XENOBIOTICS, XENOESTROGENS AND REPRODUCTION DISTURBANCES IN FISH

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Fish maturation and reproduction are complex biological processes that are regulated by endogenous substances (hormones), and synchronized by exogenous factors (photoperiod and temperature), thus ensuring that reproduction occurs at a time of the year optimal for survival of the offspring. The survival of any fish species is ultimately determined by the ability of its members to reproduce successfully in a fluctuating environment and thereby maintain a viable population. Several reports have documented that many compounds introduced into the environment by human activity either deliberately or unintentionally are capable of affecting reproductive processes in fish. Zonagenesis and vitellogenesis (eggshell protein and egg yolk precursor production, respectively) are two estrogen-regulated processes that are integral aspects of fish oogenesis. Several *in vivo* and *in vitro* studies have reported that some xenobiotics (xenoestrogens) possess the ability to mimic natural estrogens and therefore initiate precocious or unscheduled zonagenesis and vitellogenesis. Aspects of these effects and other xenobiotically-induced responses will be discussed here, with special reference to their possible consequences for fish populations.

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INTRODUCTION

Fish (*Pisces*) are the most numerous and diverse of all the vertebrate groups. They dominate the waters of the world through a variety of morphological, physiological, and behavioral adaptations. Their diversity is reflected in the large number of living species. Over 21 700 have been described so far, and the number may eventually increase to around 28 000 (NELSON 1984). These fishes occupy an extraordinary array of habitats, from streams, desert springs, open oceans, deep oceanic trenches, cold mountain lakes, saline coastal embayments, to a list of other aquatic environments (MOYLE & CECH 1988). Because of the fluctuating environmental conditions, fishes have adapted different life-history strategies as a means of solving the problems of successful reproduction (THORPE 1989, 1994). These fishes are also a vital source of proteins and lipids for humans and domestic animals, forming the basis for economically important fisheries and aquaculture.

Our environment, and especially the aquatic environment, has been under focus within the past decades because of the large amount of chemical substances released into it. Thousands of synthetic chemical compounds are currently registered for use in industry and agriculture, and thousands of tons of these are produced annually (for review see, AHLBORG & al. 1995; CALDWELL 1985). In addition, several tons of more unintended byproducts accompany these synthetic compounds. Regardless of the source or original intended use, substantial amounts of these chemicals end up in the aquatic environment due to physico-chemical, hydrologic and atmospheric processes (BARRIE & al. 1992; GUARDANS & GIMENO 1994; AYOTTE & al. 1995; BJERREGAARD 1996).

As a consequence of this, large efforts have recently been devoted to dissecting the mechanisms of action of xenobiotics in wildlife, with the ultimate aim of detecting, controlling and possibly intervening in chemical exposure and its effects on ecosystems and humans. In this context, we ought to be concerned with the health and safety of aquatic species *per se*, as well as a resource for human needs.

A wide range of man-made chemicals used for several industrial and household activities have been shown to disturb normal physiology and endocrinology in living organisms. One important class of these compounds

(the xenoestrogens) is able to mimic the natural hormone estrogen. Among these are synthetic steroids such as those used in the contraceptive pill (Pelissero & al., 1993), some organochlorine pesticides (dichlorodiphenyltrichloroethane, DDTs, hexachlorocyclohexanes, HCHs; Wester & Canton 1986; Donohoe & Curtis 1996; Palmer & Palmer 1995), surfactants and detergents (alkylphenol polyethoxylates, APEs; Soto & al. 1991; Jobling & Sumpter 1993; Jobling & al. 1996; White & al. 1994; Arukwe & al. 1997a & b), plasticizers (phthalates), polychlorinated biphenyls, PCBs (McLachlan 1985), and some natural chemicals such as phytoestrogens and mycoestrogens (Pelissero & al. 1991a & b). In many cases, the estrogenic activity of these chemicals have been discovered accidentally (Soto & al. 1991). Estrogenic responses in fish, like the induction of *zona radiata* proteins (*Zr*p) and vitellogenin (Vtg) by these compounds have recently received great attention (Sumpter & Jobling 1995; Arukwe & al. 1997b) and will be discussed in more detail below.

APEs represent an important class of non-ionic surfactants that are widely used as detergents, emulsifiers, wetting and dispersing agents, and also in plastic products for industrial, agricultural and domestic use (Ahel & al. 1994b). Alkylphenols (APs) are formed by microbial degradation of APEs. Recent studies have identified APs as the most critical metabolites of APEs because of their enhanced resistance toward biodegradation, toxicity, estrogenic effects, and ability to bioaccumulate in aquatic organisms (Ahel & al. 1994a). A complex microbial degradation pattern, characterized by the formation of several metabolic products that are more toxic than the parent compound, has been established for APEs (Naylor & al. 1992; Ekelund & al. 1990).

In this review, we want to discuss the basis of xenoestrogen-induced disturbances in fish reproduction and the possible consequences for fish populations. This will include energetic issues, sexual maturation, zonagenesis and vitellogenesis, and effects of xenoestrogens observed in the laboratory and in field situations. In addition, other xenoestrogen-induced non-vitellogenic responses will also be discussed.

The liver is a central and important organ that plays a significant role in the life of an organism (Hinton & Lauren 1990). These include the production of *Zr*p and Vtg for the ovary (synthetic activities) and biotransformation of gonadal hormones and xenobiotics (metabolic activities). However, there are myriads of other factors than can modulate the effects of xenobiotics in the liver. These are only mentioned briefly, since they are outside the scope of this paper.

ENERGETIC ADAPTATION

Given that an organism can only acquire a limited amount of energy for which several processes compete directly, an increase in the energetic allocation to one process must result in a decrease in energy allocation to others (Ware 1980, 1982; Sibly & Calow 1983), as illustrated in Fig. 1. The concepts of optimal foraging and life-history provides the physiological basis for the fate of food energy ingested by animals. The sexual maturation process is energetically expensive and is reflected in the general finding that female fish mature later than males (Thorpe 1994). It requires generally a greater energy accumulation to develop ovaries and eggs than to develop testes and sperm. In order to meet their standard metabolism (maintenance) and activity costs, fish must transform ingested food into net (usable) energy (Ware 1980).

