VITELLOGENIN AS A BIOMARKER FOR ENVIRONMENTAL ESTROGENS

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ABSTRACT

Much interest has recently focused on the discharge of 'xenoestrogens' to the environment. The potential for these chemicals to disrupt normal hormonal function in wildlife has led to the development of techniques to monitor the effects of these discharges particularly in aquatic environments. Increased concentrations of the egg yolk precursor protein, vitellogenin (Vtg), in the plasma of fish and other vertebrates has been identified as a potentially useful biomarker of exposure to compounds with estrogen-like properties. In this review we discuss the mechanisms underlying the production of Vtg and methods for the analysis of this protein. We also review laboratory and field studies that have measured production of Vtg in response to chemical or effluent exposure. These studies indicate that the production of Vtg is species dependent and may be a useful biomarker of exposure to estrogenic chemicals. However, a greater understanding of the mechanism of action of these chemicals is required to better explain the observed responses. Studies relating more ecologically relevant endpoints such as reproduction to observed levels of Vtg induction have to date been limited to single chemical laboratory exposures.

Key words: vitellogenin, biomarker, fish, mechanism, estrogen.

INTRODUCTION

Since the late 1980s there has been a dramatic increase in scientific regulatory and public awareness of the presence of endocrine disrupting chemicals (EDCs) in the environment. Much of this interest arose from the publication of the book "Our Stolen Future" (Colborn et al. 1996), which highlighted some of the possible effects of exposure to EDCs. This increase in awareness has lead to numerous investigations of the effects of these "endocrine disruptors" on wildlife (Ankley et al. 1998) and humans (Kavlock et al. 1996). To date much of the focus of these studies has remained at the level of the steroid "sex hormones", particularly estrogen, and the term "xenoestrogen" has been applied to these compounds (White et al. 1994; Guillette 1995; Sumpter and Jobling 1995; Harries et al. 1996, 1997; Nimrod and Benson 1996). However, the US-EPA's Endocrine Disruptor Screening and Testing Committee (EDSTAC) also considered potential effects on androgen receptor and thyroid receptor-mediated effects (US-EPA 1998). The situation concerning EDCs in the USA has been further complicated by the reauthorisation of the Clean Water Act and by legislation in the Food Safety Act (Safe Drinking Water Act Amendments 95-1995 - Bill number S.1316; Food Quality Protection Act of 1996 - Bill number PL 104-170), which mandate the screening of large numbers of chemicals for endocrine-disrupting effects.

Several environmental contaminants have been identified as xenoestrogens because of their ability to induce responses similar to those caused by 17β-estradiol (E2). These compounds include significant concentrations of the therapeutic natural estrogens E2 and estrone, as well as the synthetic estrogen, ethinylestradiol (EE2) (Snyder et al. 1999). In addition to these pharmaceuticals, which are relatively strong estrogen agonists, several less potent synthetic chemicals have been found in waste waters. Environmental pollutants that exhibit "estrogenic" action include some organochlorine insecticides such
as chlordane (Eroschenko 1981), o,p'-DDT and its derivatives or metabolites (Fry and Toone 1981), and some industrial chemicals like phthalate plasticizers (Jobling et al. 1995), nonylphenol (NP) (Soto et al. 1991; Jobling and Sumpter 1993; White et al. 1994), octylphenol (OP) (Arnold et al. 1996), bisphenol A (Feldman et al. 1993), and polychlorinated biphenyls (PCBs) and their derivatives (Korach et al. 1988; Purdom et al. 1993). Although these compounds differ structurally from one another as well as from endogenous steroid estrogens, it has been suggested that some of them mimic estrogenic actions in the reproductive tracts of laboratory animals by binding with estrogen receptors (ER) (Eroschenko 1981; Gray et al. 1997). While based on their affinity for the ER and their relative potency in in vitro expression assays, these compounds are considerably less potent than E2, some can occur in surface waters at sufficiently great concentrations to be of potential concern (Vileneuve et al. 1998). One such class of compounds are the alkylphenol ethoxylate (APEO) non-ionic surfactants and their intermediate degradation products, the alkylphenols (APs) particularly NP and OP (Ashfield et al. 1998). These intermediate transformation compounds are weak estrogen agonists and have been proposed to act as estrogen mimics by direct action at the ER (Talmage 1994; White et al. 1994; Routledge and Sumpter 1996; Rudel et al. 1998). Approximately 350 000 tons of APEO are manufactured annually in the USA and Western Europe for use in industrial and household cleaning agents, agricultural chemical formulations, and in emulsion polymerisation processes (Talmage 1994).

