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野生生物への影響

Effects on Wildlife

The Significance of Interactions Between Endocrine Disruption and Other Mechanisms of Toxicity

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The issue of endocrine disruption continues to be a cause for concern to environmental agencies and the public world-wide. Extensive efforts are being made to identify actual and potential causative agents, and to develop appropriate screening and toxicity assessment systems. The unprecedented expenditure of resources on this topic reflects the widely held view that adverse effects on biota may be occurring via endocrine mechanisms at pollutant exposure concentrations well below those that initiate other pathways of toxicological damage. In this paper, this fundamental assumption is re-examined. The criteria for identifying different mechanism of toxicity are considered in the light of our improved understanding of biochemical and physiological responses of cells, organs and whole organisms to toxicant exposure. The inter-relations among different components of cellular detoxification and excretory systems will be outlined. Consideration of much of the endocrine disrupter literature highlights the difficulty of ascribing effects to specific mechanisms of endocrine disruption. For example, changes in growth rate, development and reproductive success might well arise as a result of altered endocrine function, but it is well known that metabolic toxicity (e.g. enzyme inhibition) and genotoxicity (e.g. altered gene expression) can produce similar consequences. New evidence will be presented which demonstrates that even the proven endocrine disrupter, tributyl tin not only affects the hormone systems of molluscs, but also is a genotoxin at extremely low concentrations. These observations highlight the need to identify specific mechanisms by which effects arise if we are to gain an holistic view of toxicity and the relative contributions of different toxicity mechanisms. How this might contribute to the planning and instigation of sensible management actions will be discussed. For example, a chemical, which is capable of disrupting endocrine function, may be a cause for concern, but if the same chemical simultaneously acts as a genotoxin and immunotoxin, at the same low exposure concentrations then even firmer management action may be warranted. Furthermore, it might also influence the choice of endpoints to be included in monitoring programmes. New approaches which take into account the biocomplexity of toxicity will be proposed.

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Endocrine Disruptor Susceptibility Genes: Molecular Analysis of Aryl Hydrocarbon Receptors and Dioxin Sensitivity in Wildlife

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Numerous environmental contaminants are distributed globally, accumulate in tissues of wildlife, and have the potential to disrupt the reproduction and development of animals. Assessing the risk of chemical exposure to wildlife is complicated by the dramatic differences in chemical sensitivity among species. Many of these environmental chemicals act by interfering with specific receptors for hormones, growth factors, and other signaling molecules. We propose that species-specific cDNA cloning and characterization of proteins involved in toxicity can contribute to risk assessment by linking mechanistic studies in rodents to epidemiological findings in wildlife. To investigate the role of receptors in species differences in toxicity, we have focused on the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor (receptor) through which planar halogenated aromatic hydrocarbons (PHAHs) such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) cause altered gene expression and developmental/ reproductive toxicity. We have undertaken the molecular characterization of AHRs from fish, birds, and marine mammals.

Fish. Many species of fish are highly sensitive to PHAHs. However, in contrast to mammals, which express a single TCDD-binding AHR, the Atlantic killifish *Fundulus heteroclitus* and several other species of bony and cartilaginous fish possess two AHR genes (AHR1 and AHR2) (1-3). Both fish AHRs bind TCDD with high affinity, but these two AHRs are highly divergent and exhibit different patterns of tissue-specific expression, suggesting distinct functions. The role of AHR polymorphisms in evolved dioxin resistance in fish is currently being investigated (4).

Birds. There are dramatic differences in sensitivity to PHAHs among species of birds (5). For example, common terns are ~80-fold less sensitive than chickens to effects of PHAHs (5, 6). To investigate the molecular mechanism of differential PHAH sensitivity, we have cloned and sequenced AHR cDNAs from white leghorn chicken *(Callus gallus)* and common tern *(Sterna hirundo).* The chicken AHR cDNA encodes a protein of 858 amino acids (96.2 kDa); the tern AHR is 859 amino acids (96.3 kDa) (7). Chicken and tern AHRs share 93% amino acid identity overall, and 98% in the ligand binding domain. Chicken and tern AHRs synthesized by *in vitro* transcription and translation exhibited specific binding of [^{3}HJTCDD. However, saturation binding analysis (0 - 10 nM J^{3}HJTCDD) showed that the binding affinity of the tern AHR was approximately 7-fold lower than that of the chicken AHR. We conclude that differences in the TCDD-binding affinity and other properties of the common tern AHR contribute to the reduced sensitivity of this species to PHAH effects.