Fig. 1 shows an illustrated presentation of the fate of surplus energy (i.e. absorbed energy minus energy used for respiration and standard metabolism) in organisms. If a proportion q of surplus energy in food is allocated to growth, then $1 - q$ can be allocated to reproduction (Sibly & Calow 1983). Both growth and reproduction has the optimal aim of maintaining parental and offspring fitness. Comparatively, growth will either stop or gradually diminish with age as increasingly more energy is invested in reproduction (Schaffer 1981; Ware 1982; Stearns 1983; Thorpe 1994). Typically, there is a power allocation trade-off between reproduction and growth, condition and survival, current and future reproduction, quantity and quality of progeny. Since all components of power allocation trade-off will increase fitness, the two components of fitness are age specific fecundity and survival. Interested readers are referred to Roff (1992) and references therein for details on trade-offs.

SEXUAL MATURATION

Gonadal development is regulated through the so-called hypothalamus-pituitary-gonadal-hepatic axis (Fig. 2), because these organs produce substances influencing each other, leading to gonadal development and spawning. In teleosts, the hypothalamus releases gonadotropin releasing hormone (GnRH) which stimulate the release of gonadotropins (GtH I and II) in a dose-dependent manner from the pituitary (Peter 1983; Sherwood 1987; Habibi & Peter 1991). However, the gonadotropic control of oocyte maturation in fishes is best understood in salmonids (Redding & Patino 1993). The primary action of GtH I is to induce estradiol-17β (E_2) synthesis by ovarian follicular cells, which initiates hepatic synthesis of eggshell *Zr*p and Vtg (Mommsen & Walsh 1988; Oppen-Berntsen & al. 1992; Hyllner & al. 1994). GtH I also induces testosterone production by testicular cells

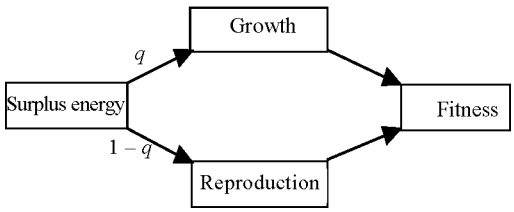

Fig. 1. Fate of surplus energy (i.e. absorbed energy minus energy used for respiration and standard metabolism). If a proportion q of surplus energy in food is allocated to growth, then $1 - q$ can be allocated to reproduction. Both growth and reproduction increase fitness.

(Sherwood 1987; Redding & Patino 1993). Fish oogenesis can be divided into two main events (1) zonagenesis and (2) vitellogenesis. Both processes are stimulated by increasing plasma and cellular E_2 levels.

ZONAGENESIS AND VITELLOGENESIS

Zonagenesis and vitellogenesis are E_2-regulated hepatic synthesis of eggshell (*Zr*p) and egg yolk protein precursor (Vtg), respectively, their secretion and transport in blood to the ovary and their uptake into maturing oocytes in oviparous vertebrates. The liver of oviparous vertebrates has proved to be an excellent model for the studies of molecular mechanisms of steroid hormone action (Wahli 1988; Tata & Smith 1979). *Zr*p (also referred to as chorion, eggshell proteins or major vitelline envelope proteins) are comprised of 3-4 protein monomers with molecular weights in the range of 50-60 kDa (Oppen-Berntsen & al. 1992; Hyllner & al. 1994). Thus, *Zr*p shows clear structural and functional similarities with the *zona pellucida* (ZP); a much thinner, transparent extracellular envelope that lies immediately outside the plasma membrane in the eggs of placental mammals. This envelope is composed of a relatively small number of glycoproteins termed ZP1, ZP2 and ZP3 with a molecular mass of around 200, 120 and 83 kDa respectively (Bleil & Wassarman 1980; Wassarman 1988). Vitellogenin is a bulky (MW; 250-600 kDa) and complex calcium-binding phospholipoglycoproteins (Schneider 1996; Tyler & al. 1991).

A simplified general model of the molecular mechanisms that leads to the production of *Zr*p and Vtg in the hepatocyte is shown in Fig. 3. E_2 produced by the ovarian follicular cells in response to GtH I enters the cell by diffusion. There, the E_2 is retained in target cells by high affinity binding to a specific steroid-receptor protein (such as the E_2-receptor, ER; 1). The hormone-receptor complex binds tightly in the nucleus at estrogen responsive elements (ERE) located upstream of, or within the estrogen-responsive genes in DNA. This results in the activation or enhanced transcription of Vtg genes (2) and

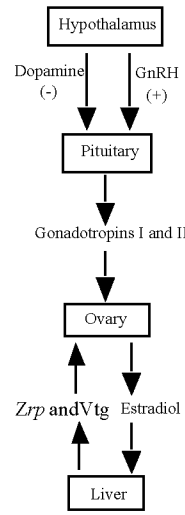

Fig. 2. Simplified schematic diagram of the hypothalamus-pituitary-ovarian-hepatic axis during zonagenesis and vitellogenesis in female teleosts; GnRH = gonadotropin releasing hormone; *Zr*p = *zona radiata* proteins; Vtg = vitellogenin; + and - = indicate stimulatory and inhibitory effects, respectively.

subsequent increase and stabilization of Vtg messenger RNA (mRNA). At present, ERE for *zona radiata* protein genes have not been identified in fish. Given the speculation that different EREs on the DNA may be temporarily masked by associated proteins, thus resulting in sequential or partial induction of various estrogenic responses (Ruh & al. 1988), it is possible that there may be subtle differences in the responsive elements for *Zr*p and Vtg. *Zr*p (3) and Vtg (4) precursors are modified extensively in the rough endoplasmic reticulum (RER) and modified *Zr*-protein (5) and Vtg (6) are secreted into the serum for transport to the ovary. Several changes in hepatic morphology such as proliferation of RER and Golgi apparatus also accompany estrogen stimulation and this accounts for the drastic increases in liversomatic index (LSI; liver % of total bodyweight) usually recorded during oogenesis in fish (Mommsen & Walsh 1988; Tata & Smith 1979).