As a result of interest in EDCs a large effort has been expended by many organisations to monitor the presence of these chemicals in surface waters and their effects on biota (Miyamoto and Klein 1998). This focus stems from early observations of greatly increased concentrations of the egg yolk precursor protein vitellogenin (Vtg) in the blood of fish exposed to effluents containing EDCs (Sumpter et al. 1990). The increase seen in this protein rapidly gained acceptance as a "biomarker" for exposure to these chemicals, and some studies suggested that elevated levels of this protein could lead to adverse effects in exposed fish.

It was believed that great concentrations of Vtg in serum resulted in kidney malfunction. Vitellogenin is under E2 control but other processes, such as development of the reproductive tract, and behaviour could also be disrupted by compounds interfering with E2 actions (Van Der Kraak et al. 1998). Therefore, Vtg was also proposed as a biomarker of adverse effects in exposed fish. In addition, fish living in the vicinity of or caged below waste-water treatment plants (WWTP) have been found to have altered hormone status or gonadal histology (Purdom et al. 1993; Heppell et al. 1995; Folmar et al. 1996; Harries et al. 1996, 1997; Knudsen et al. 1997; Ly et al. 1997; Soimiasuo et al. 1998).

In contrast to the responses discussed above, similar studies in North America have failed to demonstrate consistent increases in serum Vtg in fish exposed to WWTP effluents (Nichols et al. 2000; Folmar et al. 1996). This may relate to several differences in the etiology of exposure as well as differences in WWTP technologies used between these different regions of the world.

These preliminary studies of Vtg have lead to an increased interest in the endocrine regulatory system in fish - providing research and understanding that was previously lacking. In addition, better studies explaining Vtg production have increased. Given this increased knowledge, it seems pertinent to review the use of Vtg as a biomarker response. In this paper we also review methods used for Vtg measurement and discuss situations where Vtg has been used as a biomarker.

**VITELLOGENIN**

Vitellogenin is a phospholipoglycoprotein synthesised in the liver of all oviparous vertebrates. It is released to the blood stream for transportation to the developing eggs. After receptor-mediated uptake, Vtg is cleaved into two daughter yolk proteins phosvitin and vitellin. While the subunit molecular weights for fish Vtgs are in the range 150 to 180 kDa, the native molecular weight of Vtg is species dependent and in fish ranges from 350 to 600 kDa (Specker and Sullivan 1994) due to its occurrence in multimeric forms in the blood. In ripening female fish, Vtg reaches circulating concentrations of 10 to 100 mg/mL. Its high phosphorus content makes it active in Ca++ transport while Vtg lipophilic moieties may be involved in the transport of lipophilic contaminants and hormones (Giesy and Snyder 1998). Production of Vtg is under the control of E2, produced mainly in the developing ovary. Ovarian E2 produced in response to a surge in gonadotrophin results in increased Vtg synthesis by the liver with subsequent transport to the ovary. Production of E2 is regulated by gonadotropin releasing-hormone (GnRH), which is itself regulated by various environmental signals transmitted through the hypothalamic-pituitary-gonadal (HPG) axis. Signals transmitted through the HPG axis result in changes in circulating Vtg concentrations due to season, sex, age (Wallace and Babin 1994), temperature (MacKay and Lazier 1993) and chemical signals such as Ca++ (Ye and Mugiya 1997), Al (Mugiya and Tanahashi 1998), and contaminants such as cyanide (Ruby et al. 1986, 1987). In addition to these external signals, Vtg production is
also modulated by other endogenous hormones such as cortisol (Ding et al. 1994) and androgens (Lazier et al. 1996).

Mechanisms of endocrine disruption
There is a range of possible mechanisms by which EDCs could disrupt the normal endocrine system homeostasis. As well as directly interacting with hormone receptors, xenoestrogens could effect changes by altering biochemical processes that change the pools of available substrates, or by eliminating hormones. Exposure to general stress either from environmental or chemical stressors can result in a "general adaption syndrome", which alters the organism's hormonal status - it follows from this that all stressors are ultimately endocrine disruptors. Finally, stressors that cause adverse effects, lesions or damage to structure or function (in tissues responsible for hormone production, metabolism or as specific targets for hormonal signals) will alter the organism's hormonal status and thus have endocrine-disrupting effects.

The ability of some chemicals to mimic the effects of estrogen has been known for decades (Fry and Toone 1981). In addition, human production and release of a variety of chemicals have both increased in the recent past. Therefore, while exposure to persistent organochlorine chemicals such as p,p-DDT and PCBs is generally decreasing, exposure to industrial chemicals such as NPAFEOS, OP, bisphenol A and phthalates is continuing or increasing. In addition to these anthropogenic chemicals, organisms are exposed to a variety of natural products, including phytoestrogens (eg. flavonoids, genistein, stiosterol) (Safe and Gallo 1998), natural "estrogens" and related pharmaceuticals such as EE2, in complex sewage effluents (Snyder et al. 1999).

