Chronic Toxicity of Environmental Contaminants: Sentinels and Biomarkers

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Due to the use of a limited number of species and subchronic exposures, current ecological hazard assessment processes can underestimate the chronic toxicity of environmental contaminants resulting in adverse responses of sentinel species. Several incidences where sentinel species have responded to the effects of chronic exposure to ambient levels of environmental contaminants are discussed, including the development of neoplasia in fish, immunosuppression in marine mammals, pseudohermaphrodism in invertebrates, teratogenicity in amphibians, and aberrations in the sexual development of fish and reptiles. Biomarkers of chronic toxicity, including DNA mutations, alterations in specific protein and mRNA levels, and perturbations in metabolism, are presented. The incorporation of appropriate surrogate species and biomarkers of chronic toxicity into standard toxicity characterizations is proposed as a means of significantly refining the ecological hazard assessment process. — Environ Health Perspect 105(Suppl 1):65–80 (1997)

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Introduction

An integral early component of all chemical hazard assessment processes is the characterization of toxicity associated with the chemical. In assessments of hazards to humans, this characterization largely involves defining acute and chronic toxicity of the chemical to surrogate mammalian species such as rat and mouse and the use of uncertainty factors to account for species extrapolations. Uncertainty associated with toxicity characterization is significantly enhanced in ecological hazard assessments, since the target of toxicity is not a single species (i.e., human) but complex assemblages of species including microorganisms, plants, invertebrates, and vertebrates. Accordingly, chemical toxicity to select species representing various levels of phylogenetic organization (i.e., plant, invertebrate, fish, bird, mammal) is typically assessed and results extrapolated to represent the plethora of species that compose ecosystems.

Procedures for the characterization of acute toxicity (defined as toxicity elicited as a result of exposure to the chemical for a short duration) of environmental chemicals are standardized and amenable for use with a wide array of species [e.g., Stephan (*1*)]. Extensive characterization of the acute toxicity of chemicals has allowed the successful implementation of chemical discharge limitations that have been largely successful in protecting against acute toxicity of these contaminants. Definitive assessments of the chronic toxicity (defined as toxicity elicited as a result of exposure to the chemical over the life cycle of the organism) of chemicals is a more complex undertaking that is often encumbered by limited knowledge of the full life cycle of the organism, the inability to maintain many species in the laboratory over their life cycles, failure to induce reproduction under laboratory conditions, and the duration of time required to assess chemical toxicity over the full life cycle of the organism. To circumvent such difficulties, a limited number of species that are easily reared in the laboratory are routinely used in chronic toxicity evaluations. Abridged life-cycle (subchronic) exposures are often used as surrogates to full life-cycle testing, since comparative studies have shown that assessment of a chemical's toxicity during the early life stages of many species provides results identical to full life-cycle toxicity assessments for approximately 80% of the chemicals evaluated (*2*). It is noteworthy that not all chemicals elicit chronic toxicity. Perhaps the 20% of chemicals for which subchronic testing does not accurately predict chronic toxicity represents those chemicals that are truly chronically toxic. Thus, the use of a limited number of species and subchronic exposures may cause the underestimation of chronic toxicity of environmental contaminants. Such underestimation could result in the establishment of acceptable levels of contaminants in the environment that are actually detrimental because of chronic toxicity.

Verification and monitoring is peripheral, though integral, to the U.S. Environmental Protection Agency's (U.S. EPA) framework for ecological risk assessment (*3*). Recognizing the potential for error in risk assessment, environmental monitoring following implementation of regulatory guidelines is imperative to verify the validity of the risk assessment process, to identify deficiencies in the assessment, to evaluate the effectiveness of the policy decision, and to point out the need for improved or novel methodologies to be incorporated into the process (*3*). Should deficiencies exist in the assessment of chronic toxicity of environmental contaminants, then the monitoring process should reveal resulting consequences. Such toxicity may not be blatantly evident because of the potential species specificity of such effects and the subtle nature of the effects (*4*). The identification of sentinel species of such effects as well as sensitive biomarkers of chronic toxicity is imperative to thoroughly evaluate environmental health.

This review will

- Identify incidences where sentinel species (defined as species that have been shown to elicit responses to contaminants present in the environment) appear to be signaling the occurrence of unacceptable toxicity in the environment.
- Define the nature of the toxicant effects to which these species are responding.
- Highlight biomarkers that have been used to detect and characterize the specific modes of toxicity described.

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Abbreviations used: DES, diethylstilbestrol; DTH, delayed-type hypersensitivity; PAH, polycyclic aromatic hydrocarbon; PAPS, 3'-phosphoadenosine-5'-phosphate; PCB, polychlorinated biphenyl; PCR, polymerase chain reaction; RPSI, relative penis size index; SDS, sodium dodecyl sulfate; UV, ultraviolet; VDSI, vas deferens sequence index.

Such information will

- Assist in tailoring future monitoring endeavors to maximize detection sensitivity of the process.
- Define sensitive species that should be considered for use in toxicity assessments, especially when precedence indicates that the species would be particularly sensitive to the type of chemical under evaluation.
- Identify appropriate end points (i.e., biomarkers) that should be incorporated into laboratory assessments of chemical toxicity and field monitoring of environmental effects.

Neoplasia in Fish

Evidence implicating environmental contaminants as causative agents in the occurrence of neoplasia in fish has accrued over the past four decades. Three early reports linking environmental contamination and neoplasms in feral fish came from investigators in both California and Maryland. The first study, by Russell and Kotin (*5*), showed a high incidence of papillomas in white croakers (*Genyonemus lineatus*) from the Los Angeles harbor. Although no conclusive supporting evidence was provided, the investigators concluded that exposure to sewage outfalls in the area may have been responsible for the tumors. A second study also reported a high incidence of papillomas in white croakers and Dover sole from several sites in Southern California (*6*). Again, exposure to sewage outfalls was considered responsible for the papillomas. The third report, also in 1964, demonstrated a higher level of hepatic neoplasms in white suckers (*Catostomus commersoni*) in Deep Creek Lake, Maryland (*7*). From these three early reports, further investigations were undertaken to determine whether tumors in feral fish could be due to exposure to environmental contaminants [for reviews see Black and Baumann (*8*) and Mix (*9*)].

Studies of fish neoplasia in the Fox River in Illinois were some of the first to correlate neoplasm prevalence with water quality. The Fox River flows through heavy manufacturing areas in northern Illinois from which toluene, benzene, chlorinated hydrocarbons, triazines, and organophosphate insecticides were known to contaminate the river. Seventeen species of fish, including walleye (*Stizostedion vitreum*), northern pike (*Esox lucius*), brown bullhead (*Ictalurus nebulosus*), carp (*Cyprinus carpio*) and hogsucker (*Hypentelium nigricans*), were collected from the Fox River and a relatively pristine site, Lake of the Woods, Ontario, Canada, in 1973. In all species, the prevalence of tumors was higher in fish from the Fox River (*10*). A second study published in 1977 showed essentially the same frequencies of tumors (*11*).

