Fish Zona Radiata (Eggshell) Protein: A Sensitive Biomarker for Environmental Estrogens

Augustine Arukwe,[1] Frank R. Knudsen,[2] and Anders Goksøyr[1]

[1]Laboratory of Marine Molecular Biology, Department of Molecular Biology, University of Bergen, Bergen, Norway; [2]Department of Biology, University of Oslo, Oslo, Norway

Environmental estrogens have recently caused great concern because of their ability to mimic natural hormones and influence vital endocrine functions in humans and wildlife. The induction of vitellogenin (Vtg) synthesis by environmental estrogens in viviparous vertebrates has been proposed as an effective and sensitive biomarker of estrogenicity. Immunochemical analysis of plasma from Atlantic salmon (*Salmo salar*) exposed to 4-nonylphenol (NP) or to effluent from oil refinery treatment plant (ORTP), shows that NP and ORTP effluent induces Vtg and zona radiata proteins (Zrp) in a dose-dependent manner. However, Zrp-β cross-reactive proteins are more responsive than Zrp-α, Zrp-γ, and Vtg. The sensitivity of Zrp induction points to the zona radiata proteins as alternate biomarkers of estrogenicity. *Key words*: aquatic ecotoxicology, biomarkers, eggshell protein, fish, nonylphenol, vitellogenin. *Environ Health Perspect* 105:418–422 (1997)

One category of endocrine disrupting xenobiotics that has recently raised considerable concern recently consists of chemicals with the ability to act as either estrogen agonists or antagonists. By disrupting reproduction and developmental processes, xenobiotic estrogens in the environment pose an insidious risk to both wildlife and humans (*1–4*). Due to the lipophilic and persistent nature of most xenobiotic estrogens and their degradation products, many of these compounds bioaccumulate and biomagnify. Surprisingly, a wide range of man-made chemicals released either deliberately or unintentionally into the aquatic environment are estrogenic. These include some organochlorine pesticides, polychlorinated biphenyls (PCBs), surfactants, plasticizers, and some natural chemicals such as phytoestrogens and mycoestrogens (*5*). Nonylphenol (NP) is a breakdown product of alkylphenol polyethoxylates and is widely used in many detergent formulations for domestic and industrial use. NP has been used as a model xenoestrogen in several studies of endocrine disruption (*6–8*).

One of the most important responses to estrogen is the induction of protein transcription and translation (*9*). Particularly well known among these responses is the estrogenic induction of vitellogenin (Vtg) in females of lower viviparous vertebrates (*9*). Vtg is the complex phospholipoglycoprotein synthesized by the liver in response to estrogen stimulation. It is secreted by the liver and transported in the blood to the ovary, where it is sequestered and cleaved into the yolk proteins lipovitellin and phosvitin, which are stored in the yolk and serve as food reserve for the developing embryo (*10*). More recently, it was discovered that the fish vitelline envelope or zona radiata protein (Zrp), composed of a small number of proteins, is synthesized in the teleost liver in response to an estrogen signal (*11,12*).

Teleostean Zrp forms the eggshell that provides protection against mechanical disturbances for the developing embryo during the first fragile period but it is shed when hatching is induced by developmental and environmental signals (*13,14*). Furthermore, the eggshell plays an important role during fertilization by preventing polyspermy because sperm entry to the fish egg is restricted to a predetermined site (micropyle) at the animal pole (*15*). After fertilization, the eggshell undergoes a calcium-dependent hardening process that induces a 10-fold increase in the mechanical strength of the eggshell (*16*). The eggs of placental mammals are surrounded by a much thinner, transparent extracellular envelope that lies immediately outside the plasma membrane of the egg. This envelope is called the zona pellucida and 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 (*17,18*). The zona pellucida plays a significant role during fertilization and in the prevention of polyspermy (*19*), as does the teleostean zona radiata proteins, thus showing clear functional similarities. Very little Vtg or Zrp, if any, can be detected in male and in juvenile fish, presumably because of low estrogen concentrations, but it is known that these proteins are synthesized by the liver cells (*in vivo* and *in vitro*) of male and juvenile fish treated with 17β-estradiol (*12,20,21*).

The aim of this study is to comparatively evaluate the response of vitellogenin and zona radiata proteins as environmental estrogen biomarkers in viviparous species using immunoassay methods.