Methods for the determination of vitellogenin
Non-specific techniques used to measure Vtg range from the measurement of acid labile phosphorus in the blood since Vtg is a highly phosphorylated protein (Mitchell and Carlisle 1991; Kramer et al. 1998) or assessment of protein species in electrophoretic gels (Janssen et al. 1997; Ding et al. 1994; Heck et al. 1997;Mugiya and Tanahashi 1998) or by HPLC. Acid labile phosphorus is determined by releasing the phosphate attached to the plasma Vtg by acid digestion and then determining the content of inorganic phosphorus in the extract. In simple electrophoresis methods, proteins are separated based on molecular weight, and the relative intensity of the protein band at the molecular weight corresponding to Vtg is assessed either qualitatively or quantitatively by densitometry. These simple non-specific methods are relatively easy and inexpensive as no specialised reagents such as antibodies are required. They are generally less sensitive and specific than immunochemical methods and can also be subject to interference from non-Vtg proteins. These methods are therefore more applicable when the degree of Vtg induction is high and where differences between exposure groups are relatively great. The specificity of the electrophoretic procedures can be improved by transferring the separated proteins to a blotting membrane and performing Western-blotting procedures with an antibody specific for Vtg.

Immuonassay procedures range from simple immunodiffusion or "rocket" immunoassays (MacKay and Lazier 1993), radioimmunoassay (Copeland et al. 1986; Copeland and Thomas 1988) to enzyme-linked immunosorbent assays (ELISA) (Bon et al. 1997; Mosconi et al. 1998; Wahl et al. 1998; Lomax et al. 1998; Korsgaard and Pedersen 1998; Sherry et al. 1999a; Monteverdi and Di Giulio 1999). All of these methods are based on the use of antibodies specific to Vtg and, therefore, are generally not subject to interference from other proteins. However, the specificity of the antibodies sometimes can lead to a high degree of species specificity preventing the use of assays across species, particularly when using monoclonal antibodies. However, some polyclonal antibodies do react to Vtg from a range of species on a limited basis, such as those used by Nichols et al. (2000) that detect Vtg from most cyprinid fishes as well as a number of freshwater and marine species from other genera. The wider specificity range of polyclonal antibodies is also an advantage if the detection of the Vtg daughter proteins is desired. In recent years the ELISA has been the preferred method for immunoassay detection due to its relative simplicity, use of non-radioactive reagents, amenability to automation and good sensitivity, generally in the ng/ml range. In general, the method involves coating a 96-well plate with Vtg to act as a "capture surface". Sample containing Vtg is then added to a Vtg-specific antibody (usually IgG) solution and the mixture is added to the wells of the 96-well plate. The Vtg in the sample binds the antibody preventing its binding to the Vtg on the plate surface. Antibody that does bind to the plate (ie. in excess of Vtg in the sample) is detected with a detection system specific for IgG antibodies. The amount of Vtg in the sample is inversely proportional to the amount of antibody binding to the plate. All immunological methods require specialised facilities and procedures to produce the required antibodies and some specialised equipment to permit quantitation of assay results. However, once antibodies have been produced and methods standardised, the ELISA is relatively simple and inexpensive and kits are now available commercially.
Nucleic acid methods for the measurement of Vtg induction

Changes in Vtg mRNA are generally seen as one of the earliest signals of Vtg induction that can be detected at the subcellular level (Le Guellec et al. 1988). One of the most commonly used methods to quantify mRNAs is Northern blotting (Olsson et al. 1995; Mellanen et al. 1999; Miller et al. 1999). Initially, vitellogenesis was studied in egg-laying species such as chicken (Maeenpaa 1976) and the African clawed toad (Xenopus spp.) (Wahl et al. 1979) and their Vtg gene sequences were among the first to be described. Based on these nucleotide sequences several authors have used Xenoopus or other amphibians as an initial test system to elucidate the mechanisms of steroid-induced Vtg expression (Riegel et al. 1986; Carnevali et al. 1992; Klosa et al. 1999). One of the first teleost Vtg genes to be (partly) sequenced was that of rainbow trout (Onchorhyncus mykiss) (Le Guellec et al. 1988), therefore, most of the Vtg mRNA analyses in fish have been carried out using this species (Mackay and Lazier 1993; Olsson et al. 1995; Mellanen et al. 1999; Perazzolo et al. 1999). As more information on Vtg sequences has become available, Northern blotting has also been used on other species such as tilapia (Oreochromis sp.) (Ding et al. 1990; Lazier et al. 1996).