The Puget Sound, Washington, has supported heavy manufacturing industries, chemical plants, wood product plants, and a large shipping industry (*12*). Studies of English sole (*Paropyrys vetulus*) in the Duwamish River unveiled a tumor incidence that appeared to correlate with polychlorinated biphenyl (PCB) levels (*13,14*). Further investigation demonstrated that English sole, Pacific tomcod (*Microgadus proximus*), and rock sole (*Lepidopsetta bilineata*) from contaminated areas had neoplasm prevalence of 2.4, 3.4, and 2.1%, respectively, whereas those fish collected from control areas had no tumors (*15*). A supplementary study showed that 12.9% of the English sole and 1.1% of the starry flounder from the PCB-contaminated Duwamish River contained hepatomas, as did 8.2% of the English sole from the Lake Washington ship canal (*16*). In contrast, no hepatomas were found in fish from the control site (*16*). Six separate studies performed by the National Marine Fisheries Service from 1979 to 1984 showed a statistically significant correlation between sediment polycyclic aromatic hydrocarbon (PAH) concentrations or bile fluorescent aromatic hydrocarbon concentrations and hepatic lesions (*17*). These studies also showed a progression of stepwise changes in hepatic lesions ultimately leading to hepatic neoplasms similar to experimental rodent models of carcinogenesis. Laboratory studies confirming these observations were conducted by injecting English sole with an extract from either the contaminated or reference sediment or the model hepatocarcinogen benzo[*a*]pyrene. The results demonstrated that injections with benzo[*a*]pyrene or the contaminated sediment extract increased the incidence of hepatotoxic lesions, including basophilic foci, a preneoplastic lesion (*18*). Follow-up studies showed that the risk of hepatic lesions in English sole, starry flounder, and white croaker increased in urban sites in which the fish contained high levels of PAHs, PCBs, and DDT (*19*). The investigators formulated a model to determine prevalence associated with species of fish, type of exposure, location of capture, age, and gender. For example, an English sole from Elliot Bay, Washington, was 734 times more likely to have a neoplasm than a fish from a reference site, with controls for both age and gender.

A study of tumor incidence among fish in the Black River in Ohio, which empties into Lake Erie, showed that in 1980, 1.2% of the 2-year-old and 33% of the 3-year-old brown bullheads collected from the river had liver tumors compared to a 0% incidence among fish in Buckeye Lake, the control site (*20*). In 1983 a coking plant on the river closed, and as a result, studies after 1983 demonstrated a decrease in PAH levels in both the river sediment and in the tissues of the brown bullheads of almost 99% by 1987. Prevalence of liver cancer in 3-year-old fish decreased to 10% in 1987 versus 39% in 1982 (*21*). This is the first study to show a decrease in neoplasm prevalence associated with a decrease in environmental contaminants, which greatly strengthens the contention that neoplasms in feral fish are good indicators of chronic exposure to contaminants within a defined environment.

Fish neoplasia resulting from chronic exposure to environmental pollutants is not only relevant from a historical perspective. Studies conducted during the current decade continue to demonstrate that fish populations are responding to environmental carcinogens. Mummichog (*Fundulus heteroclitus*), small nonmigratory fish from the Elizabeth River, Virginia, were sampled from an area adjacent to a wood treatment facility (*22*) in which the sediments were contaminated with creosote (*23*). Ninety-three percent of the mummichog collected next to the plant had visible hepatic lesions and 33% had hepatocellular carcinoma. There were no hepatic lesions in the mummichog captured from two control sites. As mummichog have a home range of only 30 to 40 m (*24*), this study demonstrates how species of limited range can serve as sentinels of contamination within a defined area—such as the receiving waters for chemical wastes.

Over 90% of the older Atlantic tomcod (*Microgadus tomcod*) sampled from the Hudson River in 1994 had hepatocellular carcinoma compared to <5% sampled from control areas [summarized in Wirgin et al. (*25*)]. Interestingly, there was a very small number of 2-year-old fish and a complete absence of fish younger than 2 years old in the Hudson River. This suggests the possibility of tumor-associated mortality in contaminated areas that may result in underestimates of the effects of pollutants on these fish. Baumann (*26*) showed that the highest prevalence of liver tumors in

brown bullheads from Lake Erie was in 4- to 5-year-old fish, but that 6- to 7-year-old fish were completely absent from the lake and their age group represented 18% of the total catch from uncontaminated sites. Thus, neoplasia-induced mortality may result in underestimates of tumor incidence among fish populations.

In summary, the evidence demonstrates that elevated tumor incidence in fish populations has heralded and continues to herald the presence of chemical carcinogens in the environment. Aquatic environments serve as major repositories for the accumulation of environmental contaminants, including carcinogens, and thus inhabitants of these environments serve as sentinels for the presence of toxic quantities of contaminants in the environment.

Biomarkers of Carcinogenicity

By far the simplest and most definitive biomarker of neoplasia in fish is the visual detection of tumors. However, the detection of frank tumors in fish populations is a late measure of response to environmental contaminants with sensitivity only slightly greater than the use of mortality as a biomarker. Histopathologic and molecular measures of early events in the process of carcinogenesis can increase the sensitivity of detection and possibly signal the occurrence of chemical exposure in the absence of overt disease among the exposed population.

The liver is a major site of contaminant accumulation and biotransformation to reactive metabolites. The liver is thus a common target at which chemical-induced alterations leading to neoplasia can be detected. For example, the development of altered foci is an early event in the development of hepatic neoplasia. Altered foci in fish typically exhibit enhanced cytoplasmic staining with hematoxylin and often are characterized by many cellular alterations, including glycogen depletion (*27*), increased levels of cytoplasmic RNA (*28*), and large aberrant-shaped nuclei (*29*). Detailed descriptions of altered hepatic foci in fish can be found in Hinton et al. (*30*) and Hinton and Laurén (*31*).

Greatest sensitivity in the detection of chemical-induced carcinogenic lesions can be gained by analyses for the presence of initial molecular lesions elicited by the toxicant. Common molecular targets of chemical carcinogens that contribute to the process of tumor generation are mutations of protooncogenes and tumor suppressor genes such as c-*ras* and *p53*, respectively. The advent of polymerase chain reaction (PCR) technology has allowed for the selective enrichment of the mutated genes of interest from samples derived from the toxicant-exposed organisms. Once amplified sufficiently, samples can be analyzed for the presence of the mutations using a variety of techniques, as discussed by Shugart et al. (*32*) that include oligonucleotide hybridization (*33*), sequencing (*34*), restriction analysis (*35*), RNase mapping (*36*), and gel retardation (*37*).

Immunosuppression in Marine Mammals

Large-scale mortality due to infectious agents has been noted in many marine mammal populations that inhabit areas containing industrial contaminants. These observations have led to speculation that pollution-induced immunosuppression is contributing to such incidence. During 1988 and 1989, nearly 18,000 harbor seals (*Phoca vitulina*) died in the North, Irish, and Baltic seas (*38*) because of to a morbillivirus-related distemper virus (*39*) now called phocine distemper virus (*40*). Mortality was highest in areas with high levels of pollutants (*41*) and analyses of dead seals revealed the presence of high tissue levels of PCBs and other contaminants (*42*). Many industrial chemicals such as PCBs (*43*), hexachlorobenzene (*44*), dieldrin (*45*), and DDT (*46*) have been shown to cause immunosuppression in laboratory animals. To determine if immunosuppressive chemicals contributed to the seal mortality, harbor seals were fed herring for a total of 93 weeks. The herring was caught either from a relatively pristine area or from a polluted coastal area. The estimated daily intake of organochlorines from the contaminated herring was 3 to 10 times higher than from the uncontaminated herring (*47*). Immunological analyses revealed that natural killer cell activity, which provides a first line of defense against viral infections (*48*), and lymphocyte proliferative responses, which are a measure of T-cell function (*49*), were significantly lower in the seals fed contaminated fish (*47*). The same investigators also discovered that seals fed herring from the contaminated area for 2.5 years exhibited reduced lymphocyte proliferative responses over the course of the exposure. When these animals were immunized with rabies virus antigen and tetanus toxoid, lymphocyte proliferative response was again compromised (*50*). These seals also had significantly lower responses to a challenge with ovalbumin, showing decreased intradermal swelling after the challenge in a delayed-type hypersensitivity (DTH) reaction. There was an inverse relationship between DTH swelling and total Ah receptor-binding contaminant levels. Furthermore, the serum antibody titers to ovalbumin were approximately 37% lower in the seals fed the contaminated herring than those fed the control herring (*51*). Earlier studies had shown that seals fed PCB-contaminated fish had significantly lower serum retinol levels, which when converted to vitamin A, can play an important role in resistance to microbial infections (*52,53*). Thus, chronic exposure to environmental chemicals can compromise the immune system of marine mammals and increase susceptibility to infectious agents.