In the ovary *Zr*p forms the eggshell that prevents polyspermy during fertilization and provides protection against mechanical disturbances for the developing embryo (Hyllner & al. 1991; Oppen-Berntsen 1992, 1994). Vitellogenin is processed by enzymatic cleavage into lipovitellin I and II and phosvitin (Mackay & Lazier 1993) that serve as nutrient reserves for the embryo. During a normal reproduction cycle of female salmonids, *Zr*p can be detected in the plasma several months before Vtg is appearing (Hyllner & al. 1994; Oppen-Berntsen & al. 1994). This is in accordance with their different

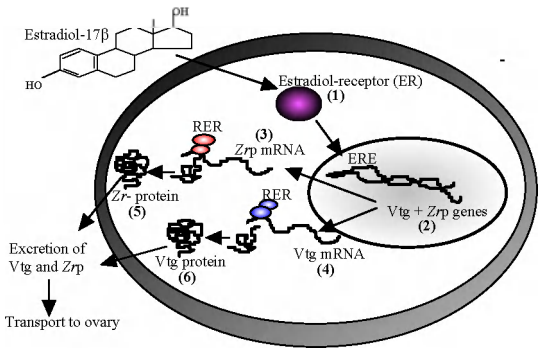

Fig. 3. Simplified schematic representation of estradiol-17β (E_2) stimulated zonagenesis and vitellogenesis (eggshell *zona radiata* proteins and egg yolk protein precursor, vitellogenin, respectively, their secretion and transport in blood to the ovary and their uptake into maturing oocytes) in female teleosts. Number indicate individual steps and are referred to in the text.

function in oogenesis (eggshell or yolk production), and it suggests a differential response to low levels of estradiol.

EFFECTS OF XENOESTROGENS

In classical pharmacology, an agonist is defined as a ligand that can bind to a receptor and 'turn it on' and the potency of an agonist depends on its ability to turn on the receptor (i.e. its affinity). For the ER, E_2 is the natural agonist. On the other hand, an antagonist is a ligand that blocks responses elicited by agonists (Nimrod & Benson 1996). An antagonist can be competitive (i.e. competes for the same binding site as the agonist) or functional (i.e. through a non-receptor mediated mechanism). Xenobiotics (foreign compounds) with the ability to mimic natural estrogens have generally been referred as xenoestrogens (for review, see Colborn & Clement 1992; McLachlan 1985). The general mechanisms by which xenoestrogenic compounds mediate their effects is not well understood, but it is known that they can bind with high affinity to the ER (agonists) and initiate the processes shown in Fig. 3, which is typical of natural estrogens. Some compounds also have the ability to bind to the receptor, but not eliciting estrogenic activities (antiestrogens or antagonists), thereby blocking the binding site of natural estrogens (Safe 1995; Safe & Krishnan 1995; Ahlborg & al. 1995).

During ovarian recrudescence, incorporation of vitellogenin accounts for the major growth of the developing oocytes. A probable indirect measure of altered hepatic vitellogenin synthesis in fish exposed to xenobiotic is reduced or increased gonadosomatic index (GSI). A more direct quantification of these alterations can be obtained from plasma, hepatic and ovarian vitellogenin concentrations (Kime 1995).

LABORATORY STUDIES

Laboratory studies have been conducted to evaluate the impact of fish exposure to toxicants on ovarian development. Several effects have been observed and these includes inhibition of oocyte development and maturation, increased folicular atresia of both yolked and previtellogenic oocytes, abnormal yolk deposition and formation within oocytes, and abnormal egg maturation and production (for reviews, see Lam 1983; Susani 1986; Kime 1995). In developing a bioassay for estrogenic effects, Vtg induction in males and juveniles of oviparous vertebrates has been reported as a sensitive biomarker for estrogenicity (Sumpter & Jobling 1995; Palmer & Palmer 1995), and antibodies against fish Vtg has been developed for this purpose (Heppell & al. 1995; Nilsen & al. 1998). Recently, we have shown that *Zr*p is more sensitive to xenoestrogen exposure than Vtg, and as such provides an alternative means for monitoring environmental estrogens (Arukwe & al. 1997b).

In a three-month exposure study using medaka (*Oryzias latipes*) and β-HCH, Wester & Canton (1986) observed the development of testis-ova in males and induced vitellogenesis in either sex, demonstrating estrogenic effects of this compound. A similar response was observed when medaka was exposed to 4-nonylphenol (NP) in a more recent study (Gray & Metcalfe 1997).

In a two week *in vivo* study using juvenile Atlantic salmon (*Salmo salar*) and different doses of NP, we saw that NP treatment significantly elevated plasma levels of *Zr*p and Vtg (FIG. 4), with the former showing more sensitivity to the xenoestrogen compound (Arukwe & al. 1997b). Higher sensitivity of *Zr*p when compared with Vtg evaluated with indirect ELISA have also been observed in our laboratory with juvenile Atlantic salmon treated with different doses of an oil refinery treatment plant effluent (Arukwe & al. 1997b) and with E_2 (Celius & Walther 1998). In both these studies, induced *Zr*p levels were apparent at lower E_2 doses, while Vtg was only induced at higher E_2 doses, thus showing differential induction of both proteins as was observed using NP (Arukwe & al. 1997b). Induction of *Zr*p by γ-HCH and other HCH-isomers has also been observed in our laboratory (Olsen & al. 1994).

Furthermore, several *in vivo* studies have also reported Vtg induction by xenobiotic estrogens in fish, these include Jobling & al. (1996) using rainbow trout (*Oncorhychus mykiss*) and alkylphenolic chemicals; Donohoe & Curtis (1996) using juvenile rainbow trout, *o,p'*-DDT and *o,p'*-DDE; Schwaiger & al. (1997) using rainbow trout, common carp (*Carpio carpio*) and NP; and Janssen & al. (1997) using flounder (*Platichthys flesus*) and polluted harbour sediment. All studies showed

significant elevations of Vtg at the tested dose of the chemicals. In addition, cytochrome P4501A-inducers such as polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs) have been shown to modulate estradiol-17β-induced Vtg synthesis in both *in vitro* and *in vivo* studies using rainbow trout (Villalobos & al. 1996; Anderson & al. 1996a & b).