Materials and Methods

Chemicals. 4-nonylphenol (85% of *p*-isomers) was purchased from Fluka Chemika-Biochemika (Buchs, Switzerland). The impurities in 4-nonylphenol consist mainly of phenol (8–13%), tripropylene (~1%), and 2,4-dinonylphenol (~1%). Aprotinin, 17β-estradiol (E_2), Ponceau S, *o*-phenylenediamine dihydrochloride (OPD), *N,N,N′,N′*-tetramethylethylenediamine (TEMED), and 4-chloro-1-naphthol were purchased from Sigma Chemical Co. (St. Louis, MO). Equipment and other chemicals [goat anti-rabbit-horseradish peroxidase (GAR-HRP)] for Western blotting and ELISA were purchased from Bio-Rad Laboratories (Hercules, CA). MaxiSorp microtiter plates were purchased from Nunc (Roskilde, Denmark). All other chemicals were of the highest commercially available grade.

Experiment 1. In experiment 1, juvenile Atlantic salmon, *Salmo salar*, approximately 1+ years old and with a body weight of 75–100 g, were purchased from Sævareid smolt producer A/S in Fusa county (near Bergen, Norway). The fish were a homogenous group belonging to the Norwegian salmon strain (NLA-stamme). They were maintained at the Industrial Laboratory (ILAB) at the High Technology Center in Bergen (HIB) under natural photoperiod (Bergen, 60°N) and continously running sea water with salinity of 35% (ppt) at a constant temperature of 10°C. At the start of this experiment, 36 individuals from the group were randomly selected and transferred to a separate tank. They were further divided into six subgroups of six fish each and acclimatized to sea water with salinity of 34% (ppt) at 10°C ± 0.4°C for 1 week

Address correspondence to A. Arukwe, Laboratory of Marine Molecular Biology, University of Bergen, HIB, N-5020 Bergen, Norway.

We thank the Norwegian Research Council (NFR, Program for Ecotoxicology) and the European Environmental Research Organisation (EERO Environmental Toxicology-Network) for financial support. We also thank Kjersti Milde for rabbit immunization and Rong Chunjung for the zona radiata protein antiserum. The excellent technical assistance/support of M.L. Wiborg, E. Mona, and J. Stenersen at the Biology Department of Oslo University and Ø. Sundberg at Esso Slagentangen are greatly appreciated. We also want to thank Bernt Walther for comments on the manuscript.
Received 13 September 1996; accepted 5 November 1996.

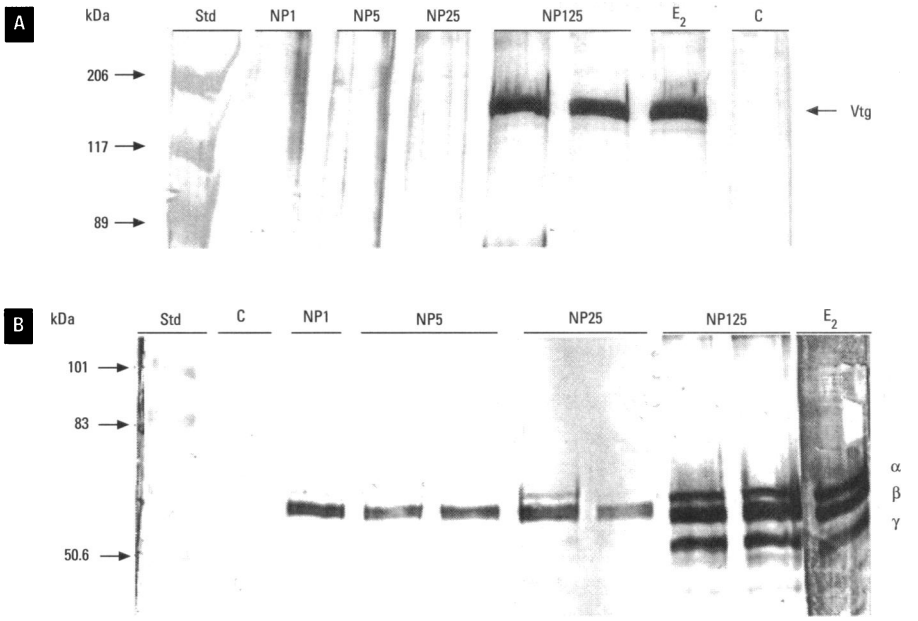

Figure 1. Western blot analysis of vitellogenin (Vtg) and zona radiata proteins (Zrp) in plasma of control (C), 4-nonylphenol (NP; single ip injection at 1, 5, 25, and 125 mg/kg body weight), and 17β-estradiol (E_2); 5 mg/kg body weight treated juvenile salmon. High range prestained molecular weight standards (Std) were run in the first lane. (A) Vtg (180 kDa) and (B) Zrp (zrp-α, 60 kDa; zrp-β, 55 kDa; zrp-γ = 50 kDa) were probed with homologous primary polyclonal rabbit anti-salmon Vtg and Zrp antibodies, respectively. Goat anti-rabbit horseradish peroxidase (GAR-HRP) was used as a secondary antibody in both cases. Plasma from control and NP treated (50 nl) and E_2 treated (10 nl) salmon was applied per well (each lane represents plasma from an individual fish).