Since 1987, the east coast of the United States (*54*) and the Gulf of Mexico (*55*) have experienced high incidences of mortality of bottlenose dolphins (*Tursiops truncatus*). Similarly, high mortality of striped dolphin (*Stenella coeruleoalba*) populations inhabiting the Mediterranean Sea have been noted (*56*). Immunosuppression leading to infection (*57,58*) due to high body burdens of organochlorine contaminants (*56,59*) has been implicated in the dolphin mortality. Indeed, an inverse correlation was demonstrated between lymphocyte proliferative responses and levels of pentachlorinated and hexachlorinated PCBs in the blood sampled from dolphins along the west coast of Florida (*60*).

Beluga whales (*Delphinapterus leucas*) from the St. Lawrence estuary were overhunted in the early 1900s, which dropped their population from 5000 to 500 (*61*). Despite heroic efforts to protect the whales, the population has not recovered during the last 40 years (*61*). High levels of organochlorines have been measured in the tissues of these whales (*62,63*). Many of the whales show a high prevalence of lesions associated with mildly pathogenic bacteria, which suggests that immunosuppression from the organochlorines may be occurring. Furthermore, these whales exhibit very high levels of neoplasms (*64,65*), which could either implicate the organochlorines as tumor promoters or implicate immunosuppressive agents that decreased the surveillance for tumors by natural killer cells (*66*).

Biomarkers of Immunosuppression

The immune system is composed of a complex array of components that provide both cell-mediated and humoral-mediated defenses against foreign materials. The

complexity of the system provides many targets amenable to analyses as biomarkers of toxicant exposure and effect. These include analyses of both structural and functional components of the immune system, as discussed by Weeks et al. (*67*) and summarized in Table 1. Both cell and humoral components of the immune system have been shown to be susceptible to perturbation by environmental contaminants. For example, cellular responses have been observed following exposure to PAHs (*68,69*) and PCBs (*70*). Humoral responses have been measured following exposure to metals (*71,72*), pentachlorophenol (*73*), and petroleum hydrocarbons (*74*).

Pseudohermaphrodism in Invertebrates

In 1970, Blaber (*81*) observed that dogwhelks along the English coast exhibited a pseudohermaphroditic condition whereby some females possessed an appendage similar to a penis. While hermaphrodism is a reproductive strategy common to many mollusk species [i.e., prosobranchs, opisthobranchs (*82*)], the dogwhelk is dioecious. This condition, characterized by the imposition of male genitalia onto a female, was termed imposex (*83*). Definitive evaluations of gastropod populations since this initial discovery have revealed that imposex is a global phenomenon, with over 45 affected gastropod species (*84*); it has been documented along the coasts of England (*85*), Scotland (*81*), Australia (*86*), France (*87*), Canada (*88*), Japan (*89*), and the United States (*90,91*).

Most evident among affected females is the presence of a penis (Figure 1). Depending on the severity of the condition, females may also possess a vas deferens and seminiferous tubules (*92*). The consequences of this condition vary among species. In the nassariid mud snails, the development of a penis and vas deferens apparently does not interfere with normal female physiology (*91*); similar conditions among muricid whelks prevents the release of egg capsules from the ovaries and results in infertility (*93*). Imposex has also been considered responsible for excess female mortality, reduced fecundity, population declines, and local extinctions of affected gastropod populations (*94*).

A relationship between imposex and pollution was suggested when Smith (*91*) noted that imposex was prevalent among mud snail populations inhabiting marinas and that the incidence was negligible in pristine coastal areas. Further, a large percentage of mud snails collected from pristine areas and transplanted to marinas developed imposex. Laboratory exposures of snails to a variety of marina-associated contaminants revealed that paints containing tin were capable of inducing imposex (*91*). Subsequent confirmation of these observations (*95*) definitively implicated the chemical tributyltin as the cause of imposex among dioecious gastropods.

Tributyltin is a biocide that has been used extensively in a variety of products

Table 1. Immunoparameters that have been used as biomarkers of toxicant-mediated immune dysfuntion in vertebrates.

Assay	Reference
B-cell function	(*75*)
Natural killer cell activity	(*76*)
Lymphocyte count	(*77,78*)
Mitogenic response of lymphocytes	(*79*)
Macrophage count	(*80*)
Macrophage function	(*68*)
Granulocyte and macrophage hypertrophy	(*80*)

