Frog Deformities: Role of Endocrine Disruptors During Development

Project Scope

The high incidence of developmentally deformed frogs (e.g., multiple limbs, incomplete limbs) observed in North America, coupled with the worldwide decline in the occurrence of amphibian species, suggests that environmental change is negatively affecting amphibian populations. Although the cause of amphibian declines in relatively pristine environments remains unknown, there is an emerging consensus that the increasing prevalence of deformed frogs is the result of waterborne contamination that has appeared or reached a critical concentration in recent years.

The aim of this project was to assess endocrine disrupting chemicals (EDCs) that activate retinoid signaling pathways for their role in causing limb developmental deformities in frogs and to understand their mechanism(s) of action. The objectives of this project were to:

- Characterize the formation of bony triangles (a specific type of deformity) in response to retinoid exposure using the highly active retinoid TTNPB[1];
- Determine whether other commonly used chemicals induce bony triangles;
- Screen and fractionate field samples collected from deformed frog sites to identify the agent(s) causing abnormalities in the wild.

Grant Title and Principal Investigator

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Key Findings

- All of the limb deformity phenotypes observed in wild populations of frogs can be induced in the laboratory by TTNPB exposure at appropriate stages of development.
- There are multiple developmental windows of sensitivity during limb bud development, and these windows are remarkably short.
- At high doses and long exposures, TTNPB is acutely toxic at all stages of development, causing death within a few weeks of exposure.
- Using the parameters established from studies of TTNPB effects on stages of *Xenopus* limb development, a developmental toxicology protocol to screen the activity of a large number of known pesticides was established.

Project Period: October 1999 to September 2002

Preliminary studies of a variety of vertebrate species by several investigators have discovered a frequent yet novel malformation termed a bony triangle. Triangles have been seen before in several species of animals and in humans, but only as a result of exposure to retinoids. The central hypothesis here is that inappropriate activation of retinoid signaling pathways at sensitive life-stages causes deformities and that finding of bony triangles in field populations are indicative of exogenous retinoid exposures. Mechanistically, it has been proposed that triangles arise as a consequence of changes in pattern specification along the proximal-distal limb axis, mediated by retinoid-induced alterations in the expression of genes important for establishment of limb pattern. It is further proposed that bony triangles in deformed frogs in the wild can arise as a result of exposure to EDCs acting on retinoid signaling pathways.

[1] The full chemical name of TTNPB is (**E**)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthylenyl)-1-propenyl] benzoic acid.

Project Results and Implications

Skeletal dysplasias were observed in severely affected frogs from Minnesota and two classes of common limb abnormalities were identified. Supernumerary or absent limbs suggested that the process of limb initiation is being affected. Skeletal abnormalities, including truncated and phocomelic limbs, suggested that limb growth and pattern formation also are being modified. In the phocomelic limbs, the skeletal elements are folded back on themselves, such that the proximal and distal ends of the bone lie adjacent to one another and the mid-portion of the bone projects laterally, forming a "bony triangle."

Because most deformed frogs come from agricultural areas, it is plausible that a commonly used agrochemical is involved. Chemicals that test positive for retinoid action in the *Xenopus* assay were retested using a native frog species (*Rana pipiens*) known to develop deformities in the wild. Lake water collected from deformed frog sites were screened and fractionated to test for the agent(s) that could be causing abnormalities in the wild. A high-throughput bioassay-guided screen for fractions that can activate retinoid receptors in cultured cells was used. Positive fractions were assayed for their ability to induce bony triangles in *R. pipiens.*

Relevance to ORD's Multi-Year Research Plan

This project contributes to two important long-term goals of the ORD's MYP: (1) to support EPA's screening and testing program, and (2) to determine the extent of the impact of endocrine disruptors on humans, wildlife, and the environment.

This research improved the understanding of environmental EDCs that activate the retinoic acid receptor (RAR) in amphibians, likely causing many of the limb malformations observed in frog populations in the wild. This project has contributed to developing an explanation of: (1) the types of a limb malformations observed in *Xenopus laevis* (including bony triangles), and (2) the steady decline in the occurrence and abundance of natural amphibian populations. In addition, this project developed a protocol to identify compounds from water samples that activate the RAR and, thus, are suspected to induce limb malformation in natural populations of frogs in particular.

Metamorphosing *Xenopus laevis* larvae were treated with receptor-selective retinoids to establish the time, dose, stage, and signaling pathway involved in bony triangle formation. Downstream target genes of TTNPB (i.e., those involving in the induction of each of the various limb malformations) and the stage-specific expression of molecular markers of limb development (e.g., such as Bm-4, Shh, Gdf-5, Xhoxa11, Xhoxa13, Meis-2, Wnt-14, Xhoxd-11 and Xhoxd-13) were examined. Using the parameters established from the studies of TTNPB effects on stages of *Xenopus* limb development, a developmental toxicity assay to screen the activity of a large number of known pesticides was established. A large number of chemicals were selected (see Table 1) by a variety of criteria, including known effects on retinoid-sensitive signaling pathways and known presence in water samples from a central Minnesota lake (Minnesota Crow Wing County B [CWB] site), which has a high frequency of frog limb malformations.