Marine mammals. Some marine mammals accumulate extremely high concentrations of PHAHs in their tissues, but the sensitivity of marine mammals to these chemicals is not well known and cannot be determined

directly. To infer the sensitivity of marine mammals from biochemical data, we have cloned AHRs from an odontocete, the beluga Delphinapterus leucas, and a pinniped, the harbor seal Phoca vitulina. The beluga AHR cDNA encodes an 845 amino acid protein that shares 85% identity with the human AHR and 75% identity with the mouse AHR Ah^{b-1} allele (8). Beluga AHR protein synthesized *in vitro* bound [^{3}HJTCDD with an affinity that was at least as high as that of the mouse AHR and significantly greater than that of the human AHR. Comparing the beluga AHR affinity with concentrations of AHR ligands (TCDD-EQs) in beluga tissues suggests that levels of receptor occupancy are sufficient for effects to occur. Measurement of competitive AHR binding affinities for a series of PHAHs is providing an estimate of beluga-specific relative potencies ("TEFs"). The harbor seal AHR contains 843 amino acids and shares 82% and 79% identity with beluga and human AHRs, respectively (9). Like the beluga AHR, the seal AHR bound [^{3}H]TCDD with high affinity, consistent with experimental studies showing that seals may be sensitive to PHAH effects.

Invertebrates. AHR homologs have been identified and cloned from *Caenorhabditis elegans* (2, 10), *Drosophila melanogaster* (11), and the soft-shell clam, *Mya arenaria* (12). However, when expressed by *in vitro* transcription and translation, these AHR homologs lack the ability to bind [^{3}HJTCDD or [^{3}Hjb-naphthoflavone (BNF) (12). The absence of specific, high-affinity binding of the prototypical AHR ligands TCDD and BNF distinguishes invertebrate AHR homologs from vertebrate AHRs, and suggests that many invertebrates may be less sensitive to PHAHs.

Together, these results show that the use of *in vitro*-expressed proteins is a promising approach for understanding and predicting the molecular basis of PHAH toxicity in wildlife. (Supported by the U.S. National Institutes of Health grant ES06272 and U.S. National Oceanic and Atmospheric Administration Sea Grant NA46RG0470 (R/P-58) and NA86RG0075 (R/P-64)).

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The Effects of Endocrine Disruptors on Fish Maturation and Reproduction -A Focus on Projects Underway at the Ministry of Agriculture, Forestry, and Fisheries-

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1. Introduction. Endocrine-disrupting effects that certain chemical substances cause in fish appear in reports published outside Japan on the ovaries and testes of roach and flounder, the development of male sex organs in female mosquito fish, and enlargement of the thyroid gland in salmonids. These reports warn of the undesirable effects of these chemical substances on maturation and reproduction in these fish. Japanese reports on the effects of endocrine disruptors on fish-which document carp in the Tama River and the marbled sole in Tokyo Bay-are fragmentary, and details have yet to be elucidated. The effects on sexual differentiation and gonad development, the mechanism of action on behavior, and influence on reproduction in fish are unknown. It is feared that endocrine disruptors may travel through the food chain to concentrate in marine life, detracting from its safety as a source of food.

This is the situation faced by the water-area team in "the integrated research program for effects of endocrine disrupters on agriculture, forestry and fisheries and their action mechamisms on domestic animals and fishes (environmental hormone research) funded by the Ministry of Agriculture, Forestry, and Fisheries," whose mission is to promote research into both the endocrine and reproductive functions and reproductive process in the wide variety of marine life that constitutes the aquatic ecosystem and the behavior of chemical substances in bodies of water, including the concentration of chemicals in living creatures accumulated via the food chain. To this end, three sub-teams were formed: (1) the conditions of influence sub-team, (2) the environmental kinetics sub-team, and (3) the action mechanism sub-team. These three are currently working on research on 24 topics (8 per sub-group). As the title suggests, this paper will present the results of research conducted over the past two years of the project, focusing on the effects of endocrine disruptors on fish maturation and reproduction.

2. Vitellogenin Assay. Systems to measure vitellogenin (Vg) using ELISA in chub, carp, mudskippers, spiny goby, gray mullet, white herring, stone flounder, ridged-eye flounder, right-eyed flounder, and three-lined tongue sole have already been established by Matsubara et al. (Hokkaido National Fisheries Research Institute) Ito et al. (National Research Institute of Fisheries Science) and Yurimoto et al. (Seikai National Fisheries Research Institute). Blood Vg concentrations throughout the year have been measured in carp, chub, mudskippers, and other fish. The measurements indicate seasonal variation in males. The base blood Vg concentration in males of certain fish varies. Threshold values for endocrine-disrupting effect in Hokkaido ranging from 100 ng/ml spiny goby to 10/ μ g/ml or above in chub have been proposed.