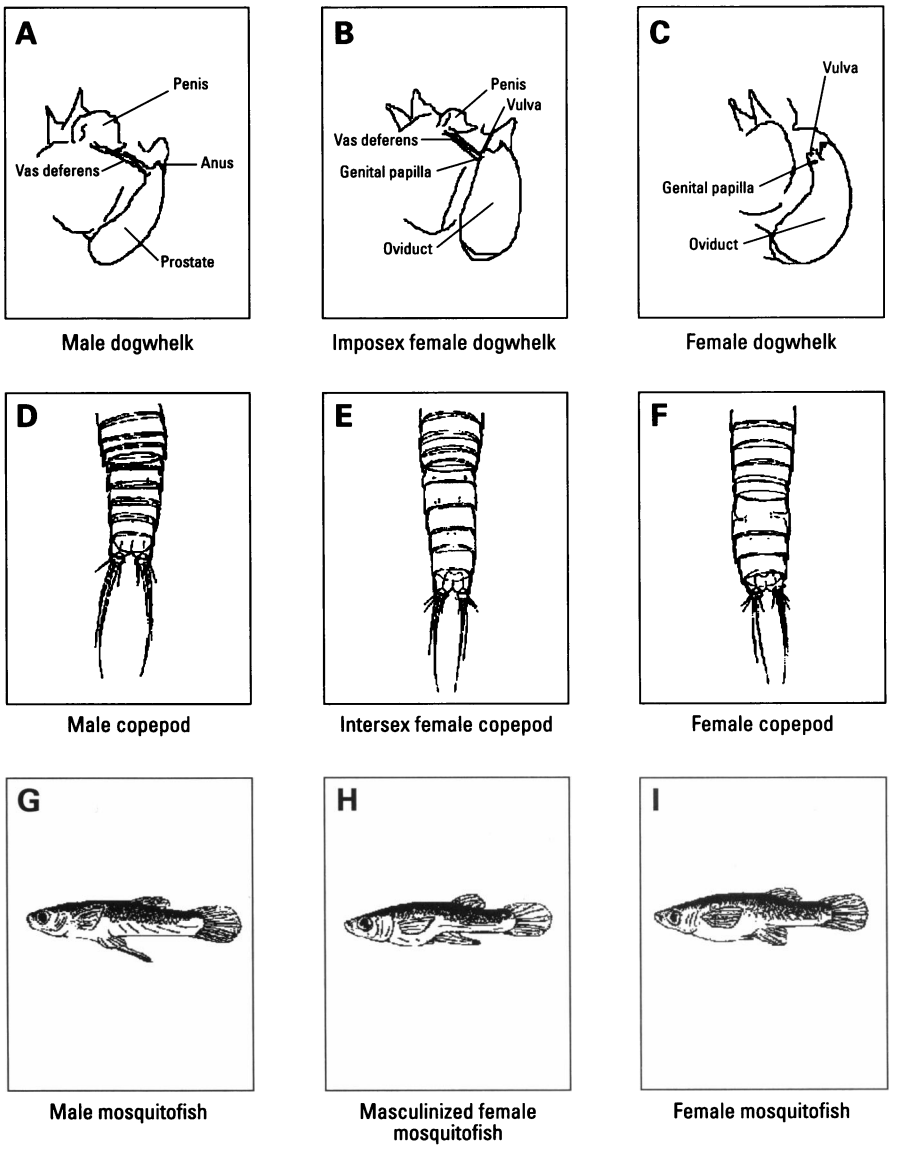

Figure 1. Sexual aberrations in dogwhelk (*A–C*), copepod (*D–F*), and mosquitofish (*G–H*) following exposure to environmental contaminants. Tributyltin caused the development of a vas deferens and a penis in female dogwhelks (imposex). Sewage effluent appeared responsible for the masculinization of the abdominal segments of female copepods. Kraft pulp mill effluent caused alterations in the anal fin of female mosquitofish resulting in a gonopodiumlike appendage characteristic of male fish. Diagrams based on information in Gibbs et al. (*92*), Moore and Stevenson (*106*), and Denten et al. (*168*).

including marine paints, fish-farming cages, disinfectants, and preservatives for wood and fiber (*96*). Its utility stems from its high toxicity. Tributyltin is acutely toxic to most aquatic organisms at ppb (µg/liter) concentrations (Table 2). However, only following the observed response of the sentinel gastropods was tributyltin found to be capable of causing imposex at concentrations as low as 1 ppt (ng/liter) (*85*). By comparison, in 1986, tributyltin concentrations were found to be as high as 1 to 2 µg/liter and in excess of 15 ng/liter in marina and nonmarina waters, respectively, along the U.S. Chesapeake Bay (*97*). Tributyltin does not affect only females. Male snails (*Ilyanassa obsoleta*) typically lose their penises upon completion of reproductive cycles. However, in areas contaminated with tributyltin and where females exhibited imposex, males were found to retain their penises following reproduction (*90*).

Tributyltin appears to elicit its effect by interfering with normal endocrine control of masculinization. Laboratory exposures of female dogwhelks to tributyltin caused a significant increase in testosterone levels commensurate with the development of a penis but with no appreciable effect on 17β-estradiol or progesterone levels, as measured by radioimmunoassay (*98*). Studies of the effects of tributyltin on testosterone metabolism have indicated that tributyltin decreases the metabolic clearance of testosterone while enhancing the conversion of testosterone to other androgenic steroid hormones (*99*).

Reports of abnormal occurrences of pseudohermaphroditic conditions are not restricted to gastropods but have also been reported among populations of crustaceans, including copepods (*106*) (Figure 1), amphipods (*107,108*), isopods (*109,110*), and penaeid shrimp (*111*). While the occurrence of intersex among crustacean populations is typically low (<1%), a 93% incidence of intersex was observed among copepods inhabiting an area receiving sewage discharge (*112*). This high incidence led the investigators to speculate that pollutants in the discharge were responsible for the pseudohermaphroditic condition. Laboratory experiments have shown that exposure of the crustacean *Daphnia magna* to a variety of environmental chemicals, including fungicides (Figure 2), detergents, and agricultural effluent significantly inhibited the metabolic clearance of exogenously administered testosterone and enhanced the production of androgenic derivatives (*113–117*). This phenomenon of metabolic androgenization is identical to that observed with tributyltin and gastropods and suggests that a variety of environmental chemicals have the potential to upset the hormonal balance of sensitive species.

Table 2. Toxicity of tributyltin to aquatic organisms.

Species	Acute toxicity, µg/liter	Chronic toxicity, µg/liter	Imposex, µg/liter	Reference
Daphnid	1.7	–	–	(*100*)
Polychaete worm	–	0.10	–	(*101*)
Copepod	1.0	0.023	–	(*102,103*)
Oyster	1.3	0.25	–	(*104,105*)
Dogwhelk	–	–	≤0.0010	(*85*)

Abbreviations: LC_{50}, median lethal concentration; LOEC, lowest observed effect concentration. Acute toxicity values are presented as the LC_{50} and chronic toxicity values are presented as the LOEC.

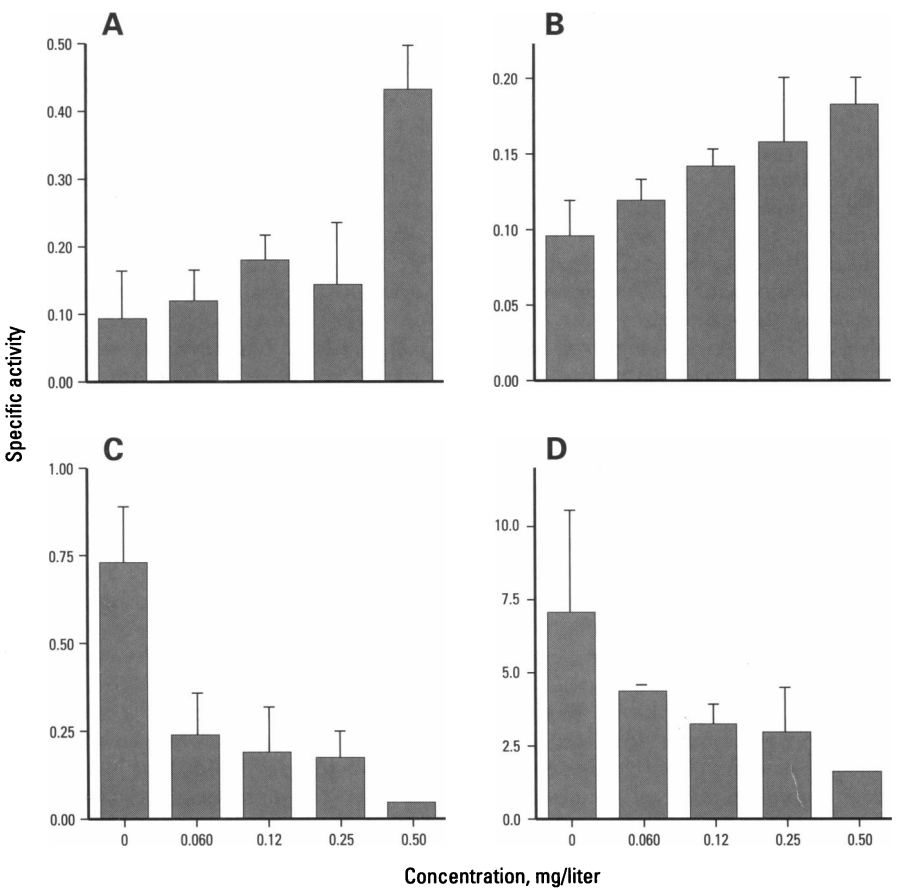

Figure 2. Altered *in vivo* metabolism of [^{14}C]testosterone by *Daphnia magna* following exposure to pentachlorophenol. Exposure to the fungicide caused an increase in the rate of synthesis of apolar testosterone derivatives [(*A*) androstenedione, (*B*) androstanediols] and a decrease in conjugated elimination products [(*C*) testosterone-glucose, (*D*) testosterone-sulfate]. Specific activities for (*A*), (*B*), and (*C*) are presented as nmol product formed/hr/mg soluble protein associated with the daphnids. Specific activity for (*D*) is presented as pmol product formed/hr/mg soluble protein associated wth the daphnids. Data are from Parks and LeBlanc (*113*) and LeBlanc (unpublished data).