Elsewhere, Sumpter & Jobling (1995), Pelissero & al. (1993), Jobling & Sumpter (1993) have reported the *in vitro* estrogenic potencies (in a dose-dependent manner) of several environmental chemicals, including APE metabolites. Using Vtg as a marker of estrogenic potency, these authors showed that NP, octylphenol (OP), *o,p'*-DDT, Aroclor 1221, bisphenol-A and all five chemicals combined (in order of increasing potency at 1 µM), significantly induced Vtg synthesis in cultured hepatocytes of rainbow trout.

Both *in vitro* and *in vivo* studies have been used to study zonagenesis and vitellogenesis in fish. Given that *in vitro* systems lack the complex metabolic processes that are typical of *in vivo* systems, the former system should be used as a supplement to the latter system. In this respect, behavioral and growth parameters deserve more attention.

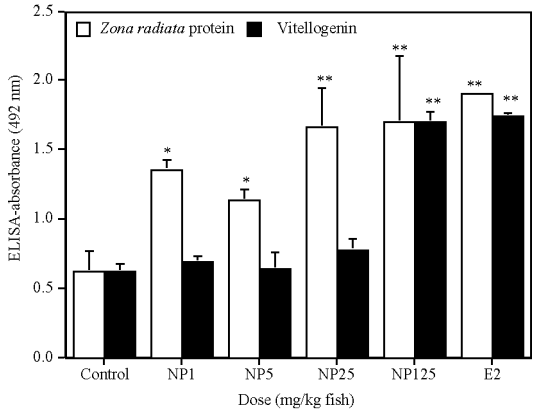

Fig. 4. Immunochemical analysis using indirect ELISA with homologous antisera against Atlantic salmon *zona radiata* proteins (*Zrp*) and vitellogenin (Vtg) from control juvenile salmon or after 2-week exposure to 4-nonylphenol (NP; single intraperitoneal (*ip*) injection at 1, 5, 25 and 125 mg/kg body weight), and estradiol-17β (E_2; positive control). Data are given as mean ELISA absorbance values (492 nm) ± SD (n=6 per treatment group). Data were log-transformed before analysis using Dunnett's tests for comparison with control group. *p<0.001; **p<0.0001. Reproduced with permission from Arukwe & al. (1997b).

FIELD STUDIES

In the literature, there are several reports of xenobiotic-induced reproductive disturbances in aquatic organisms, including fish, living in polluted environments (for reviews, see Colborn & Clement 1992; McLachlan 1980; 1985; Guillette & al. 1995a). Table 1 shows a list of selected field study examples, including those mentioned in the text, of pollutant-induced reproduction disturbances in fish. See Kime (1995) for an extensive overview of other effects and in addition, laboratory study examples.

Several methods and parameters have been used for assessing reproductive success of feral fish species. These includes reduced viable hatch in the Baltic flounder (*Platichthys flesus*) and Baltic herring (*Clupea harengus*) in correlation with elevated PCB concentrations in the eggs (von Westernhagen & al. 1981; Hansen & al. 1985), high egg mortality of Lake Geneva charr (*Salvelinus alpinus*) in correlation with elevated PCB and DDT in charr eggs (Monod 1985), reduced fertilization success and viable hatch in female starry flounder (*Platichthys stellatus*) from contaminated areas of San Francisco Bay (Spies & Rice 1988).

In studies conducted by Dethlefsen, Cameron and coworkers between 1984 and 1995 on developmental effects in eggs of dab (*Limanda limanda*), whiting (*Merlangius merlangus*), cod (*Gadus morhua*), flounder and plaice (*Pleuronectes platessa*) in the southern North Sea, highest incidences of embryo malformations were observed near coastal waters known to receive high pollution loads (Dethlefsen & al. 1996; Cameron & al. 1996; Cameron & von Westernhagen 1997). Developmental defects were evaluated using deviation of life stage morphology from normal morphological differentiation. Common defects recorded by these authors include blister proliferation in early and late embryos, failure to close the blastopore and deformation of the notochord. However, significant correlations were only found for malformations of dab and concentrations of *p,p'*-DDE residues. Further, Cameron & al. (1988) have reported chromosomal and embryo malformations in fish caught in the North Sea. These authors found a positive correlation between anaphase aberrations and the levels of organochlorines such as PCBs, DDT and DDE in gonads and livers of whiting with highest malformation rates from stations near the coast of The Netherlands and off the Rhine River Estuary.

Elsewhere, altered ovarian development in plaice exposed to crude oil as a result of the *Amoco Cadiz* oil spill near Brittany, France have been reported by Stott & al. (1983). Furthermore, the grounding of the tanker *Exxon Valdez* in 1989 that spilled 42 000 000 l of crude oil into the Prince William Sound in Alaska, has resulted in severe effects on the reproductive success of pink salmon (*Oncorhynchus gorbuscha,* Wertheimer & Celewycz 1996) and Pacific herring (*Clupea pallasi*,

Table 1. Selected field study examples of pollutant-induced reproduction disturbances in fish.