before the start of the experiment. The groups were given single intraperitoneal (ip) injections of NP [1, 5, 25, and 125 mg/kg body weight (bw)], 17β-estradiol (5 mg/kg bw; positive control), and vehicle (negative control), respectively. The nonylphenol doses were chosen to span a wide range of doses that are environmentally relevant. All the subgroups were kept in separate 150-liter tanks. NP was dissolved in 1:1 ratio of acetone/Echantillon Alkamuls EL-620 (vehicle), Rhone-Poulenc (Paris, France). The animals were starved during the 14-day experimental period. Fish were anesthetized with benzocaine (50 mg in 1 liter sea water) and blood was collected from the caudal vessel in heparinized precooled syringes and immediately centrifuged (5,000 rpm for 5 min). Plasma samples collected from the centrifuged blood were stored at -20°C until analyzed.

Experiment 2. Experiment 2 was performed using juvenile Atlantic salmon (mean weight 25 g) obtained from Drammen County Fish Administration (Drammen, Norway) and kept in high quality running fresh water at 10°C and a 12 hr light:12 hr dark photoperiod at the Biology Department, University of Oslo. Four tanks receiving high quality drinking water from a municipal water source were installed at the oil refinery treatment plant. One of these tanks received drinking water throughout the experimental period, whereas the remaining three received effluent from the oil refinery treatment works mixed with drinking water to a final concentration of 1, 10, and 50%, respectively. Twenty fish were transported from the Biology Department of Oslo University, released into each of the four tanks, and kept there for 4 weeks. They were fed every second day throughout the experimental period. After 4 weeks, the fish were anesthetized and blood was collected. Plasma samples collected from the centrifuged blood were stored at -20°C until analyzed.

An oil refinery treatment plant (ORTP) receives waste water from the oil refining process containing oil remnants and chemicals used in the oil refining process. Among these chemicals are alkylphenols used as emulsifying agents in the oil refining process. The basis for the ORTP doses comes from a previous study at the same site (Knudsen et al., unpublished data) where rainbow trout (*Oncorhynchus mykiss*) were exposed to 2.5, 10, and 20% ORTP effluent for a period of 3 weeks. A significant increase in plasma Vtg levels was observed only in the group exposed to 20% effluent. There were no significant differences in mortality between the different groups of fish in the experiment. The recorded 30% mortality was probably caused by stress from handling during blood sampling rather than by toxic effluent.

Production of polyclonal vitellogenin antiserum. We isolated, purified, and produced polyclonal rabbit antibodies to salmon vitellogenin. Vitellogenin synthesis was induced in juvenile fish by weekly ip injections of 17β-estradiol (dissolved and sonicated in peanut oil) at a dose level of 10 mg/kg bw/week. Blood samples were collected 27 days after the first injection from the caudal vessel using heparinized precooled syringes. To avoid Vtg proteolysis, the fish were injected with a trypsin inhibitor, aprotinin [10–20 trypsin inhibitor units (TIU/ml) dissolved in 0.9% NaCl], injected in the caudal vessel 20 min before sampling. The blood was immediately centrifuged (5,000 rpm for 5 min). All preparative procedures were carried out at 4°C. Vtg was purified by selective precipitation as described by Wiley et al. (*22*) and Norberg and Haux (*23*). Additional protection against Vtg degradation was provided by adding 100 µl of aprotinin to the Vtg solution before dissolving it in NaCl. Polyclonal Vtg antibody was prepared in rabbit from nitrocellulose-blotted Vtg fractions, essentially as described by Diano et al. (*24*). Production of a polyclonal antibody using this method gave a highly specific polyclonal antiserum, thus avoiding the long purification of antigenic protein and the use of Freund's adjuvant. We have tested several dilutions of this antibody and its ability to cross-react with Vtg degradation products; dilutions of up to 1:11,000 were able to detect the Vtg and its degradation products in plasma (results not shown). The polyclonal salmon Zrp antibody was a gift from Chunjung Rong (Bergen, Norway) and was prepared against solubilized and purified salmon zona radiata proteins.