Biomarkers of Pseudohermaphrodism and Metabolic Androgenization

The most commonly used indicators of imposex in gastropods are the relative penis size index (RPSI) or the vas deferens sequence index (VDSI). Briefly, individuals are narcotized and removed from their shells. The sex of the gastropod is determined by

the presence of typical sex organs (i.e., testis/prostate and penis in males; capsule gland, sperm-ingesting gland, albumen gland, and ovary in females). Quantification of imposex among females by the RPSI is accomplished by measuring the bulk of the female penis expressed as a percentage of the mean bulk of the penises of normal males (*85*). Penis bulk is calculated as the cube of its length. Using the VDSI, the stage of vas deferens development is scored on an established scale of 1 to 6 (*87*).

Should causality be established between metabolic androgenization and imposex, then this parameter may prove useful as a biomarker of pseudohermaphrodism in neogastropods and perhaps other species. Metabolic androgenization has generally been detected by administering [^{14}C]testosterone to the organisms and monitoring the rate of elimination of polar testosterone metabolites and/or accumulation of [^{14}C]testosterone and its apolar derivatives by the organisms (*99,118,119*). The apolar metabolites of testosterone typically consist of ethyl acetate-extractable metabolites that have mobilities during high-pressure liquid chromatography or thin-layer chromatography similar to those of testosterone. These products may be androgenic (i.e., dihydrotestosterone) or serve as substrates for synthesis of androgens (i.e., androstenedione). Polar metabolites of testosterone include glycosyl- or sulfate-conjugated derivatives. These metabolites are not ethyl acetate extractable but can be extracted following acid or enzymatic hydrolysis (*118*). The ratio of the rate of apolar to polar metabolites of testosterone produced provides a metabolic–androgenization index that can be used as a relative monitor of the degree of androgenization (*116,117*).

Amphibian Development

Since 1990, several studies have established that amphibian populations are declining on a global scale (*120–122*). Habitat loss from the destruction of wetlands is regarded as a major reason for these declines (*123*); however, ample evidence also suggests that amphibian populations are announcing the decline in environmental quality. Amphibians are unique as environmental sensors in that they are exposed to multiple environments through their life cycle. Eggs are generally deposited in aquatic habitats where the developing embryos are susceptible to insults of aqueous origin. The adults inhabit both aquatic and terrestrial environments. The moist, unprotected skin of many amphibians contributes to respiration and likely has reduced resilience to environmental insults when compared to feather-, fur-, or shell-bearing organisms.

Ample evidence suggests that environmental contaminants are contributing both directly and indirectly to the demise of some amphibian populations. Organophosphate pesticides constitute the most abundant class of chemicals currently used to control insect infestation. Laboratory experiments have demonstrated that many organophosphate pesticides alter the development of amphibian embryos (*124*). Observed effects include abnormal pigmentation, abnormal gut development, and notochordal defects. Most larvae exhibiting the latter defect developed into adults with deformed or missing limbs. Developmental effects occurred at exposure concentrations an order of magnitude below concentrations that elicited overt toxicity to the larvae. Recent experiments were reported in which frog and toad eggs were exposed to environmentally relevant concentrations of organophosphate, organochlorine, and carbamate pesticides commonly used in apple orchards (*125*). Growth inhibition of green frog tadpoles was noted, with the dithiocarbamate Dithane DG (Rohm and Haas Co., Philadelphia, PA) as the most potent formulation. Many frogs exposed as embryos and raised to adulthood exhibited developmental abnormalities. Teratogenic effects similar to those caused by pesticides have also been observed among frog embryos exposed to surface waters downstream from a lead and zinc mining operation (*126*). Effects included abnormal pigmentation and gut development. Heavy metals were implicated as causative agents, since water samples subjected to ion-exchange chromatography were no longer teratogenic.

The sensitivity of frogs to teratogenic effects of some pesticides, metals, and other environmental contaminants may explain the local demise of amphibian populations and may be indicative of mechanisms operative against amphibian populations on a more global scale. Increased penetration of ultraviolet (UV)-B radiation due to the depletion of stratospheric ozone poses the risk of increased incidence of a variety of maladies to both plants and animals (*127*). Chlorofluorocarbons released into the atmosphere are considered primarily responsible for ozone depletion and pose the threat of adverse effects on a global scale. A recent study documented a temporal increase in UV-B radiation ranging from 7% (summer) to 35% (winter) per year (*128*).

Amphibian eggs exposed to UV-B radiation in the laboratory hatched as larvae with many of the deformities as described following exposure of eggs to pesticide (*129*). Blaustein et al. (*130*) quantified the level of photolyase, a key UV-damage-specific DNA repair enzyme, in eggs of several amphibian species and observed a more than 80-fold interspecies difference in the level of expression of the enzyme. Perhaps significantly, photolyase levels were appreciably lower in species known to be experiencing population decline than in species showing stable population levels. Experiments were then undertaken to evaluate whether environmental exposure levels of UV-B radiation would be sufficient to adversely affect the survival of frog embryos. The hatching success of eggs exposed to ambient levels of UV-B radiation was positively correlated to photolyase activities measured in the different species. Further, the production of viable offspring was significantly increased among frogs with low photolyase activity when the eggs were shielded from UV-B radiation (*130*). Taken together, these observations suggest that increased UV-B radiation may be a global factor causing increased incidence of teratogenic damage to some frog populations and resulting in their decline.

The metamorphosis of amphibian larvae into adults may also increase the susceptibility of these organisms to environmental insult. Amphibian metamorphosis is triggered by an increase in circulating thyroxine levels (*131*). Studies in mammals have shown that ubiquitous and persistent environmental pollutants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (*132–134*) and some PCB isomers (*135*) significantly lower circulating levels of this hormone. Proposed mechanisms for the hypothyroxinemic effects of these chemicals include increased metabolic clearance and competitive displacement of the thyroxine from plasma binding proteins (*136*). Indeed, many industrial chemicals have been shown to be capable of displacing thyroxine from plasma binding proteins, resulting in decreased circulating hormone levels (*137*). Such effects on amphibians may result in delayed metamorphosis. Delayed metamorphosis was indicated in a population of salamanders inhabiting a lagoon contaminated with treated residential sewage and industrial waste (*138*). Delayed metamorphosis decreases the chance of survival by increasing susceptibility to predators (*139*). Further, many amphibian species undergo metamorphosis before the

onset of winter. Delayed metamorphosis may be lethal to such species by forcing the exposure to winter conditions of ill-equipped larvae (*140*).