One of the main advantages of the Northern-blotting methodology is its great specificity. Using a specifically labelled probe, the size and the amount of the desired gene product can be quantified and expressed relative to an internal standard or constitutively expressed gene such as actin. The method can also be used to assess differences in RNA splicing involved in the production of mRNA. One of the major disadvantages of the Northern-blotting approach is that the degree to which toxicants cause alterations in mRNA concentrations is narrow (usually less than 10-fold), which makes it difficult to establish dose responses. These relatively limited alterations are difficult to quantify due to the relatively great amount of variability in the quantitation of mRNA.

Recently the usefulness of Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) to measure Vtg gene expression has been demonstrated for rainbow trout (Lech et al. 1996; Ren et al. 1996a). The main advantage of this technique is its greater sensitivity than Northern blotting. In theory, a single mRNA copy can be amplified and detected from a total pool of mRNAs. Using RT-PCR the existence of precursor Vtg mRNA has been demonstrated in males and females, which is transformed into mature Vtg mRNA following exposure to E2 or NP (Ren et al. 1996b). This suggests that these chemicals might interact with post-transcriptional regulation processes of the Vtg metabolism.

One limitation of the RT-PCR approach is that, although "house keeping genes" such as $\beta$-actin or transcription factors can serve as internal standards, the technique is only a semiquantitative analysis of specific gene transcripts. Exact quantitation of mRNAs through RT-PCR is difficult to standardise and, therefore, any chemical-induced effects are generally expressed relative to the control. This makes it difficult to compare the observed data with other published effects data in the literature. As with the Northern analysis, the RT-PCR shows that Vtg mRNA levels are only moderately affected due to xenoestrogen exposure. However, combining RT-PCR with real-time PCR techniques will allow for precise quantitation of the message, since each individual PCR product is quantified with labelled probes.

**VITELLOGENIN AS A BIOMARKER**

Several different approaches have been used to assess the utility of Vtg as a biomarker to determine the relative potencies of weak estrogen agonists or, for use in environmental monitoring, for exposure to these compounds. While some studies have been conducted in the laboratory using E2 injections or water-borne exposures, the focus of this article is on responses of Vtg to exposure to environment estrogen agonists. Laboratory studies have the advantage of allowing controlled exposure to single chemicals and permit assessment of sensitive reproductive endpoints that would be difficult to measure in field situations (Nichols et al. 2000). While field studies and particularly caging studies provide valuable information on in vivo responses at relevant environmental exposures, difficulties exist in linking observed Vtg responses to ecologically relevant endpoints.

Here we summarise studies that have measured Vtg as a result of chemical exposure (Tables 1, 2 and 3). We have not included a discussion of exposures where E2 was used solely to induce Vtg for purification since they have little or no environmental relevance. However, we have included several studies with E2 that were used to demonstrate modulation of E2-induced Vtg expression by exogenous estrogens or xenoestrogens.