Immunochemical studies. Immunochemical analysis of plasma samples was performed using Western blotting and indirect ELISA. For Western blotting, proteins were separated using 4% stacking and 9% separating sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (*25*) and blotting was performed as described by Towbin et al. (*26*). Indirect ELISA was performed essentially as described by Goksøyr (*27*). Cross-reactions of Vtg and Zrp were probed with homologous primary polyclonal rabbit anti-salmon antibodies (diluted 1:2,000). Peroxidase conjugated GAR-HRP (Bio-Rad) diluted 1:3,000 and H_2O_2/4-chloro-1-naphthol (HRP color development reagent; Bio-Rad) were used as the detection system. Statistical analysis of ELISA absorbance was performed on

log-transformed data using Dunnett's test. The level of significance was set at $p \leq 0.05$, unless otherwise stated, with JMP Software (version 3.1.6) for Statistical Visualization (SAS Institute, Cary, NC).

For validation of the ELISA assay, pooled plasma samples from control and 5 mg and 125 mg NP/kg fish were serially diluted in coating buffer (50 mM Na-bicarbonate buffer, pH 9.5) in eppendorf tubes. The diluted samples were adsorbed to microtiter wells and incubated with homologous rabbit anti-salmon vitellogenin and zona radiata protein antibodies, respectively, as described above.

Results

In plasma from Atlantic salmon (*Salmo salar*) exposed to different doses of NP via ip injection, a cross-reacting Vtg protein (180 kDa) was detected only in plasma from the group treated with the highest dose (125 mg NP/kg fish, Fig. 1A) using Western blotting with homologous polyclonal antibodies. At this dose, Zrp-α (60 kDa), Zrp-β (55 kDa) and Zrp-γ (50 kDa) were also detected; at the lower doses, only a cross-reacting zrp protein (possibly a double band) was detected in plasma at the β-position (Fig. 1B). Consistent with the immunoblot analysis, an indirect ELISA of Vtg and Zrp levels reflected the differences in inducibility of these proteins (Fig. 2). Results of the ELISA assay validation tests are presented in Figure 3. Immunoreactive Vtg and Zrp proteins were dominant in plasma from 125 mg NP treated fish (also Zrp immunoreactive proteins in 5 mg NP treated fish). This assay method revealed the absence of Vtg and Zrp in control fish (also Vtg in 5 mg NP treated fish). A parallel decrease in absorbance with the antigen (Vtg and Zrp) dilution was observed (Fig. 3).

For validation of the laboratory experiment results, we performed a second study under semifield conditions, exposing juvenile salmon to ORTP effluent. Again, Vtg (Fig. 4A) and Zrp-α and Zrp-γ (Fig. 4B) were detected only in plasma from the group exposed to 50% mixture (v/v) of ORTP effluent, whereas Zrp-β reaction was detected in the groups exposed to 1 and 10% ORTP effluent (Fig. 4B). Indirect ELISA analysis of these samples again confirmed the immunoblot analysis (Fig. 5), although the Zrp-β response to 1% ORTP, readily visible on Western blots, was less apparent in ELISA.

Discussion

Immunochemical analysis, using Western blotting and ELISA with polyclonal homologous antisera to Atlantic salmon Vtg and Zrp, was used in this study to measure plasma levels of Vtg and Zrp of control, NP, 17β-estradiol, and ORTP effluent-treated juvenile salmon. The specificity of these antibodies has been verified by several immunological techniques such as immunohistochemistry, immunoblotting, and immunoprecipitation with very high antigenic recognition, in addition to crossreactivity towards heterologous Atlantic cod (*Gadus morhua*) Zrp (*21,28*) and Vtg (results not shown). Validation of the ELISA assay used in this study shows a parallel decrease in absorbance with antigen dilution. However, the Vtg antiserum shows higher affinity for its antigen than the Zrp antiserum (Fig. 3). Similar results were also observed using serial dilution of the Vtg and Zrp antisera respectively (results not shown).

Our results represent the first report on the comparative estrogenic effects of an