Biomarkers of Amphibian Toxicity

While many factors are undoubtedly contributing to the global decline of amphibian populations, susceptibility to teratogenesis may prove to be primarily responsible for the unique susceptibility of some amphibians to diverse environmental stresses. Standardized methods for the assessment of teratogenic insult on the developing amphibian embryo are available (*141,142*) and have gained popularity as a rapid assessment method for characterizing teratogenic properties of individual chemicals and chemical mixtures (*143–147*). Amphibian teratogenicity assays are typically performed with a single species, the African clawed frog (*Xenopus laevis*), because of its availability and amenability to laboratory manipulations. Of the 10 amphibian species assayed, *Xenopus* was found to have the lowest activity of the DNA-repair enzyme photolyase (*130*), suggesting that *Xenopus* would be a suitably sensitive model for assessing DNA lesions normally repaired by this enzyme (Table 3). However, photolyase repairs DNA lesions caused primarily by UV radiation and not by chemical teratogens. Further study is necessary to evaluate the relative susceptibility of *Xenopus* to chemical teratogens and to possibly identify amphibians that are more highly susceptible to chemical teratogenesis in order to exploit these species as sentinels of chemical toxicity.

Feminization of Male Fish

Feminization is characterized by the acquisition of female traits by males. Feminization is typically indicative of exposure to estrogenic xenobiotics or increases in endogenous estrogen levels due to aberrations in hormone homeostasis. Salmonids in the river Lea and other rivers of the United Kingdom had been first noted by local fishermen to exhibit abnormal sexual characteristics (*148*). Caged male fish placed in the affected rivers were found to produce vitellogenin (*149*). Vitellogenin is a lipoglycophosphoprotein that is produced by the liver of oviparous vertebrates under the control of estrogen (*150*). Vitellogenin is normally transported to the ovaries, where it is incorporated into developing oocytes and constitutes a major portion of the yolk protein (*151,152*). Exposure of male fish to xenoestrogens can result in the production of vitellogenin by the liver and its accumulation in the blood.

Examination of potential sources of estrogenicity associated with sewage discharge implicated degradation products of alkylphenol-polyethoxylate nonionic surfactants (*148*). Alkylphenols such as nonylphenol are generated by the microbial degradation of alkylphenol polyethoxlates during sewage treatment. The alkylphenol polyethoxlates are components of cleaning products, textiles, agricultural chemicals, plastics, paper products, and personal care products (*153*). Concentrations of nonylphenols in the aquatic environment vary. Analyses of the Saginaw River, Michigan, revealed the presence of 1 ppb nonylphenol in the water column (*154*), whereas 45 ppb was measured in the Glatt River, Switzerland (*155*).

Using rainbow trout hepatocytes, Jobling and Sumpter (*156*) demonstrated that 4-nonylphenol and other degradation products of the alkylphenol polyethoxylates stimulate vitellogenin production at low micromolar concentrations. Further studies demonstrated that concentrations of the alkylphenol polyethoxlates that stimulated vitellogenin production in male trout also inhibited testicular growth (*157*). Thus, concentrations of nonylphenols and related compounds found in some aquatic environments may be sufficiently high to elicit reproductive dysfunction in fish. Consideration should also be given to the possibility that the effects of alkylphenol polyethoxylates may be extended to fish-eating vertebrates, including humans. Administration of 4-octylphenol to pregnant rats at environmentally relevant doses resulted in a significant reduction in testis size and sperm production among male offspring (*158*).

Table 3. Relative specific photolyase activity of amphibians.

Species	Relative specific activity
Dunn's salamander	<1
African clawed frog	1
Rough-skinned newt	2
Southern torrent salamander	3
Western redback salamander	5
Long-toed salamander	8
Northwestern salamander	10
Western toad	13
Cascades frog	24
Pacific tree frog	75

Data summarized from Blaustein et al. (*130*).

Demasculinization of Alligators

Lake Apopka, the fourth largest lake in the state of Florida, has experienced a precipitous decline in its alligator population since 1980 despite the concurrent maintenance of stable populations in other Florida lakes (*159*). Compared to a reference lake (Lake Woodruff), male alligators from Lake Apopka were found to have diminutive phalli, poorly organized testes, and significantly lower plasma testosterone levels (*160*). Testes from Lake Apopka and Lake Woodruff alligators were evaluated for *in vitro* steroid biosynthesis (*161*). No differences were observed in the synthesis of testosterone, though testes from Lake Apopka alligators produced significantly more 17β-estradiol compared to testes from Lake Woodruff alligators. These observations suggest male alligators from Lake Apopka have reduced plasma testosterone due to enhanced biotransformation of testosterone of other steroid hormones (i.e., 17β-estradiol) and/or elimination products (i.e., testosterone-glucuronide).

Lake Apopka is located adjacent to a U.S. EPA-designated Superfund site contaminated with the organochlorine pesticides dicofol and DDT. Alligator eggs sampled from the lake were found to contain up to 5.8 ppm of the persistent DDT metabolite *p,p'*-DDE (*162*). *p,p'*-DDE has been shown to bind the androgen receptor with 50% displacement of androgen occurring at a concentration of 5 μM (*163*). However, *p,p'*-DDE is not androgenic. Using an androgen receptor/luciferase reporter gene construct, this compound was shown to inhibit androgen-receptor-mediated transcription. Administration of *p,p'*-DDE to rats inhibited androgen-dependent processes such as ventral prostate and seminal vesicle growth (*163*). Thus, the observed demasculinization of alligators in Lake Apopka is likely to be due, at least in part, to contamination by *p,p'*-DDE.

Altered Steroid Hormone Homeostasis in Fish

Field surveys in Florida revealed the presence of mosquitofish (*Gambusia affinis*) populations that contained females expressing male anatomical and behavioral characteristics (*164*). Specifically, masculinized females possessed a modified anal fin resembling a gonopodium (Figure 1), the intromittent organ of males, and exhibited reproductive behaviors such as mating attempts. Detailed surveys of the location of these populations demonstrated that

masculinized females were located exclusively downstream of kraft pulp mills, suggesting that components of the mill effluent were responsible for this phenomenon (*164*). Indeed, laboratory exposure of female mosquitofish to kraft pulp mill effluent caused the structural modification of the anal fin to a degree intermediate between normal and masculinized females captured in the wild (*165*).

Modification of the anal fin to a gonopodium is under the regulatory control of androgen and laboratory studies have shown that exposure of female mosquitofish to aqueous concentrations of the androgens androstenedione, androstanol, and methyltestosterone masculinized the anal fin (*166*) in a manner similar to that of kraft pulp mill effluent. While androgenic steroids are not considered to be a direct component of kraft pulp mill effluent, phytosterols, which are abundant in tall oil of pine trees, may be converted to C-19 steroids by the degradative action of bacteria [Figure 3 (*167*)]. Some of these steroids may be androgenic in fish. Incubation of a phytosterol mixture consisting of β-sitosterol and stigmastanol with the bacterium *Mycobacterium* produced degradation products that masculinized female mosquitofish (*168*). Thus, while the kraft pulp mill effluent appears innocuous with respect to masculinization, the action of bacteria on effluent components once released into the environment results in the production of compounds that can have profound effects on the reproductive capacity of an exposed population.