**Laboratory exposures**

Laboratory exposures to chemicals are often limited to exposure to single compounds to ascertain the relative potency of potentially estrogenic compounds. The actual number of these studies is considerable and we cannot comment on each study individually; however, the experiential studies are outlined in Table 1. While these studies are useful in describing the potential effects of exposure, they do not expose the organism to the usual range of other environmental conditions.
<table>
<thead>
<tr>
<th>Species</th>
<th>Chemical</th>
<th>Method</th>
<th>Vtg response</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown trout female</td>
<td>Sex maturation</td>
<td>RIA</td>
<td>Varied</td>
<td>Norberg et al. (1989)</td>
</tr>
<tr>
<td>Catfish</td>
<td>Methoxychlor, BNF, MCX</td>
<td>ELISA</td>
<td>+; = MCX alone</td>
<td>Schlenk et al. (1997)</td>
</tr>
<tr>
<td>Duck</td>
<td>Lindane</td>
<td>ALP</td>
<td>-</td>
<td>Chakravarty et al. (1986)</td>
</tr>
<tr>
<td>Fathead minnow</td>
<td>NP</td>
<td>ELISA</td>
<td>Varied</td>
<td>Nichols et al. (2000)</td>
</tr>
<tr>
<td>Frog <em>Xenopus</em></td>
<td>E2, tamoxifen, 4OH-tamoxifen</td>
<td>Western, Northern mRNA and protein</td>
<td>Riegel et al. (1986)</td>
<td></td>
</tr>
<tr>
<td>Lizard</td>
<td>Captivity</td>
<td>PAGE, Dot Blot</td>
<td>- captivity, + E2</td>
<td>Morales and Sanchez (1996)</td>
</tr>
<tr>
<td><em>Oreochromis aureus</em></td>
<td>Cortisol</td>
<td>Northern, PAGE</td>
<td>+</td>
<td>Ding et al. (1990, 1994)</td>
</tr>
<tr>
<td><em>Oryzias latipes</em></td>
<td>E2</td>
<td>Western</td>
<td></td>
<td>Murata et al. (1994)</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>E2, season</td>
<td>ELISA</td>
<td></td>
<td>Bon et al. (1997)</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>E2, T</td>
<td>Rocket, slot blot</td>
<td>+ but varied with T</td>
<td>Mackay and Lazzier (1993)</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>E2 and Cd</td>
<td>PAGE, Northern Cd inhibits Vtg transcription</td>
<td>Olsson et al. (1995)</td>
<td></td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>Effluents</td>
<td>RIA</td>
<td>+ or =</td>
<td>Knudsen et al. (1997)</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>Cellular develop.</td>
<td>Northern/ in situ + with cell size</td>
<td>Perazzolo et al. (1999)</td>
<td></td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>β-sitosterol</td>
<td>Northern    + β-sitosterol</td>
<td>Mellanen et al. (1999)</td>
<td></td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>E2</td>
<td>Northern analyses time course</td>
<td>Le Guellec et al. (1988)</td>
<td></td>
</tr>
<tr>
<td>Salmon</td>
<td>o,p-DDT, p,p-DDT, E2</td>
<td>Western, ELISA Vtg =</td>
<td>Cellius and Walther (1998a,b)</td>
<td></td>
</tr>
<tr>
<td>Spotted seatrout</td>
<td>E2, reprod. cycle</td>
<td>RIA         + E2; rise at spawn</td>
<td>Copeland and Thomas (1988)</td>
<td></td>
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<tr>
<td>Striped bass</td>
<td>TCB</td>
<td>RIA         =</td>
<td>Monsson et al. (1996)</td>
<td></td>
</tr>
<tr>
<td>Sturgeon</td>
<td>phyto-estrogens</td>
<td>ELISA</td>
<td>Coumestrol strongest</td>
<td>Pelissero et al. (1991)</td>
</tr>
<tr>
<td>Tilapia</td>
<td>17alphaMT</td>
<td>PAGE, Northern + E2; other - analyses</td>
<td>Lazier et al. (1996)</td>
<td></td>
</tr>
<tr>
<td>Turtle</td>
<td>E2, ovariectomydised</td>
<td>Western     +</td>
<td>Selcer and Palmer (1995)</td>
<td></td>
</tr>
<tr>
<td>Zoarcus viviparans</td>
<td>NP</td>
<td>ELISA       + gravid and embryo</td>
<td>Korsgaard and Pederson (1998)</td>
<td></td>
</tr>
<tr>
<td>Zoarcus viviparans</td>
<td>NP, E2</td>
<td>ELISA       +</td>
<td>Christiansen et al. (1998)</td>
<td></td>
</tr>
</tbody>
</table>

*Methods described in the text; + induction, - inhibition, = no effect.

T: testosterone; 17alphaMT: 17 alpha-methyltestosterone; BNF: beta-naphthoflavone; MCX: methoxychlor; TCB: 3,3',4,4'-tetrachlorobiphenyl
### Table 2. Field studies using Vtg as a biomarker.

<table>
<thead>
<tr>
<th>Species</th>
<th>Chemical</th>
<th>Method</th>
<th>Vtg response</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown trout</td>
<td>E2</td>
<td>Immunohistochemical</td>
<td>+ in lab, + downstream WWTP</td>
<td>Wahl et al. (1998)</td>
</tr>
<tr>
<td>Duck</td>
<td>Lindane</td>
<td>ALP</td>
<td>–</td>
<td>Chakravarty et al. (1986)</td>
</tr>
<tr>
<td>Frog</td>
<td>Seasonal changes</td>
<td>Immunoelectrophoresis</td>
<td>E2, Vtg coincides in liver, plasma, ovary, not fat body</td>
<td>Varriale et al. (1988)</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>E2 season</td>
<td>ELISA</td>
<td>+</td>
<td>Bon et al. (1997)</td>
</tr>
<tr>
<td>Salmon</td>
<td>Dioxin</td>
<td>ELISA</td>
<td>+</td>
<td>Von der Decken and Waters (1993)</td>
</tr>
<tr>
<td>Trout</td>
<td>Seasonal cycle</td>
<td>PAGE</td>
<td>Reprod., season. control of lipoproteins</td>
<td>Wallasert and Babin (1994)</td>
</tr>
<tr>
<td>Whitefish</td>
<td>PandP mill effluent</td>
<td>Northern</td>
<td>+</td>
<td>Mellanen et al. (1999); Soimasa et al. (1998)</td>
</tr>
</tbody>
</table>