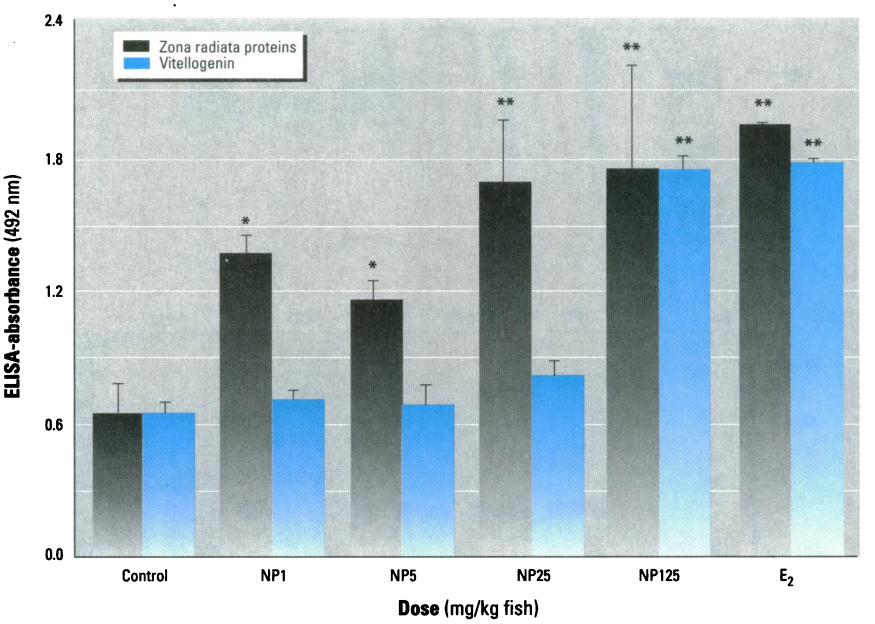

Figure 2. Indirect ELISA analysis of vitellogenin (Vtg) and zona radiata proteins (Zrp) levels in plasma of control (C), 4-nonylphenol (NP; single ip injection at 1, 5, 25, and 125 mg/kg body weight), and 17β-estradiol (E_2) treated juvenile salmon. Data are given as mean ELISA absorbance values (492 nm) ± standard deviation ($n = 6$ per treatment group).
*$p<0.001$.
**$p<0.0001$.

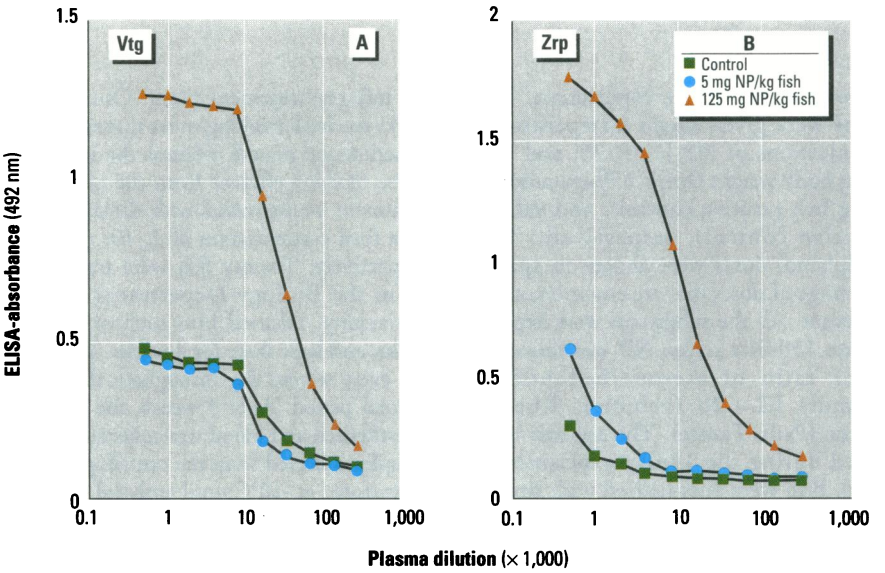

Figure 3. Validation of ELISA assay for vitellogenin (Vtg) and zona radiata proteins (Zrp). The ELISA responses (absorbance at 492 nm) are plotted against total plasma dilution (log-scale). Absorbances are shown as absolute values since blanks were not subtracted.