Effect	Species	Pollutant	Source	Reference
Vitellogenesis	Rainbow trout	Alkylphenols	Sewage effluent	Purdom & al. 1994; Harries &al. 1996; 1997; Lye & al. 1997
Male secondary sex morphological characters (gonopodium-like structure)	Female mosquitofish	KME[a]	Discharge	Davis & Bortone 1992; Rosa-Molinar & Williams 1984
Higher eggs and larvae mortality, reduced number of eggs and larvae produced.	Sand goby	Sewage sludge	Discharge	Waring & al. 1996
Reduced viable hatch	Baltic flounder and herring	PCB[b]	Discharge	Hansen & al. 1985
Decreased hatch, malformed embryos	Whiting, dab, flounder, plaice	North Sea	Discharge	Dethlefsen 1989;Dethlefsen & al. 1996
High egg mortality	Charr	PCB, DDT[c]	Discharge	Monod 1985
Reduced viable hatch and fertilization	Starry flounder	Organic contaminants	Discharge	Spies & Rice 1988
Chromosomal aberration and embryo malformations	Whiting	PCBs, DDT, DDE[d]	Discharge	Cameron & al. 1988; Cameron & von Westernhagen 1997
Precocious sexual maturation and inhibited gonadal development	English sole	PCBs, PAHs[e]	Urban discharges	Collier & al. 1998
Altered ovarian development	Plaice	crude oil	Oil spill (*Amoco Cadiz*)	Stott & al. 1983
Inhibited gonadal recrudescence and lower plasma estradiol levels	English sole	PCBs, PAHs	Discharge	Johnson & al. 1988; Casillas & al. 1991
Premature hatch, egg and larval mortality, morphological deformities, cytogenetic abnormalities	Pacific herring	Crude oil	Oil spill (*Exxon Valdez*)	Hose & al. 1996; Kocan & al. 1996; Norcross & al. 1996
Thyroid hyperplasia and hypertrophy, reduced fertility, embryo mortality, low plasma steroid levels	Great Lake salmons	Contaminants (PCBs?)	Discharge	Leatherland 1992
Decreased plasma estradiol levels	English sole	sediment extract	Injected	Stein & al. 1991
Reduced plasma sex steroid, sperm motility and GSI	White sucker	KME	Discharge	McMaster & al. 1992
Lowered response to GtH and GnRH	White sucker	KME	Discharge	Van der Kraak & al. 1992
Decreased plasma androgens	Atlantic Salmon, Flounder	Crude petroleum	Oil slick	Truscott & al. 1983
Over-ripe eggs, fry deformity, low fertilization rate	Coho salmon	Contaminants	Discharge	Flett & al. 1991
Oocyte atresia	Air-breathing fish	Textile-mill effluent	Discharge	Murugesan & Haniffa 1992
Retarded ovarian growth	Freshwater murrel	Vegetable oil factory effluent	Discharge	Saxena & Bhatia 1983
Decreased gonadal lipid, increased fatty acids	Air-breathing fish	Vegetable oil factory effluent	Discharge	Kondal & al. 1989

[a]KME = Kraft mill effluent; [b]PCB = Polychlorinated biphenyls; [c]DDT = 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; [d]DDE = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene; [e]PAH = Polycyclic aromatic hydrocarbons

Hose & al. 1996; Kocan & al. 1996; Norcross & al. 1996). Parameters used in evaluating Pacific herring reproductive success include egg and larval mortality, morphological deformities, cytogenetic abnormalities and premature hatch. Significant correlations were found between these effects and crude oil exposure. Nevertheless, there have also been reports of no clear effects on fish reproductive activity, of exposure to contaminants from petroleum production sites in the Gulf of Mexico (Stott & al. 1980, 1981).

In 1994, Purdom and coworkers (Purdom & al. 1994) showed that sewage effluent contain estrogenic substances that induce Vtg synthesis in male trout and this response was attributed to alkylphenolic compounds. Since then, several studies have been performed in the United Kingdom (UK) freshwater and marine environments with the aim of identifying rivers and estuaries where these compounds occur (Sumpter 1995; Harries & al. 1996, 1997; Jobling & Sumpter 1993; White & al. 1994; Lye & al. 1997). A unique feature of all UK river and estuarine systems where estrogenicity occur is that they are recipients of sewage treatment work (STW) effluents. In Norway, STW effluents have been shown to be estrogenic to rainbow trout (Elsrud Schou & al. 1996) in a similar manner. In addition, we have shown that effluents from an oil refinery treatment plant (ORTP) was estrogenic (using *Zr*p and Vtg as markers) to juvenile salmon in a dose-dependent manner (Arukwe & al. 1997b), again with *Zr*p as the more sensitive biomarker.

Johnson & al. (1988) and Casillas & al. (1991) have reported the effects on ovarian development in English sole (*Parophrys vetulus*) from Puget Sound, Washington (USA). One significant finding of these authors was that female English sole from sites heavily contaminated with polychlorinated biphenyls (PCBs) and PAHs had lower plasma estradiol levels and were significantly less likely to undergo gonadal recrudescence than females from the less contaminated sites. Recently, Collier & al. (1998) have also reported precocious juvenile sexual maturation and inhibited gonadal development in female flatfish from the Hylebos Waterway, in central Puget Sound known to be severely contaminated by variety of organic and inorganic contaminants.

In another study, epizootics of thyroid hyperplasia and hypertrophy that affected 100 % of the pink (*Oncorhynchus gorbuscha*), coho (*O. kisutch*) and chinook (*O. tshawytscha*) salmon populations taken from the Great Lakes (North America), in addition to reduced fertility, high prevalence of embryo mortality and low plasma steroid hormone levels, when compared to Pacific Northwest populations, were reported by Leatherland (1992) and Drongowski & al. (1975). Regardless of the method used, it is evident that there are effects on the reproductive abilities of the feral fish species mentioned above, and more of not yet studied ones.

Masculinization responses involving the development of male secondary sex morphological characters (such as modified anal fin into a gonopodium-like structure) was observed in female mosquitofish (*Gambusia affinis*, Poecilliidae) sampled from streams receiving kraft mill effluent (KME, Davis & Bortone (1992); Bortone et al. (1989); Rosa-Molinar & Williams (1984); Howell & al. (1980). These effects were irreversible and often concomitant with male fish behavior, including mating attempts, and have been associated with androgenic properties of compounds in the KME.

Another important aspect of reproduction disturbances in marine organisms is the occurrence of the imposex phenomenon, which is the development of male sexual characteristics (such as penis and vas deferens) in female neogastropod molluscs. The imposex phenomenon, which was first described for dogwhelk *Nucella lapillus* L., an estuarine snail (Babler 1970), is caused by pollution of the marine environment by organotin compounds, such as tributyltin (TBT), which have been used in antifouling paints for ships, boats and fishing nets (Din & Ahamad 1995; Horiguchi & al. 1994; Bryan & al. 1986; 1987; Gibbs & Bryan 1986). Imposex was reported for the first time in the open ocean in female whelks (*Buccinum undatum* L.) from the North Sea by ten Hallers-Tjabbes & al. (1994). However, the use of TBT as antifouling paint have been banned for small boats and fishing nets in most countries.