### Table 3. In vitro studies using production of Vtg as a biomarker.

<table>
<thead>
<tr>
<th>Species/Cell type</th>
<th>Chemicals</th>
<th>Method</th>
<th>Vtg response</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carp hepatocytes</td>
<td>E2, Bisphenol A, TCDD</td>
<td>ELISA</td>
<td>+</td>
<td>Sneets et al. (1999)</td>
</tr>
<tr>
<td>Eel</td>
<td>E2, GH, prolactin</td>
<td>Western+35Smet</td>
<td>–</td>
<td>Kwon and Mugiya (1994)</td>
</tr>
<tr>
<td>Frog hepatocytes</td>
<td>E2, Growth hormone</td>
<td>ELISA</td>
<td>Multihormonal control of Vtg</td>
<td>Carnevali et al. (1992)</td>
</tr>
<tr>
<td>MCF7 and HeLa cells</td>
<td>TCDD</td>
<td>Cell bioassays</td>
<td>Varied</td>
<td>Nodland et al. (1997)</td>
</tr>
<tr>
<td>Rainbow trout hepatocytes</td>
<td>E2, OP, NP, BPA</td>
<td>ELISA</td>
<td>+</td>
<td>Schrag et al. (1998)</td>
</tr>
<tr>
<td>Rainbow trout hepatocytes</td>
<td>Acetaminofen</td>
<td>ELISA, Northern</td>
<td>–</td>
<td>Miller et al. (1999)</td>
</tr>
<tr>
<td>Rainbow trout hepatocytes</td>
<td>PCB</td>
<td>ELISA, Northern</td>
<td>+ with OH-PCB</td>
<td>Andersson et al. (1999)</td>
</tr>
<tr>
<td>Rainbow trout hepatocytes</td>
<td>NP, OP, DDT, PCB, BPA</td>
<td>ELISA</td>
<td>+</td>
<td>Sumpter and Jobling (1995)</td>
</tr>
<tr>
<td>Rainbow trout hepatocytes</td>
<td>E2 and Ca</td>
<td>PAGE</td>
<td>Max at 5 meq Ca/L</td>
<td>Yeo and Mugiya (1997)</td>
</tr>
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<td>Rainbow trout hepatocytes</td>
<td>E2</td>
<td>PAGE</td>
<td>+</td>
<td>Mugiya and Tanahashi (1998)</td>
</tr>
<tr>
<td>Trout hepatocytes</td>
<td>OP, NP, NP derivatives</td>
<td>RIA</td>
<td>+</td>
<td>White et al. (1994)</td>
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<tr>
<td>X. laevis hepatocytes</td>
<td>E2, NP, Bisphenol A</td>
<td>RT-PCR</td>
<td>E2&gt;NP&gt;Bisphenol</td>
<td>Kloas et al. (1999)</td>
</tr>
<tr>
<td>X. laevis hepatocytes</td>
<td>E2</td>
<td>mRNA degradation</td>
<td>–</td>
<td>McKenzie and Knowland (1990)</td>
</tr>
</tbody>
</table>

* Methods described in the text;  *b* induction, – inhibition, = no effect; BaP: benzo(a)pyrene; DES: diethylstilbestrol; GH: growth hormones; BPA: Bisphenol A; PandP mill: pulp and paper mill.
variables that could modulate the Vtg response. In addition, exposures in the environment are rarely if ever "single chemical" exposures, therefore, possible interactive and ameliorative effects are generally not evaluated.

Laboratory exposures have been used to demonstrate that a variety of chemicals are capable of inducing Vtg production in fish, these include nonylphenol, octylphenol, DDT residues and PCB metabolites (Table 1). Laboratory studies have also helped to define the underlying mechanisms for increases in Vtg production at the molecular level (Ren et al. 1996a) and to investigate the interactive effects of chemicals (Ruby et al. 1987) and hormone systems on Vtg production (Ding et al. 1994). Of particular use are those studies that investigate Vtg induction in relation to other possibly more ecologically relevant endpoints (Giese et al. 2000).

Field exposures

The exposure of fish to complex effluents in situ or under laboratory conditions has been used to assess the estrogenic effects of exposure on plasma Vtg concentrations at environmentally relevant concentrations (Table 2). Immunohistochemical staining was used to demonstrate increased Vtg production in male and female brown trout (Salmo trutta fario) collected downstream of sewage treatment works in rivers in Switzerland (Wahl et al. 1998). Four-to six-fold increases in concentrations of Vtg mRNA in liver have been reported in whitefish (Coregonus lavaretus L. s.l) caged in the vicinity of pulp and paper production facilities in Finland (Soimasauro et al. 1998; Mellanen et al. 1999). In similar studies using rainbow trout caged downstream of sewage treatment plants in the United Kingdom, a 500- to 50,000-fold increase in plasma Vtg concentrations was observed (Sumpter and Jobling 1995). In contrast to the above results of Soimasauro et al. (1998) from Finland, Vtg induction was not detected in brown trout exposed to Canadian pulp and paper mill effluent by either waterborne exposure (static renewal in laboratory) or intraperitoneal injection of concentrated effluent extracts (Sherry et al. 1999b). Similarly, a recent survey of waste water treatment plants in lower Michigan, USA, demonstrated only limited Vtg increase in in situ-exposed fathead minnows (Pimephales promelas) (Nichols et al. 2000). Also, inconsistent responses of plasma Vtg were observed in feral fish collected from the vicinity of Minnesota municipal WWTPs (Folmar et al. 1996). In contrast, significant time- and dilution-related increases of plasma Vtg have been demonstrated for rainbow trout exposed to Norwegian sewage waste waters and, at high concentrations, oil refinery effluent (Knudsen et al. 1997). This result is again in conflict with the results of Sherry and co-workers (1999b) who found no Vtg induction in Canadian refinery effluents. The reasons for these regional differences in response are unclear but probably relate to differences in treatment plant processes, effluent dilution in receiving waters, and absolute and relative concentrations of compounds in effluents.