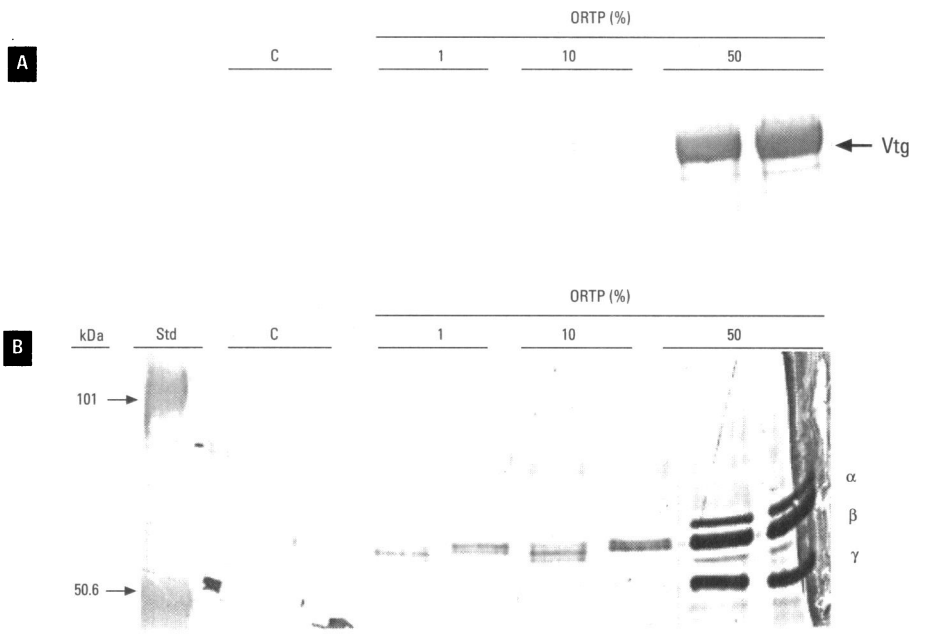

Figure 4. Western blot analysis of vitellogenin (Vtg; 180 kDa) and zona radiata protein (Zrp; zrp-α, 60 kDa; zrp-β, 55 kDa; zrp-γ = 50 kDa) levels in plasma from juvenile Atlantic salmon after 4-week exposure to 1, 10, and 50% mixture of oil refinery treatment plant (ORTP) effluent. (A) VTG and (B) Zrp were analyzed using homologous antibodies as described (see legend to Fig. 1). Plasma (50 nl) was applied per well.

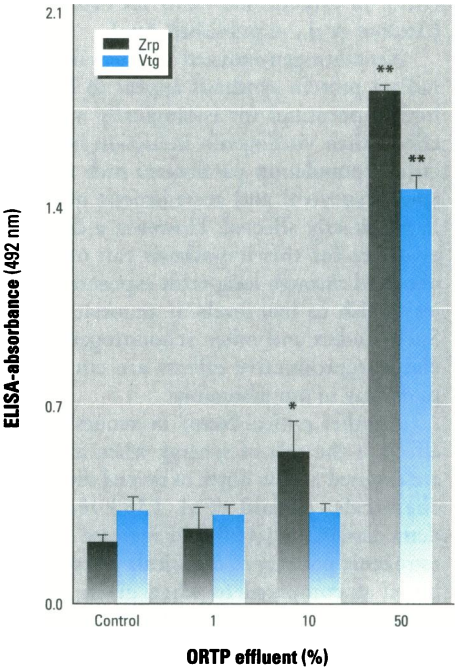

Figure 5. Immunochemical analysis using indirect ELISA of vitellogenin (Vtg) and zona radiata protein (Zrp) in plasma from control juvenile Atlantic salmon or after 4-week exposure to 1, 10, and 50% mixture of oil refinery treatment plant (ORTP) effluent. Data are given as mean ELISA absorbance values ± standard deviation (n = 6 per treatment group). For further explanation, see legend to Figure 2.
*p<0.001.
**p<0.0001.

environmental estrogen on the induction of Vtg and Zrp in fish or lower vertebrates. The detection of Vtg and Zrp in plasma of nonylphenol-treated juvenile salmon shows that Zrp-β is more responsive than Zrp-α, Zrp-γ, and Vtg, as these proteins were only detectable in the groups treated with 125 mg NP and 50% ORTP effluent. Induction of an immunoreacting protein band (or double band) at the β-position of Zrp suggests that these proteins are synthesized first during zonagenesis (hepatic synthesis of zona radiata proteins and transport in the blood for deposition in the ovaries). The reproducibility of the laboratory experiment was verified by performing a second independent experiment in the laboratory. In the second experiment, juvenile salmon from the same group used in experiment 1 were exposed to a single ip injection of NP at 25 and 125 mg/kg fish for 1 week. Again, Vtg and the three Zrp monomers were induced in the group treated with 125 mg, whereas only Zrp-β was induced in the group treated with 25 mg NP (results not shown). Furthermore, these results have also been analyzed using monoclonal anti-salmon vitellogenin antibody produced in our laboratory (Nilsen et al., unpublished data) with similar results.

Although these results are in accordance with the estrogenic effects of NP and other alkylphenol polyethoxylates (APEs) reported earlier (*7,8,29,30*), we were able to detect Vtg only at very high NP concentrations. In contrast, Zrp-β was detectable at lower NP concentrations. In the literature, the induction of vitellogenin has been singled out as the most effective biomarker of estrogenic contaminants in viviparous vertebrates (*31,32*). The sensitivity of Zrp cross-reaction demonstrated in this report points to the zona radiata proteins as a more sensitive biomarker for estrogenic effects of environmental pollutants. The sensitivity of Zrp at low NP doses is probably a result of the physiological function of the eggshell as protector of the growing embryo. It has been demonstrated that the formation of the eggshell envelope precedes the active uptake of vitellogenin during oocyte development (*12,33*).