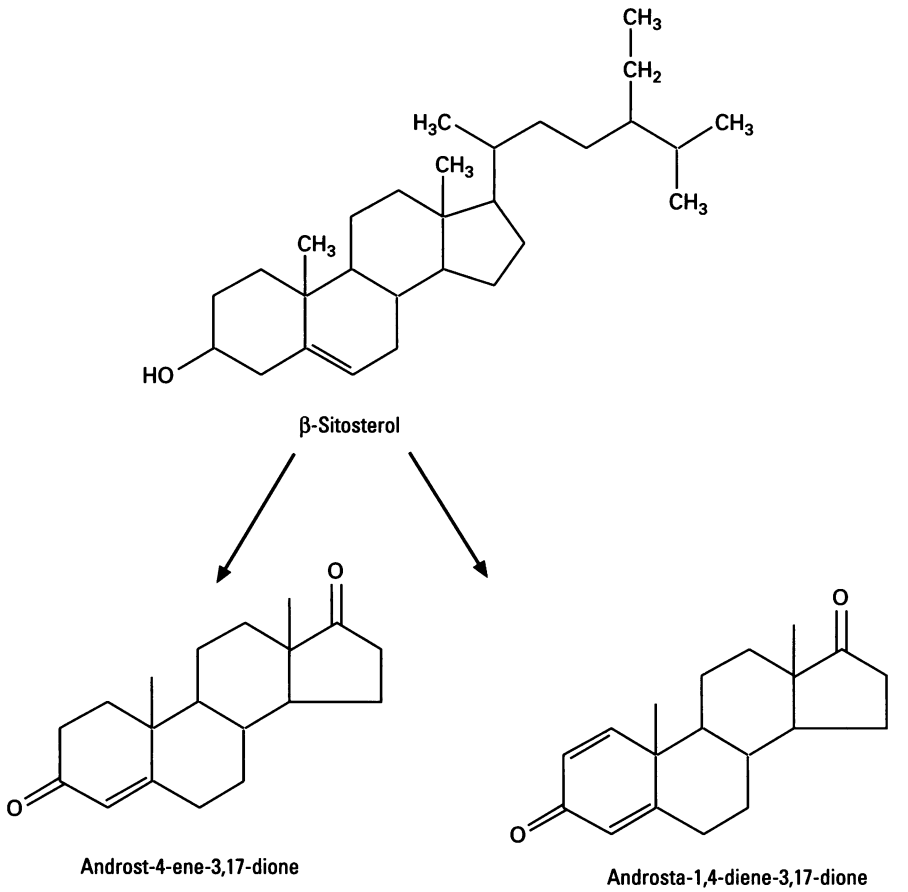

Figure 3. C19 androgens that have been shown to be generated from the microbial degradation of β-sitosterol (*167*). Androgenic degradation products are likely responsible for the masculinizing effects of kraft pulp mill effluent on female mosquitofish.

Kraft pulp mill effluents, with the ability to alter steroid hormone homeostasis by the introduction of xenoandrogens to the environment, also have been shown to reduce levels of endogenous steroid hormones in exposed fish. White sucker fish (*Catostomus commersoni*) collected from a kraft pulp mill effluent receiving area had lower serum 17β-estradiol, testosterone, 17α,20β-dihydroprogesterone, and 11-ketotestosterone levels compared to fish collected from reference sites (*169,170*). Similar observations were made among lake whitefish (*Coregonus clupeaformis*) collected from the same locations (*171*). The direct involvement of kraft pulp mill effluent was demonstrated when laboratory exposure of rainbow trout (*Onorhynchus mykiss*) to kraft pulp mill effluent was shown to reduce plasma testosterone levels by approximately 50% (*172*).

Interestingly, β-sitosterol, the phytosterol likely to be responsible for the masculinizing effect of kraft pulp mill effluent following its conversion to steroidal androgens, may also be responsible for the ability of the effluent to lower endogenous steroid hormone levels in exposed fish. Goldfish exposed to β-sitosterol experienced a reduction in plasma levels of testosterone, 11-ketotestosterone, and 17β-estradiol (*173*). This effect was not due to interference with pituitary function but may have been due to effects on gonadal steroid hormone biosynthetic capacity. Alterations in steroid hormone homeostasis by kraft pulp mill effluents may be responsible for a variety of abnormalities documented among fish in areas receiving effluents, including increased age to maturity (*170*), smaller gonads (*170*), reduced fecundity (*170*), absence of secondary sex characteristics in males (*170*), and fewer eggs at maturity (*169*).

Biomarkers of Sexual Aberration

Abnormalities in primary and secondary sex characteristics can serve as definitive, albeit insensitive, biomarkers of alterations in the regulation of sexual function. As discussed above, these effects include alterations such as modification of the anal fin of female *Poeciliidae* fishes (androgens) and reduced phallus size (antiandrogens) in reptiles. However, more sensitive biomarkers of altered sexual development are required for application to the analyses of field populations prior to eliciting overt modifications of the reproductive systems in these populations. Precedence dictates that once the exposure to environmental contaminants has caused major modifications of the reproductive system, declines in fecundity are also likely to occur.

As salmonids inhabiting contaminated regions of the United Kingdom demonstrate, elevated plasma vitellogenin levels in males of oviparous organisms can serve as sensitive biochemical markers of exposure to xenoestrogens. Vitellogenin in vertebrates is regulated by estrogen (*174*) both at the level of gene transcription and at

mRNA stabilization (*175*). Vitellogenin accumulates in the blood of estrogen-exposed males and can be readily assayed by radioimmunoassay, enzyme-linked immunosorbent assay, or immunoblotting (*176*). As discussed by Specker and Sullivan (*177*), antibodies for use in such assays have been generated to quantify vitellogenin derived from a variety of species including cartilaginous fishes, bony fishes, reptiles, amphibians, and crustaceans (Table 4). Recent studies have shown that antibodies developed to phylogenetically conserved regions of the vitellogenin protein can be used to recognize vitellogenin from organisms as diverse as fish, amphibians, reptiles, and birds (*178*).

The protein lactoferrin has shown potential as a biomarker of exposure to estrogenic chemicals in mammals. Lactoferrin, an iron-binding glycoprotein originally discovered in bovine whey, is also present in human milk, wet surface mucosa, tears, and saliva (*194,195*), and secondary granules of mature neutrophils (*196*). Lactoferrin is also found in organs of reproduction such as mammary glands (*197*) and the uterus (*198,199*). The known biological functions of lactoferrin are the stimulation of DNA synthesis (*200*), the transport of iron through the fetal intestine (*201*), the modulation of the immune system (*202,203*), and bacteriostatic/bactericidal activities by chelating iron (*204*). Lactoferrin is believed to play a role in the uterus by one or more of the following mechanisms: as an iron reservoir (*205*), by maintaining uterine proliferation (*206*), by tissue remodeling through its intrinsic RNase activity (*207–209*), or by fighting infections through its bacteriostatic (*194*) and bacteriocidal (*204*) activities.

Table 4. Species with which antibodies have been used to quantify vitellogenin levels.

Species	Reference
Little skate	(*179*)
Atlantic salmon	(*180*)
Brown trout	(*180,181*)
Spotted seatrout	(*182*)
Sole	(*183*)
Coho salmon	(*184*)
Channel catfish	(*185*)
Carp	(*186*)
Siberian sturgeon	(*187*)
European eel	(*188*)
Atlantic halibut	(*189*)
Striped bass	(*190*)
Tilapia	(*191*)
Tiger prawn	(*192*)
Blue crab	(*193*)

Like vitellogenin, lactoferrin is under the positive regulatory control of estrogen. Lactoferrin protein is normally expressed only in adult females and lactoferrin protein and mRNA levels were correlated with estrogen levels in the mouse (*210*). At diestrus, lactoferrin protein levels were very low in uterine epithelial cells and absent in uterine luminal fluid, whereas at proestrus, lactoferrin mRNA and protein levels increased in uterine luminal fluid and uterine epithelium and reached their highest levels at estrus. At early metestrus lactoferrin levels decreased, and by late metestrus, lactoferrin decreased to an extremely low level (*210,211*). The promoter region of the lactoferrin gene has been shown to contain an estrogen response element 349 base pairs (bp) upstream of the transcription start site (*212*).