If environmental retinoids are the cause of frog deformities observed in the field, then retinoids will be found at sites where deformed frogs are found. To test this hypothesis, hydrophobic substances were extracted from water samples taken from the CWB site, and then fractionated by high-performance liquid chromatography and tested for their ability to activate the retinoic acid receptor in transient transfection assays. Active fractions were purified to homogeneity as judged by ultraviolet absorption spectra and then analyzed by electrospray and electron impact mass spectroscopy for exact mass determination. Candidate compounds thus identified then were retested in the reporter and animal assay to verify biological activity. Similar activity peaks have been discovered in water samples from a vernal pond in Mission Viejo, California, where similar limb malformations have been seen in Pacific tree frogs at seasonally high frequencies, as in the CWB site in Minnesota.

Table 1. Compounds Tested for the Ability to Disrupt Retinoid-Sensitive Signaling Pathways During Amphibian Limb Development

Atrazine	Aatrax	Nonylphenol
Bromoxynil	Tetrachlorobisphenol A	Tetrabromobisphenol A
Bisphenol A	Nonylphenol plus TTNPB	Nonylphenol plus coumaphos
Nonylphenol plus DEET	Sevin (carbaryl)	Bladex
MCPA	Triphenyltin acetate	4-Heptylbenzoic acid
Phenothiazine	p-Terphenyl	Aximphos-ethyl
Permethrin	Nitrite	Maneb
Tributyltin	Triethyltin bromide	POE (8)
Polyoxyethylene (POE) (9-10)	POE (10-11)	POE (12)
Dicamba	Fenitrothion	DEET

In addition to the laboratory studies, field sites in Minnesota continue to be monitored. At the CWB site, populations of all ranid frog species have declined during the 3 years of the study. In recent years, no leopard frog or green frog calls were heard, and only scattered mink frog calls were heard. Only one leopard frog juvenile was found at the site during the entire 2000 season (compared to 562 in 1997, a year of similar sampling effort, for example). Mink frog capture success also was decreased; only 74 juveniles were captured in 2000, compared to 365 in 1997. The mink frog was the only species with enough juvenile captures to calculate meaningful malformation frequencies. The total malformation frequency among juveniles in 2000 was 18 percent, apparently lower than 1996-1999, when the frequency ranged from 50-75 percent, but still much higher than the working “background” frequency of 1 percent.

This study provides critical information about the links between deformed frogs and environmental retinoids. There are multiple developmental windows during *Xenopus* and *Rana* early limb bud development during which retinoid signaling induces multiple phenotypes. *Xenopus* limb bud development occurs in larval development stages 48 to 55. Experiments have been completed assessing the effects of a known activator of retinoic acid receptor (RAR)-mediated signaling (i.e., TTNPB) on limb bud stages of developing *Xenopus* tadpoles and in *Rana sylvatica*. All the phenotypes observed in wild populations of frogs can be induced in the laboratory by TTNPB exposure for periods as short 3 hours or as long as two weeks at appropriate stages of development.

The results indicate that there are multiple developmental windows of sensitivity during limb bud development, and that these windows are remarkably short. TTNPB is toxic at all stages of development at high doses (≥ 100nM) and long exposures (> 24 h), as evidenced by the death of virtually all larvae a few weeks after exposure. At lower doses, survival is high (comparable to negative control); and, between stages 50 and 53, the rate of malformation is greater than 50% at low doses (i.e., 40 and 80nM). Treatment at stage 48/49 has no observed phenotypic effects on surviving tadpoles. Treatment at stage 50/51 induces duplicated limb buds. Treatment with TTNPB at stage 51/52 induces bony triangles. Treatment at stage 52/53 induces the loss of distal limb structures. Although both forelimbs and hindlimbs can be affected, hindlimb development is more frequently affected in the wild. Equivalent results have been obtained with studies of *Rana*. These data demonstrate that retinoids only induce malformations, such bony triangles, during specific windows of sensitivity; in addition, different types of malformations are induced depending on the stage of development when retinoid exposure occurs. Exposures to retinoids at earlier developmental stages induced malformations in other organ systems (i.e., craniofacial, axial), but not in the limbs.

Retinoid treatment at sensitive stages mimics deformities in wild frogs. By exposing larvae to TTNPB at different stages of development, all of the limb deformities that have been observed in wild populations of frogs could be induced in the laboratory. Using the parameters established in these studies, a

developmental toxicity assay has been developed to screen EDCs for RAR activation. This assay can be used to screen the chemicals known to be present at sites where deformed frogs are found to determine the chemicals' ability to induce limb malformations.

Field studies indicate that populations of amphibians continue to decline, and that new populations of deformed frogs continue to be discovered.

Investigators

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NCER Project Abstract and Reports:

http://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/443/report/0