In addition to their implications to the individual fish exposed to xenoestrogens, these findings may also have an ecological significance. Elevated Vtg concentrations in the plasma of male fish exposed to sewage treatment effluent have earlier been reported as a feminization response (*34*). However, the ecological consequences of xenoestrogen-induced hepatic Vtg synthesis is unknown due to the absence of unequivocal evidence that fish, or any other aquatic organisms, are adversely affected by living in and accumulating estrogenic chemicals. Given the pivotal role played by endogenous estrogens in reproduction, such as sexual differentiation at the egg/embryo and adult sexual maturation stages, it is obvious that the most likely process to be affected is reproduction. In this respect, small changes in zona radiata protein synthesis might cause the thickness and mechanical strength of the eggshell to be altered, thus causing a loss in its ability to provide protection against mechanical disturbances during the very fragile period of oocyte development and the prevention of polyspermy during fertilization.

Although Vtg is a critical factor that provides the growing embryo with necessary nutrients, it could be argued that more subtle variations in Vtg content would not be of great significance to the survival of the offspring. For the male fish, accumulation of high Vtg and possibly Zrp concentrations might be deleterious because high Vtg concentrations have been associated with kidney failure and increased mortality rates (*35*). Furthermore, the inhibited testicular growth reported recently by Jobling et al. (*7*) would, although not yet demonstrated, reduce fertility. However, these effects may be related to other nonvitellogenic responses to environmental estrogens, e.g., through steroid biotransformation pathways. We have observed that low levels of NP also affect progesterone hydroxylase activities in salmon liver microsomes, in addition to their inhibiting

effect on cytochrome P450 1A expression (Arukwe et al., unpublished data).

Xenoestrogen-induced changes in zona radiata protein synthesis appear to have a higher potential for ecologically adverse effects than vitellogenin induction because critical population parameters such as offspring survival and recruitment may be more directly affected. However, a definite evidence for this hypothesis can only be obtained through long-term exposure studies of fish to low levels of xenoestrogens. Such studies and other xenoestrogen-associated reproductive effects are currently underway in our laboratory.

Another critical factor in xenoestrogen effects is the role of synergy when animals are exposed to low doses of several different chemicals. Arnold et al. (*36*) recently demonstrated a 1,000-fold increase in the estrogenic potency of chemicals when combined as compared to when given alone, using a simple yeast estrogen system (YES) containing the human estrogen receptor. Such synergistic interactions are of course of profound environmental significance because most organisms are exposed to a cocktail of different environmental chemicals rather than to single compounds.

It is impossible to determine empirically the sensitivity, susceptibility, or resistance of every species to each type of compound. Therefore, an understanding of the general principles and mechanisms that determine chemical and biological specificity of environmental estrogens and their impairment of reproductive processes in aquatic organisms is very important (*37*). These findings may also lead to the development of new and sensitive biomarkers for the effects of environmental estrogens in the ecosystem.