In situ experiments

The induction of Vtg has been used by several groups in primary cultures of hepatocytes, transformed or genetically manipulated cells (Yeo and Muglai 1997; Monteverdi and Di Giulio 1999; Anderson et al. 1999). These assay procedures rely on the production of Vtg by cell cultures as determined by ELISA (Smeets et al. 1999) or electrophoresis (Yeo and Muglai 1997). These assay systems have been used to assess the estrogenic effects of a range of environmentally relevant chemicals (Monteverdi and Di Giulio 1999) including PCBs and their derivatives (Anderson et al. 1999).

Hepatocytes from a genetically uniform strain of carp (Cyprinus carpio) (Gimeno et al. 1996) have been used to reduce variability of results due to inter-individual variation (Smeets et al. 1999). The method detection limit (MDL) for this system is 2 nM E2. This assay has been used to determine the relative potencies of several weakly estrogenic compounds. Methoxychlor, o,p'-DDT, bisphenol-A, 4,4'-pentyphenol and chlorodecone had relative potencies of 1x10^4 to 1x10^4 while dieldrin, β-endosulfan, toxaphene and o,p'-DDE did not cause detectable Vtg production. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was 10 000 times more potent than tamoxifen as an anti-estrogen in carp hepatocytes in vitro. For TCDD and other Ah-receptor agonists, the order of potency as Ah-receptor agonists corresponded to the order of potency as anti-estrogens (Smeets et al. 1999). The primary advantage of the carp primary hepatocyte assay is that the in situ responses are predictive of in vivo responses of fish. However, the assay is relatively insensitive to E2 compared to assay procedures such as the MCF-7-Luc (MVLN) (Kramer et al. 1998) or yeast-based assay (YBS) (Arndt et al. 1996). This procedure may have some limitations as it can only detect xenoestrogens with a potency of > 2x10^3 relative to E2, due to practical limits in exposure concentrations and the assay MDL for E2 of approximately 2 nM.

These assay procedures offer many opportunities to rapidly assess the potential estrogenic effects of chemicals and complex effluents. The use of primary cultures of rainbow trout hepatocytes coupled with a toxicity identification/evaluation (TIE) was successfully used with Vtg as an endpoint to identify the
estrogenicity of the active compound in the lampricide 3-trifluoromethyl-4-nitrophenol field formulation (Hewitt et al. 1998). However, there is a need to correlate responses in vitro with those observed in vivo. In an attempt to validate the predictability of in vitro assays on whole-animal effects, a series of in vitro and in vivo assays was used to determine the estrogenicity of the plant sterol β-sitosterol in rainbow trout (Tremblay and Van Der Kraak 1998). It was found that Vtg must be used along with other biomarkers, as β-sitosterol was found to exert its effects through multiple mechanisms of toxicity including the induction of cytochrome P450.

**Ecological relevance**

The physiological and ecological relevance of Vtg induction has not been widely investigated. Studies have generally been limited to laboratory exposure experiments. In one experiment, male rainbow trout were exposed to 2 ng/L E2 or 30 ng/L alkyl phenols for 5 weeks (Jobling et al. 1996). Induction of Vtg in these fish was inversely correlated with testicular development as measured by the gonado-somatic index (GSI). Alkyl phenols inhibited testicular development by 50% while the control fish GSI increased from 0.2 to 0.9 during the exposure. The potency of other chemicals to inhibit testicular development was proportional to the in vitro potency to induce Vtg relative to E2, although the concentrations required to induce the responses in vitro were two orders of magnitude lower than those required to elicit the same responses in vivo. The lowest concentration of OP required to induce Vtg was 4.8 μg/L. Based on the induction of Vtg, OP was five fold more potent than NP. While OP affected both Vtg and GSI, NP had no effect on GSI. In this experiment there was only a weak relationship between Vtg induction and reproductive effects as measured by alterations in GSI, with no apparent differences in GSI when some Vtg concentrations were the greatest (Jobling et al. 1996).