3. Choriogenin Assay. The marbled sole was the first marine fish in Japan noted to be affected by endocrine disruptors. Focusing on this species, I developed a system to measure choriogenin (Cg), whose production is induced by estrogen, as that of vitellogenin is. Chemical induction was also confirmed. The research suggests that the production ofCg may be induced by lower concentrations of estrogen-like substances than are required to induce Vg production.

4. Receptor Assay. Ikuta et al. (National Research Institute of Aquaculture) developed a receptor assay that employs rainbow trout liver cell proteins and europium-labeled E2 to establish a system for rating the estrogen activity of chemical compounds *in vitro.* This assay method was used to measure the binding capabilities of bisphenol A and nonylphenol, which were determined to be 1/67 and 1/3,094 in comparison with E2.

5. Sexual Behavior. Ikuta et al. (National Research Institute of Aquaculture) investigated the effects of endocrine disruptors on the sexual behavior of the masu salmon. A variety of steroids were administered to castrated males. Sexual behavior repressed by castration (approaching, encouraging egg laying) was restored by male hormones (T, 11KT), but no such effects were noted after E2 administration. In addition, E2, nonylphenol, or bisphenol A was administered to precocious males. Although E2 caused no pronounced results, nonylphenol and bisphenol A tended to suppress male sexual behavior. This finding suggests that the latter two substances may act as antagonists to male hormones in the brain.

6. Gametogenesis. Kagawa et al. (National Research Institute of Aquaculture) are investigating the effects of endocrine disruptors on the reproduction-governing system constituted by the hypothalamus (gonadotropin releasing hormone: GnRH), pituitary gland (gonadotropin hormone: GTH), and gonads (steroidal hormones). When various steroids were administered to immature and sperm-producing male red sea bream, the further administration of E2 or T to the immature fish suppressed spermatogenesis, but the hormones were ineffective in the sperm-producing sea bream. Expression of the GnRH precursor gene and the GTH subunit gene was unaffected in both cases. These findings lead to the conclusion that environmental hormones affect spermatogenesis, and the associated action is manifested not on GnRH or GTH production but rather on the excretion of these hormones or directly on the testes.

7. Sexual Differentiation. Nakamura (University of the Ryukyus) investigated the expression of the steroid-metabolizing enzyme cholesterol side-chain cleavage enzyme, 17 α -hydroxylase, 3 β -hydroxydehydrogenase, and aromatase accompanying sexual differentiation in all-male and all-female tilapia. The professor learned that this group of enzymes is expressed in female gonads before sexual differentiation and is rapidly activated in conjunction with sexual differentiation, meaning that the onset of E2 synthesis probably leads to differentiation into a female. These enzymes were not identified in males either before or during sexual differentiation. The administration of an aromatase inhibitor (Fadrozole) to all-female tilapia during sexual differentiation led to differentiation into males. In an experiment in which sexually differentiating, all-male amago salmon were exposed to 10 ng/L ofEE2, 20 ng/L of E2, 20 μg/L of nonylphenol, or 1 mg/L of bisphenol A, the fish became females. Differences in the duration of exposure brought about different rates of female conversion. In conclusion, the effects of these substances on sexual differentiation are great.

8. The State of Endocrine Disruptor Influence in Japan. Although research to the present has identified slightly high levels of male fish Vg in a few areas, no gonad abnormalities have been reported. However, certain locations are high in estrogen activity, according to *in vitro* bioassays (Kawai of Kobe College). These areas will be studied in further detail. Gender in some species of fish changes according to age, physical factors such as water temperature, and school sex ratio. Gender in fish is much less stable than it is in mammals. Vg and Cg differ according to species and physical factors such as water temperature. It is therefore currently impossible to set allowable ranges for substances with estrogenic activity for areas in which these substances are detected.

The first two years of this research project have been devoted to supplying researchers with the tools to accurately ascertain the level of influence of estrogen disrupters in Japan. Evaluation methods have been

developed, and normal conditions have been described so that they can be differentiated from abnormal conditions. Hereafter, the water-area team of the project will strive to promptly identify threshold values (of concentrations of Vg, Cg, and other substances) for different species of fish, seek evidence of endocrine disruptor influence wherever it may lie, and identify the substances behind any abnormalities that are observed.