References

1. Colborn T, Clement C, eds. Chemically-induced alterations in sexual and functional development: the wildlife/human connection. Princeton, NJ:Princeton Scientific Publishing, 1992.
2. Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101(5): 378–384 (1993).
3. Foster WG. The reproductive toxicology of great lakes contaminants. Environ Health Perspect 103(suppl 9):63–69 (1995).
4. Guillette LJ Jr, Gross TS, Gross DA, Rooney AA, Percival HF. Gonadal steroidogenesis *in vitro* from juvenile alligators obtained from contaminated or control lakes. Environ Health Perspect 103(suppl 4):31–36 (1995).
5. McLachlan JA, ed. Estrogens in the environment II. New York:Elsevier, 1985.
6. Routledge EJ, Sumpter JP. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ Toxicol Chem 15(3):241–248 (1996).
7. Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. Environ Toxicol Chem 15:194–202 (1996).
8. Jobling S, Sumpter JP. Detergent components in sewage effluents are weakly oestrogenic to fish: an *in vitro* study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Aquat Toxicol 27:361–372 (1993).
9. Tata JR, Smith DF. Vitellogenesis: a versatile model for hormonal regulation of gene expression. Recent Prog Horm Res 35:47–90 (1979).
10. Mommsen TP, Walsh PJ. Vitellogenesis and oocyte assembly. In: Fish physiology, vol XIA, (Hoar WS, Randall DJ, eds.), New York: Academic Press, 1988;347–406.
11. Oppen-Berntsen DO, Gram-Jensen E, Walther BT. Zona radiata proteins are synthesized by rainbow trout (*Oncorhynchus mykiss*) hepatocytes in response to oestradiol-17β. J Endocrinol 135:293–302 (1992).
12. Hyllner SJ, Oppen-Berntsen DO, Helvik JV, Walther BT, Haux C. Oestradiol-17β induces the major vitelline envelope proteins in both sexes in teleosts. J Endocrinol 131:229–236 (1991).
13. Yamagami K, Hamazaki TS, Yasumasu S, Masuda K, Iuchi I. Molecular and cellular basis of formation, hardening, and breakdown of the egg envelope in fish. Int Rev Cytol 136:51–92 (1992).
14. Helvik JV, Walther BT. Photoregulation of the hatching process of halibut (*Hippoglossus hippoglossus*) eggs. J Exp Zool 263:204–209 (1992).
15. Hart NF. Fertilization in teleost fishes: mechanism of sperm-egg interaction. Int Rev Cytol 121:1–66 (1990).
16. Lønning S, Kjørsvik E, Davenport J. The hardening process of the egg chorion of the cod, *Gadus morhua*, and lumpsucker, *Cyclopterus lumpus*. J Fish Biol 24:505–522 (1984).
17. Wassarman PM. Zona pellucida glycoproteins. Ann Rev Biochem 57:415–442 (1988).
18. Bleil JD, Wassarman PM. Structure and function of of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. Dev Biol 76:185–202 (1980).
19. Wassarman PM. The biology and chemistry of fertilization. Science 235:553–560 (1987).
20. Norberg B. Atlantic halibut (*Hippoglossus hippoglossus*) vitellogenin: induction, isolation and partial characterization. Fish Physiol Biochem 14:1–13 (1995).
21. Oppen-Berntsen DO, Olsen SO, Rong C, Swanson P, Taranger GL, Walther BT. Plasma levels of eggshell Zr-proteins, estradiol-17β and gonadotropins during an annual reproductive cycle of Atlantic salmon (*Salmo salar*). J Exp Zool 268:59–70 (1994).
22. Wiley HS, Opresko L, Wallace RA. New method for the purification of vertebrate vitellogenin. Anal Biochem 97:145–152 (1979).
23. Norberg B, Haux C. Induction, isolation and a characterization of the lipid content of plasma vitellogenin from two salmo species: rainbow trout (*Salmo gairdnerii*) and sea trout (*Salmo trutta*). Comp Biochem Physiol 81B:869–876 (1985).
24. Diano M, Le Bivic A, Hirn M. A method for the production of highly specific polyclonal antibodies. Anal Biochem 166:224–229 (1987).
25. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685 (1970).
26. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354 (1979).
27. Goksøyr A. A semi-quantitative cytochrome P450IA1 ELISA: a simple method for studying the monooxygenase induction response in environmental monitoring and ecotoxicological testing of fish. Sci Total Environ 101:255–262 (1991).
28. Oppen-Berntsen DO, Hyllner SJ, Haux C, Helvik JV, Walther BT. Eggshell zona radiata proteins from cod (*Gadus morhua*): extra-ovarian origin and induction by estradiol-17β. Int J Dev Biol 36:247–254 (1992).
29. Soto AM, Justicia H, Wray JW, Sonnenschien C. p-Nonyl-phenol: an estrogenic xenobiotic released from "modified" polystyrene. Environ Health Perspect 92:167–173 (1991).
30. White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology 135:175–182 (1994).
31. Sumpter JP, Jobling S. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ Health Perspect 103(suppl 7):173–178 (1995).
32. Palmer BD, Palmer SK. Vitellogenin induction by xenobiotic estrogens in the red-eared turtle and African clawed frog. Environ Health Perspect 103(suppl 4):19–25 (1995).
33. Hyllner SJ, Silversand C, Haux C. The formation of vitelline envelope precedes the active uptake of vitellogenin during oocyte development in rainbow trout. Mol Reprod Dev 39(2):166–175 (1994).
34. Sumpter JP. Feminized responses in fish to environmental estrogens. Toxicol Lett 82/83:737–742 (1995).
35. Herman RL, Kincaid HL. Pathological effects of orally administered estradiol to rainbow trout. Aquaculture 72:165–172 (1988).
36. Arnold SF, Klotz DM, Collins BM, Vonier, PM, Guillette LJ Jr, McLachlan JA. Synergistic activation of estrogen receptor with combinations of environmental chemicals. Science 272:1489–1492 (1996).
37. Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp P, Gray LE, Kaattari S, Lucier G, Luster M, Mac MJ, Maczka C. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. Environ Health Perspect 104(suppl 4):715–740 (1996).