Furthermore, in Lake Apopka (Florida), Guillette and coworkers (Guillette & al. 1994; 1995b) have reported impaired gonadal steroidogenesis (E_2 and testosterone; T) and abnormal gonadal morphology in juvenile alligators inhabiting the lake. When compared with alligators from control sites, Lake Apopka male and female alligators synthesized significantly higher testis and ovarian E_2 levels, respectively, while normal T levels were displayed. These authors attributed the effects to the contaminants and nutrients in the lake that are derived from the extensive agricultural activities around the lake, a sewage treatment facility associated with the Winter City Garden, Florida, and a major pesticide (dicofol; kelthane) spill from the Tower Chemical Company in 1980 (Clark 1990).

ESTROGENIC POTENCIES

All the chemicals with estrogen activity discovered to date are comparatively weak estrogens. Their potencies vary, with many orders of magnitude (3-4) less than estradiol-17β. However, it could be argued that their relative potencies depend on the assay system used to assess them, but it is interesting to note that they appear to possess the full activity and interact with the ER in exactly the same manner as the natural estrogens. It should be

emphasized that more than one estrogenic assay should be employed in determining organismal response to xenoestrogens (KORACH & MCLACHLAN 1995). This is because the sequence of estrogenic responses do not necessarily have the same time curve or the same strength of response (ARUKWE & al. 1997a & b). The utilization of *in vivo* studies is critical when compounds have the potential of enhanced estrogenicity only after metabolic activation.

Concentrations of estrogenic chemicals in fish will depend on a number of factors, such as bioavailability, bioconcentration/bioaccumulation, and biotransformation. Most of the known estrogenic chemicals are lipophilic and hydrophobic and therefore have a strong potential to accumulate in aquatic biota. Therefore, determining environmental exposures is very difficult and might not be particularly meaningful. Few attempts have been made to measure the concentrations of alkylphenolic compounds in organisms. EKELUND & al. (1990) have reported bioconcentration factors (BCFs) between 13 and 3400 in fish. One consequence of bioaccumulation is that chemicals that are not estrogenic or weakly estrogenic *in vitro* might be active *in vivo* at considerably lower concentrations, given sufficient exposure time. In addition, they may be physiologically inactive while stored in fat (adipose) tissues, but when this fat is mobilized such as during sexual maturation, the compounds may be released to give toxicity elsewhere or metabolized into other compounds that might be more toxic than the parent compound. Furthermore, alkylphenolic compounds with long ethoxylate chains are more water soluble, and will not be expected to bioconcentrate to any significant level. For example, AHEL & al. (1994b) have reported concentrations of 0.1-0.2 mg NP/kg in tissue samples taken from animals in a river with low NP contamination (3.9 µg/l).

NON-VITELLOGENIC RESPONSES

The liver cell constitutes the main site for protein synthesis (e.g. *Zr*p and Vtg) and metabolic conversion of foreign compounds (xenobiotics) and endogenous substances such as steroid hormones (GOKSØYR & FÖRLIN 1992; ZIMNIAK & WAXMAN 1993). Metabolic conversion or biotransformation of xenobiotics and steroids in the liver is mediated by specific enzymes belonging to the cytochrome P450 (CYP) superfamily (NELSON & al. 1996; LEWIS 1996; ORTIZ DE MONTELLANO 1995) and to different transferase enzyme families. Isoforms belonging to the CYP1A subfamily have been extensively studied and identified as the most important subfamily in the metabolic activation of chemical carcinogens (GOKSØYR 1995; IOANNIDES 1990), while the CYP3A subfamily (and CYP2K in fish) have been linked to the metabolism of steroids (ZIMNIAK & WAXMAN 1993; CELANDER & al. 1996). Extensive research have established that PAHs, HAHs such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds are able to induce the CYP1A genes through the aryl hydrocarbon (Ah) receptor (OKEY & al. 1994; SWANSON & BRADFIELD 1993; Hahn & CHANDRAN 1996; SCHMIDT & BRADFIELD 1996).

In mammals and fish, several constitutive cytochrome P450 forms are developmentally regulated and display sex-specific expression (ADESNIK & ATCHISON 1986; FUJII-KURIYAMA & al. 1992). In fish species that exhibit a strong sex difference, the similarities between juveniles and reproductively active males, have led to suggestions that the sex differences are due to a suppression of cytochrome P4501A isozyme expression in reproductively active females (ANDERSSON & FÖRLIN 1992). Recently, it has been shown that the antiestrogenic activity of TCDD and related compounds parallel their Ah-receptor binding and subsequent activation of CYP1A genes (SAFE & KRISHNAN 1995). Thus, it is obvious that a certain level of cross-talk exists between CYP and estrogen-responsive genes. Since the P450 system metabolizes both endogenous and exogenous substances, interactions between xenobiotics and physiological processes are possible. For example, in juvenile salmon treated with NP, elevated progesterone hydroxylase activities and reduced plasma estradiol levels were observed at low NP doses, where no *Zrp* or Vtg responses were recorded (ARUKWE & al. 1997a). In this respect, the relationships between induction of biotransformation enzymes in fish liver and altered steroid metabolism *in vitro* and *in vivo* deserves more attention.

ECOLOGICAL CONSEQUENCES

Reproductive development is a continuous process throughout ontogeny. Consequently, it is susceptible to the effects of xenoestrogens and/or xenobiotics at all stages of the life-cycle, including fertilization, embryonic development, sex differentiation, oogenesis or spermatogenesis, final maturation, ovulation or spermiation and spawning. Thus, the sensitivity to a particular compound will vary depending on the stage of reproductive development (DONALDSON 1990).