Exposure of adult fathead minnows to water-borne NP ranging from 0.05 to 3.4 μg NP/L for 42 d (Giesy et al. 2000) resulted in "inverted-U" type dose-response relationships for egg production and plasma concentrations of Vtg and E2. There was no statistically significant effect of NP on plasma Vtg in either males or females. However, in one experiment, early in the reproductive season, exposure to 0.05 μg NP/L significantly increased plasma Vtg concentrations. Plasma E2 concentrations of both males and females were significantly affected by NP. Also, NP caused a significant decrease in fecundity in one exposure study conducted early in the recrudescence cycle when endogenous E2 concentrations were greater, but a decrease in an experiment later in the reproductive season when plasma E2 concentrations were less. The effects of NP on adult fathead minnows seems not to be due to a direct-acting estrogen agonist mechanism, but rather, due to changes in the endogenous concentrations of E2 through an indirect activation mechanism of action. In these studies there was no significant correlation between effects on reproductive output and plasma Vtg. This suggests that Vtg would be a relatively poor indicator of ecologically relevant effects in this species. This is relevant because the fathead minnow is an often-used bioassay organism that has been suggested to be used for in vitro screening of compounds for their estrogenic potency towards fish.

**CONCLUSIONS**

Plasma Vtg has several advantages as a biomarker. It is a non-invasive technique integrating exposures to complex mixtures of chemicals over time and space. This is particularly relevant given the biological significance of Vtg production to reproduction. Unlike mature females, male fish exhibit little natural expression of Vtg, providing little background. Generally, Vtg regulation is under the control of the ER, which provides a relatively straightforward interpretation of responses. However, investigations into the effects of xenoestrogens on mRNA splicing (Ren et al. 1996b) raise some questions about interpretation of these responses. The direct involvement of the ER means that chemicals that bind to and activate that receptor or increase endogenous levels of E2 will cause the response. Therefore, this biomarker not only measures the direct estrogenic effects of compounds, but also any effects on ER responsiveness, or hormones that regulate E2 status. From a technical perspective Vtg is relatively easy to measure by a variety of selective, rapid, and sensitive techniques. The high levels of induction observed mean that the response is clear-cut and differences between exposed and control groups are easily quantified and interpreted. The response is also rapid, which allows rapid assessment of effects in organisms exposed for relatively short periods (days to weeks) but is not so rapid (eg. minutes or hours) that artefacts due to handling stress and sampling protocols make observation of the response technically challenging and variable, which is the case for measurement of plasma hormones.

There are some limitations to the use of plasma Vtg as a biomarker. Most significantly, there seems to be little evidence of a positive correlation between reproductive and non-reproductive responses. Furthermore, this biomarker is generally limited to males because in females Vtg undergoes cyclic seasonal regulation. The relevance of Vtg production in males.
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to other biological endpoints is even less clear than in females. As with most biological responses, the Vtg response is affected by a variety of other environmental factors. It is not surprising that Vtg production varies during the reproductive cycle in females (Copeland et al. 1986; Bon et al. 1997). However, some studies have indicated that interactive effects with other contaminants can vary with developmental stage. For example, Anderson et al. (1996) demonstrated that beta-naphthoflavone potentiated E2-mediated Vtg induction in juvenile rainbow trout while inhibiting the same response in adult fish. In addition, studies have demonstrated the ability of other endogenous hor mones such as androgens (Lazier et al. 1996) and cortisol (Ding et al. 1994) to modulate the E2-directed induction of Vtg. Exposure to other chemical agents can alter the Vtg response. For instance, co-exposure to anti-estrogens or androgens ameliorated the Vtg response in tilapia (Lazier et al. 1996).

Vitellogenin production is therefore best applied as a biomarker only of exposure to chemicals that are direct estrogen agonists or that up-regulate the endogenous levels of E2 or the E2-signalling pathway. Current evidence suggests that the contribution of actual estrogen agonists to total circulating "estrogen equivalents" is small compared to the contribution of endogenous E2. Therefore, it seems more likely that those chemicals capable of disturbing E2 homeostasis are likely to cause the greatest perturbations in Vtg status. For example, fathead minnows exposed to NP showed an increase of 900% in E2-equivalents in the plasma but only approximately 4% of this increase was due to E2-equivalents contributed by actual NP; the rest was contributed by an increase in endogenous E2 (Giesy et al. 2000).

Many methods can be used to measure Vtg production. They range from the rather simple measurement of alkaline-labile phosphate to sophisticated nucleic acid techniques. While each of these methods has advantages and disadvantages, currently the various ELISA methods appear to be the most appropriate. If an ELISA is applied, the system must be well validated to take into account interference that may be caused by plasma proteins other than Vtg at concentrations near the MDL.


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