Investigators have used the model environmental estrogen diethylstilbestrol (DES) to characterize responses in lactoferrin protein and mRNA levels in the mouse, which would be predictive of exposure to other environmental estrogens. When immature female mice were injected with DES for 3 days, the uterine levels of lactoferrin mRNA were increased 300-fold compared to those of control animals (*213*), making lactoferrin expression a very sensitive biomarker of exposure to xenoestrogens.

Male mice that were prenatally exposed to DES on days 9 to 16 of gestation, the major period of organogenesis, constitutively expressed lactoferrin in their seminal vesicles, whereas control mice expressed no lactoferrin (*214*). Furthermore, when similarly DES-treated male mice were castrated between 8 and 12 weeks of age and then injected three times with 17β-estradiol, lactoferrin mRNA expression in the seminal vesicles of DES-exposed mice was 6 times higher than that in control mice (*214*). Immunohistochemistry showed colocalization of the estrogen receptor along with lactoferrin in seminal vesicle epithelial cells of prenatally DES-exposed mice (*215*). These results show that exposure to the model environmental estrogen DES during organogenesis permanently alters seminal vesicle cells to a feminized state. Thus, increased lactoferrin protein and mRNA expression appears to be a sensitive biomarker of prenatal xenoestrogen exposure in males. Antibodies have been developed against lactoferrin, allowing for analyses of lactoferrin protein levels in target tissue using immunochemical approaches such as immunoblotting and immunohistochemistry (*197*). Lactoferrin mRNA has been assayed in target tissue using PCR techniques with the oligodeoxynucleotides TACAAGGGAGTGCCACCTGGCC and ACACCATGTACCCGGGCCTT as 5′ and 3′ primers (*216*).

Metabolic biomarkers of estrogenicity may also serve as means of detection of the feminizing effects of xenoestrogens. Hepatic steroid sulfotransferase enzymes are expressed in some species in a sexually dimorphic manner. These enzymes sulfurylate steroid hormones at a hydroxyl group. The sulfated steroid has significantly reduced affinity for the steroid receptor and the modification presumably functions to inactivate steroid hormones (*217*). Adult female rats express a hepatic steroid sulfotransferase enzyme that functions in the inactivation of androgens. Androgen sulfotransferase [also referred to in the literature as SBP31 (*218*) and 29kD androgen-binding protein (*219*)] is induced by estrogen and suppressed by androgen (*218,219*). Similarly, androgen sulfotransferase was found to be 10-fold higher in the livers of adult female mice than in adult males (GA LeBlanc, unpublished data). This dimorphism in mice is largely due to androgen suppression of the enzyme. Dramatic sexual dimorphisms in the hepatic expression of androgen sulfotransferase renders this enzyme potentially useful as a biomarker of exposure to environmental chemicals that are estrogenic, antiandrogenic, or have the potential to alter endogenous androgen to estrogen ratios.

While hepatic androgen sulfotransferase is under the negative regulatory control of androgen in some species, hepatic estrogen sulfotransferase expression is dependent on androgen. In rats, estrogen sulfotransferase [also referred to as SBP 34 (*218*) and 31 kD androgen-binding protein (*219*)] is expressed in the liver of adult males but not in that of adult females (*220*). Administration of androgen to females induced estrogen sulfotransferase (*218*). Exposure to environmental androgens would likely cause the same effect. Conversely, the exposure of males to environmental antiandrogens likely would cause a measurable decline in hepatic estrogen sulfotransferase. Finally, toxicant-mediated alterations in endogenous steroid hormone titers also could result in changes in steroid sulfotransferase levels. The detection of such effects could corroborate plasma steroid analyses and may be more sensitive than these analyses because of normal variability associated with this parameter. Administration of dexamethasone to rats completely suppressed estrogen

sulfotransferase activity concurrent with a significant reduction in serum testosterone levels (*218*). The ubiquity of these sexual dimorphisms in steroid sulfotransferase proteins among species other than rodents remains to be established.

Steroid sulfotransferases can be measured using a variety of approaches. Enzyme activities can be measured using liver cytosol, the appropriate substrate (i.e., testosterone and 17β-estradiol), and 3′-phosphoadenosine-5′-phosphosulfate (PAPS), the sulfate donor (*221*). Detection of the steroid–sulfate conjugate is typically accomplished with the use of [^{3}H] or [^{14}C]steroid hormone or [^{35}S]PAPS. Androgen and estrogen sulfotransferase proteins have been identified in liver cytosolic preparations using photoaffinity labeling techniques in conjunction with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (*218,221*). Antibodies have also been generated against some of the steroid sulfotransferase proteins, thus allowing the immunodetection of the proteins (*220,222,223*). cDNA probes have been generated for several steroid sulfotransferase proteins, which allows the direct quantification of mRNA levels (*222,224–226*).

Conclusions and Recommendations

Clearly, species ranging from invertebrates to mammals are currently experiencing chronic toxicity because of exposure to ambient levels of various environmental contaminants. In some cases, the presence of the contaminant in the environment represents usage and disposal patterns that are no longer relevant (i.e., PCBs, DDT). However, normal usage patterns of other chemicals (i.e., tributyltin, organophosphates) have also resulted in adverse, unpredicted consequences. Many of the effects elicited by these compounds at environmentally relevant concentrations were not identified during toxicity characterizations of the chemicals because insufficient data on the effects of the chemicals over the life cycle of organisms were obtained, insensitive species were used during toxicity characterizations, or improper end points of toxicity were assessed during toxicity characterizations.

How can existing toxicity testing protocols be improved to maximize the detection of chronic toxicity without having to conduct full life-cycle exposures on the many known sentinel species? As demonstrated in this review, retrospective analyses can be used to identify sentinel species and end points of chronic toxicity that are most likely to respond to chemical contamination. Having identified end points (i.e., carcinogenicity, immunosuppression, reproductive dysfunction) of chronic toxicity in the environment, biomarkers of such effects, as provided in this review, can be selected and utilized in subchronic testing protocols. Such an approach precludes the necessity of conducting full chronic toxicity assays in the absence of any evidence that the chemical indeed elicits chronic toxicity. Rather, the use of biomarkers of chronic toxicity during subchronic testing provides a means for the identification of chemicals that should be further tested to definitively evaluate and characterize chronic toxicity.

The successful use of this approach is contingent upon the selection of appropriate surrogate species during subchronic testing. The susceptibility of standard surrogate species to various aspects of chronic toxicity (i.e., carcinogenicity, immunotoxicity, etc.) must be elucidated and, where necessary, alternative species that are both amenable to laboratory testing and are sensitive to specific chronic effects of chemicals must be identified and exploited. Finally, additional research is needed in order to assess mechanisms of chronic toxicity. Such information will augment the selection of an appropriate surrogate species. For example, metabolic androgenization has been proposed as the mechanism by which tributyltin causes imposex in gastropods. While daphnids (a commonly used laboratory species) have not been shown to develop imposex, they have been shown to be sensitive to chemical-induced metabolic androgenization. Thus, this biomarker could be appropriately used in this surrogate species as an indicator of imposex in gastropods. Only with the sagacious use of sentinel surrogates and biomarkers of chronic toxicity during prospective hazard assessments will their use become minimal during retrospective assessments.

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