Fig. 5 shows the visualization of the sequential order of responses to pollutants stress within a biological system (BAYNE & al. 1985). Effects at higher hierarchical levels are always preceded by changes in 'earlier' biological processes, allowing the development of early warning biomarker signals. It is however difficult to interpret the early biological responses with regard to their significance at the population and ecosystem levels. The difficulty is attributed to, among others, that the changes in fish population and ecosystem diversity may be caused

by myriads of other factors than xenobiotics, like seasonal fluctuations in temperature, salinity, food availability, fishing intensity etc. Understanding the general principles by which chemical substances or foreign compounds (xenobiotics) interfere with fish reproduction is particularly important for meeting the larger objectives in aquatic reproductive toxicology, as it is impossible to empirically determine the biological specificity or how every compound affect the reproductive life-history strategy of every species.

Current risk assessment of the reproductive effects of xenobiotics on aquatic organisms rests explicitly or implicitly on *in vitro* and *in vivo* laboratory studies. However, the ecological consequences of xenobiotic-induced *Zr*p and Vtg synthesis are not known. Presently, there is ample evidence that aquatic organisms living in and bioaccumulating xenobiotic chemicals are adversely affected. In these respects, there is an absolute need for concern, given the several roles played by endogenous estrogens in normal physiology such as during adult sexual maturation and sex differentiation at the early life (egg and embryo) stages (Hunter & Donaldson 1983; Piferrer & Donaldson 1989). Furthermore, there are myriads of other factors than can modulate the effects of xenobiotics, and these might be difficult, if not impossible, to quantify.

Given the energetic cost of reproduction and the long decision time, it seems most likely that xenobiotically-induced hepatic *Zr*p and Vtg synthesis may cause an imbalance in the reproductive strategy of a given fish population. As discussed under energetic adaptations, Thorpe (1994) suggested that during maturation, the internal responses that are synchronized by external signals depend upon some genetically determined performance threshold, and that maturation processes will continue if this performance exceeds a set point at this critical time. Furthermore, maturation has developmental priority over somatic growth, and in salmonids survival after spawning implies a chance dependent balance between stored energy and that spent on reproduction (Policansky 1983). Therefore, xenoestrogen-induced Vtg synthesis outside normal maturation period may result in wasteful use of stored energy resources. The ecological implication of this might be failure in the reproduction of affected individual fish, and in the long-term affecting recruitment in the entire population.

Effects of environmental estrogens have mostly focused on males and juveniles, because of the very low cellular levels of estrogens (Jobling & al. 1996; Arukwe & al. 1997a). One possible deleterious effect is that high Vtg and/or *Zr*p concentrations might cause kidney failure and increased mortality rates as a result of metabolic stress (Herman & Kincaid 1988). Furthermore, although not yet demonstrated, there is a possibility that the reduced testicular growth could reduce fertility (Jobling & al. 1996). Continued synthesis of Vtg diverts available energy resources (lipids, proteins), thereby reducing chances of juvenile survival before start feeding. Loss of calcium from bones and also from the scales during active Vtg synthesis (Carragher & Sumpter 1991) may increase the susceptibility of fish to disease.

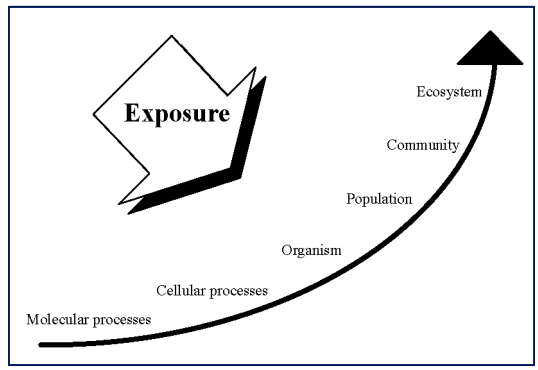

Fig. 5. Visualization of the sequential order of response to pollutant exposure within a biological system. Effects at higher levels are normally preceded by changes in the 'earlier' biological processes, allowing the development of biomarker for early warning signals of effects at 'later' response level. Modified from Bayne & al. (1985).

Xenoestrogen-induced changes in *Zr*p synthesis appear to have a higher potential for ecologically adverse effects than Vtg induction, because critical population parameters such as offspring survival and recruitment may be more directly affected. The argument for this, is that whereas subtle changes in Vtg content would not be of great significance to the survival of the offspring, small changes in *Zr*p synthesis might cause the thickness and mechanical strength of the eggshell to be altered, thus causing a loss in its ability to prevent polyspermy during fertilization and to protect the embryo during development (Arukwe & al. 1997b).

The antiestrogenic and estrogen potentiating effects of typical CYP1A-inducers reported recently by Villalobos & al. (1996) and Anderson & al. (1996a & b) were dependent upon the concentration of estradiol-17β and the CYP1A inducing agent. These responses were mediated through the ER and Ah-receptor, respectively. With regard to other reproductively important ER containing organs in fish, these modulatory effects will have some added significance. For example, ER mRNA has been detected in the hypothalamus and pituitary of rainbow trout (Pakdel & al. 1990). Anglade & al. (1994) have also reported immunolocalization of ER in three brain regions (ventral telencephalon, anterior ventral preoptic region and mediobasal hypothalamus) of rainbow trout. Further, using cultured pituitary cells from

rainbow trout, Xiong & al. (1994) have reported the identification of ERE in the proximal and distal regions of the gonadotropin II (GtH II) gene promotor. Since extrahepatic targets for ER modulating compounds exist, such compounds may have significant and deleterious consequences on neuroendocrine and endocrine regulation of fish reproduction. Such effects might include, but not be limited to, altered gonadotropin secretion, gonadal synthesis of estradiol-17β, and thereby interfering with *Zr*p and Vtg synthesis and gonadal maturation.

The physiological implications of inhibited and/or elevated P450 levels and activities for maturing fish have not been fully established. In several studies, a relationship between elevated P450 activities and disturbed physiological endocrine functions, essential for successful reproduction have also been found (Spies & al. 1985; Sivarajah & al. 1978; Thomas 1990).

