value=610 ng/g l.w.) (Tarpley et al., 1995). This observation suggests the potential hazard that these species are exposed to in the Mediterranean Sea.

The hypothesis that some Mediterranean cetaceans (*S. coeruleoalba*, *D. delphis*, *T. truncatus* and *B. physalus*) are “potentially at risk” due to organochlorines was investigated using “diagnostic” non-lethal tools. We used benzo(a)pyrene monooxygenase (CYP1A1) activity in skin biopsies (Fossi et al., 1992) as a potential indicator of exposure to organochlorines, with special reference to the compounds with endocrine disrupting capacity.

Subcutaneous tissues (skin and blubber) were obtained from *S. coeruleoalba*, *T. truncatus*, *D. delphis* and *B. physalus* from the western Ligurian Sea, between Corsica and the French–Italian coast, and from the Ionic Sea using biopsy darts launched with a crossbow (Fossi et al., 2003). Organochlorine concentrations (HCB, DDTs and PCBs) and BPMO activities, confirmed lite-

ature data and results obtained in our lab before 1994 (Fossi et al., 1992; Marsili, 2000), indicated that marked differences in levels of all contaminants exist between fin whales and odontocete species. The same was found for BPMO activity induction in which some odontocete species, such as the striped dolphin, having CYP1A1 activities four times higher than the mysticetes, binned with levels of OCs one order of magnitude higher in odontocetes (Fossi et al., 2003). The difference in organochlorine bioaccumulation and consequently in BPMO induction between the two groups is obviously related to their different positions in the marine food chain with odontocetes as terminal consumers and fin whales as macroplanktophages.

There was a linear correlation between OCs known as endocrine disruptors and CYP1A1 (BPMO) activity (Pearson test) in striped dolphins and common dolphins (Tab. 2) (Fossi et al., 2003) suggesting the exposure of these species to a potential hazard of OC-EDs. Gender differences in BPMO induction were also investigated. In striped dolphins a linear correlation was found between op'DDT/BPMO and PCB153/BPMO. In the common dolphin there were identified five linear correlations with the BPMO activity: DDTs, pp'DDE, op'DDT, PCBs and PCB153. The main result in this species was non-induction of BPMO in females with increasing levels of contaminants (Fossi et al., 2003).

These results suggest that CYP1A1 (BPMO) induction in skin biopsy may be an early sign of exposure to EDCs such as OCs and a potential alert for transgenerational effects. It is therefore a powerful “prognostic” indicator of the health of cetacean populations (Fossi and Marsili, 2003) (Table 2).

6. Conclusions

Studies on endocrine disruption in marine invertebrates are important because invertebrates represent more than 95% of the known species in the animal kingdom and large groups are of ecological relevance in the marine ecosystem. Although examples of endocrine disruption in marine invertebrates are known, the best documented of which is TBT-induced imposex in gastropods, the limited evidence for the action of EDs in invertebrates is partially due to the fact that their endocrine system is poorly understood compared to that of vertebrates,

with large diversities among groups and some of them being unique to specific phyla; in particular the presence, role and mechanisms of action of different hormones is largely unknown compared to those of vertebrates. The introduction of invertebrates in toxicity testing of EDs, although useful, consists of studies on growth and development that are based on putative mechanisms of action of both natural hormones and EDs similar to those of vertebrates, but whose existence and role has not yet been demonstrated in most invertebrate species. Therefore, in invertebrates, for most EDs a clear cause–effect relationship between exposure and specific responses is far from being established, this making the mechanistic approach utilized for vertebrate systems unfeasible. On the one hand, this aspect has been addressed in the present review, underlying the need for a critical use of biomarkers of endocrine disruption in invertebrates, like the induction of Vtg-like proteins, that is currently utilized in analogy with vertebrate systems. On the other hand, studies on steroid synthesis and metabolism and on the possible effects of EDs on functions other than reproduction, such as the immune response, may help identifying specific targets for endocrine disruption in invertebrates.

Similarly, the possible effects of EDs on certain marine vertebrates such as marine top predators represent a serious concern, especially in those areas, like the Mediterranean Sea, characterized by a high impact of contaminants and limited water exchange. The relationship between observed accumulation in large pelagic fish and cetaceans of well established EDs in vertebrates and significant effects on the endocrine system has been only recently investigated. The identification of specific biomarkers of endocrine disruption will clarify the role of ED exposure in determining worldwide depletion of these endangered species.

Acknowledgements

Work in the laboratory of L. Canesi was supported by the Italian Ministry of Scientific and Technological Research (fondi Ateneo quota ex 60%). We would like to thank Dr. C. Ciacci and Dr. M. Betti for their invaluable technical assistance. Work in the laboratory of Miren P. Cajaraville was funded by the EU BEEP project (contract no. EVK3-CT2000-00025) and by the University of the Basque Country through a grant to consolidated research groups. Work in the laboratory of C. Fossi was partially financed by grants from the Italian Ministry for the Environment and ICRAM (Istituto Centrale per la Ricerca Scientifica e Tecnologica Associata al Mare). We would like to thank Dr. S. Casini, Dr. L. Marsili, Dr. A. Ausili and Dr. G. Mori for scientific and technical support in the project activities. We would also like to thank Dr. S. Ancora and Dr. T. Romeo, for technical support in the sampling activities in the Large Pelagic Fish Project and all the researchers of the Thetys Research Institute for technical support in the sampling activities in the Marine Mammals Project.

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