Atrazine Produces Hermaphrodites in Frogs: Connecting Laboratory and Field Studies

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Recently, we showed that atrazine exposure produces hermaphrodites in larval African clawed frogs (*Xenopus laevis*) exposed to as little as 0.1 part per billion. In some cases, individuals contain as many as three ovaries and three testes. Further, the larynx in this species is androgen dependent and males have larger larynges and larger laryngeal muscles. Atrazine doses of 1 ppb and higher demasclunized males and resulted in decreased laryngeal sizes. When exposed larvae were allowed to grow for two months without exposure, the larynx did not recover. In addition, exposure to atrazine aftert metamorphosis further decreased the size of the larynx. Thus, the effect appears to be permanent. In juveniles, atrazine-exposure also resulted in decreased development of male breeding glands and an increased incidence of female-typical cloacal development. The latter effect indicates that the compound feminizes in addition to the demasculinizing effects. Exposing adult *X. laevis* to atrazine reduced androgen levels ten-fold, such that testosterone levels in exposed males were indistinguishable from females. This alteration in steroid hormone levels, suggests a mechanism for the observed effects: Atrazine appears to decrease androgen levels (potentially with a concomitant increase in estrogens). In all cases, at all developmental stages, females appear unaffected and males seem to be vulnerable.

In a continuing study, we examined the effects of atrazine on leopard frogs (*Rana pipiens*) and Pacific treefrogs (*Hyla regilla*). Characterization of effects in these two species is ongoing, but in addition to potential effects on sex differentiation, atrazine inhibited metamorphosis in *R. pipiens,* but not *H. regilla.*

The doses that produced the effects described here are ecologically relevant. Atrazine can be found at parts per million in agricultural runoff and in habitats where amphibians breed. In ground water, surface water, and even rainwater atrazine levels can exceed 1 ppb. Thus, wild amphibians may be at risk. In particular, these effects raise concern because atrazine is widespread (used for more than 40 years in the US and used in over 80 countries). The possible role of atrazine (and pesticides, in general) in global amphibian declines is another important consideration.

To address the effects of atrazine in the wild, we have collected animals from ten localities across the United States. The collections were conducted in areas where there is no reported atrazine use and areas that report the highest atrazine-use. The habitats where collections were conducted range from direct agricultural runoff, recreational areas (golf courses), wildlife refuges, rangeland, rivers, lakes, ponds, and irrigation ditches. We are currently analyzing atrazine levels in these waters in attempts to correlate developmental anomalies with atrazine levels. Further, we have collected water from the wild for conducting bioassays in the laboratory. This combination of laboratory and field studies will allow careful examination of the effects and mechanisms of atrazine action as well as an assessment of the impact effects in wild amphibians.

Beyond Estrogens -Multiple Mechanisms of Endocrine Disruption

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Wildlife and human populations are affected by contaminants in natural settings. This problem has been a growing concern over the last decade with the realization that various environmental chemicals can alter the development and functioning of endocrine organs, cells and target tissues. Documented disruptions or alterations in reproductive activity, morphology or physiology in wildlife populations have been correlated with contaminant-induced modifications in endocrine system functioning. Alterations of the endocrine system are complex, and not limited to a particular organ or molecular mechanism. For instance, contaminants have been shown to (1) alter hormone production at its endocrine source, (2) alter the release of stimulatory or inhibitory hormones from the pituitary or hypothalamus, (3) alter hepatic enzymatic biotransformation of hormones, and/or (4) alter the concentration or functioning of serum binding proteins, altering free hormone levels in the serum. New data from our laboratory implicates two of these alterations, altered hormone synthesis and hepatic biotransformation. Further, we have begun to document the endocrine disruptive effects of nitrate/nitrite exposure in several species, including the American alligator. These data suggest that concentrations of nitrate as low as 10 ppm (approved drinking water limits in the USA) can alter gonadal steroidogenesis in juvenile American alligators. An understanding of the developmental consequences of endocrine disruption in wildlife can lead to new indicators of exposure and a better understanding of the most sensitive life stages. Further, understanding the endocrine disruptive potential of environmental pollutants, other than industrial chemicals and pesticides, will allow us to calculate the true risk of environmental pollution on wildlife. Research funded in part by grants from the US EPA (#R824760-01-0;CR826357-01-1).