Recently, we have demonstrated that a xenoestrogen (4-nonylphenol) that induces *Zr*p and Vtg at higher concentrations, also elevated (at lower doses) and inhibited (at higher doses) different hepatic P450 isoforms that metabolize xenobiotics and steroid hormones (Arukwe & al. 1997a). It is a known phenomenon that E_2 inhibits the expression and activity of CYP1A (Andersson 1989; Pajor & al. 1990; Arukwe & Goksøyr 1997). Inhibition of P450 activities by environmental estrogens might result in reduced ability of the individual fish to metabolize and excrete xenoestrogens. On the other hand, elevated P450 levels might result in increased metabolism and excretion of steroid hormones necessary for active *Zr*p and Vtg synthesis. Although no links between the induction of P450 and impaired reproductive functions have yet been established, it is nevertheless important that the mechanism by which potential P450 inducers may affect sexual development and fertility is elucidated.

RESEARCH NEEDS

An interdisciplinary approach is a prerequisite in achieving an overall view on the problems of xenobiotic-induced reproductive disturbances in fish. At present, the greatest challenge to this problem is the translation of subtle functional deficits within individuals into population-level effects and this will require better field observations and laboratory studies to simulate field exposures (Kavlock & al. 1996). Furthermore, a complete risk assessment of the effects of xenobiotics or xenoestrogens on fish reproduction will require good reproductive toxicity testing, defined as the occurrence of adverse effects on the reproductive system that may result from exposure to agents from exogenous sources (Kimmel & al. 1995). The toxicity may be expressed as alterations to the reproductive organs or the related endocrine system or to progenies (Table 2).

Research needed for detection of xenobiotic-induced reproduction disturbances in fish are: (1) to establish cause and effect relationships, this implies that hypotheses generated from field studies must be tested in the

Table 2. Parental and progeny endpoints of reproductive toxicity testing in fish.

Study	Endpoints	
Parent		
	Male-specific	Female-specific
Bodyweight	Total bodyweight	Total bodyweight
Organ weight	Organosomatic index[1] (LSI and GSI)	LSI, GSI
Visual examination and histopathology	Testis (ova formation), liver, pituitary spermatogenesis, sperm number (counts) and quality (morphology, motility)	Ovarian morphology (atresia), liver, oogenesis (eggshell protein and vitellogenin synthesis and incorporation), number of eggs
Hormone concentrations and profiles	GnRH[2], GtH[3] (I & II), estrogen, steroid hydroxylases (P450 isoforms), testosterone	GnRH, GtH (I & II), testosterone, aromatase activity, steroid hydroxylases (P450 isoforms), estradiol-17β, luteinizing hormone, follicle stimulating hormone etc.
Mating time		
Progeny		
	Egg	Larvae/juvenile
	Mortality, fertilization, morphology, viability, hatchability, development.	Morphology (external, internal), age at first feeding, survival, mortality, development, gender distribution, growth, reproduction.

[1]Organosomatic index (%) = (organ weight/total body weight) x 100; LSI = Liversomatic index; GSI = Gonadosomatic index. [2]GnRH = Gonadotropin releasing hormone; [3]GtH = Gonadotropin.

laboratory and in controlled field studies; (2) development of biomarkers that must address species differences, sexual dimorphic traits, and be life stage specific. These biological markers must be applied in long-term, transgenerational studies to identify markers in progeny that can be measured shortly after exposure and be diagnostic of long-term effects; (3) data collection involving more extensive studies of highly exposed fish populations, in addition to more information on normal population variation, as well as regional and seasonal variations; (4) basic research on developmental biology to address the ontogeny of receptor systems; (5) characterization of interactions of complex chemical mixtures at environmentally relevant doses; (6) development of analytical tools (e.g. molecular probes and immunoassays) and rapid, reliable and inexpensive short-term *in vivo* and *in vitro* screening methods; (7) bioenergetic modelling; these models can incorporate chronic and transient stress effects, multiple effects and interactions, and indirect effects caused by xenobiotics. The necessity for invoking stress effects can be evaluated using healthy animals, thus the influence of a particular stressor on the energy budget can be incorporated to a mechanistic relationship into such a model (RICE 1990); (8) studies on the possible effects of xenobiotics on population heterogeneity through selection pressure. Because of the potential long-term impacts on both individuals and populations, better definition of normal variability in reproductive parameters and more comprehensive temporal data are needed, so that potential trends can be identified more readily and reliably, and hypotheses tested regarding their causation (for review, see KAVLOCK & al. 1996).

CONCLUSIONS

An increasing number of widely used chemicals and their degradation products are found to be estrogenic in animal and human systems. These effects are observed throughout the trophic levels in the aquatic environment, ranging from zooplankton to top predators. Severe impacts can occur at the level of steroidogenesis, biotransformation, gametogenesis, oogenesis and spermatogenesis. The declining semen quality, increased incidence of testicular cancer and cryptorchidism (maldescent of the testis) in man reported in the 1990s from Belgium, Denmark, France and Great Britain have been putatively attributed, at least in part, to maternal exposure to estrogenic or other hormonally active (such as antiestrogens) environmental chemicals during fetal and childhood development (TOPPARI & al. 1996). One of the possible routes of exposure to such chemicals is through the consumption of contaminated (aquatic) food resources.

In fish, convincing evidence of this effect have been obtained from studies at the molecular and cellular levels of biological organisation, in addition to reports on the individual level. In addition, there are number of reports that fish populations are adversely affected by living in and accumulating xenobiotics and xenoestrogens. In this respect, extrapolation of the effects seen at the molecular and cellular levels, to the population and community levels of biological organization is of ultimate importance. Although xenoestrogen-induced zonagenesis and vitellogenesis appear to possess a potential for ecologically adverse effects, as does inhibition and elevation of biotransformation enzymes, studies are needed of critical population parameters such as offspring survival and recruitment. Life-history strategy of fish species might be used as a basis in explaining these effects. However, a definite evidence for this hypothesis may be obtained through long-term exposure studies of fish to low levels of xenobiotics and xenoestrogens. Such studies and other studies of xenoestrogen-associated reproductive effects are underway in our laboratory.

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