Synthetic chemicals found in the environment have the capacity to disrupt the development and function of the endocrine system in both wildlife and humans. This has drawn public concern since many of these chemicals may bind to estrogen receptors (ERs) and evoke estrogenic effects. Early evidence that exposure to estrogenic chemicals during development could pose a threat to human health came from studies of a synthetic hormone, diethylstilbestrol (DES), which was used to prevent premature birth and spontaneous abortion. Laboratory experiments showed that exposure of animals to sex hormones during critical windows of perinatal life caused irreversible alterations to the endocrine and reproductive systems of both sexes. The immune and nervous systems, bone, muscle, and the liver were also affected. Although many of these chemicals can bind to ERs in wildlife and humans, the molecular basis for the action of environmental estrogens remains poorly understood. Thus, understanding the molecular mechanisms through which environmental estrogens and sex hormones act during critical developmental windows is essential.

I. Developmental origin of adult disease:
Perinatal estrogen exposure induces persistent changes in reproductive tracts

The emerging paradigm of the “embryonic/fetal origins of adult disease” provides a powerful new framework for considering the effects of endocrine disrupters on human and animal health. In 1971, prenatal DES exposure was found to result in various abnormalities of the reproductive tract in women. This syndrome was named the DES syndrome. Similar abnormalities have been demonstrated in experimental animals exposed perinatally to estrogens. Developmental estrogen exposure in mice, for example, induces persistent proliferation of vaginal epithelial cells. We found that the persistent changes in the vagina in mice exposed neonatally to estrogens result from the persistent activation of erbBs and ERα, and sustained expression of EGF-like growth factors. Currently, we are analyzing the methylation status in the mouse vagina using MeDIP (methylated DNA immunoprecipitation) coupled with a microarray (MeDIP-chip). We found several differentially methylated or demethylated DNA profiles in neonatally DES-exposed mouse vaginae and controls. We thus consider that neonatal DES exposure affects DNA methylation profiles, resulting in persistent abnormalities in mouse reproductive organs.

II. Estrogen receptors of birds, reptiles, amphibians and fishes

Steroid and xenobiotic receptors (SXR) have been cloned from various animal species (fish, amphibian, reptiles, birds and mammals) by our group and we have demonstrated species-specific differences in their responses to various environmental and endogenous chemicals (receptor gene zoo). Thus, simple predictions of chemical effects based on data from a few established model species are not sufficient to develop real world risk assessments. ER and ER-like genes have been cloned from various animal species including rockshell, Amphioxus, lamprey, lungfish, sturgeon,
gar, roach, stickleback, mosquitofish, mangrove *Rutilus*,
catshark, whale shark, Japanese giant salamander, Tokyo
salamander, newt, axolotl, toad, *Silurana tropicalis*,
American alligator, Nile crocodile, freshwater turtle,
Japanese rat snake, Okinawa habu, and vultures. Functional
studies showed that the *Amphioxus* ER sequence does not
bind estrogen but *Amphioxus* steroid receptor and lamprey
ER exhibited ligand-dependent transactivation, proving that
invertebrate and primitive vertebrates, such as the Agnatha,
have a functional ER. We found that medaka ER subtypes
have their specific functions, and medaka, zebrafish and
stickleback ERs are more sensitive to estrogen/estrogen-like
chemical exposures than other fishes by reporter gene assay.
Thus, these approaches are efficient to evaluate the
relationship between species and their sensitivities to
chemicals.

III. Evolutionary history and functional
characterization of androgen receptor
genes in jawed vertebrates

Vertebrates show diverse sexual characteristics which
are regulated by androgens. To elucidate the evolutionary
history and functional diversification of androgen
receptor (AR) genes in vertebrates, we cloned the AR
cDNAs from a shark, basal ray-finned fishes
(Actinopterygii), namely bichir and sturgeon
(Acipenseriformes), and teleosts including a basal teleost,
arrowana (Osteoglossiformes). Molecular phylogenetic
analysis revealed that a gene duplication event gave rise
to two different teleost ARs (α and β) and likely occurred
in the actinopterygian lineage leading to teleosts after the
divergence of Acipenseriformes but before the split of
Osteoglossiformes. Functional analysis revealed that the
shark AR activates the target gene via androgen response
element by classical androgens. The teleost AR
showed unique intracellular localization with a significantly
higher transactivation capacity than that of teleost ARβ.
These results indicate that the most ancient type of AR, as
activated by the classic androgens as ligands, emerged
before the Chondrichthyes-Osteichthyes split and the
AR gene was duplicated during a teleost-specific gene
duplication event.

IV. Gene zoo and receptor zoo

We are establishing cDNA library banks and receptor gene
banks of animal species including lancelet, lamprey,
sturgeon, lungfish, gar, mangrove *Rutilus*, whale shark
catshark, Japanese giant salamander, newt, *Rana rugosa*,
*Silurana tropicalis*, Japanese rat snake, Okinawa habu,
Florida red berry turtle, American alligator, Nile crocodile,
vulture and polar bear in collaboration with the University of
Pretoria, South Africa, University of Florida, Medical
University of South Carolina, San Diego Zoo, USA, and the
Asa Zoo in Hiroshima.

V. Sex differentiation mechanism in Daphnids

*Daphnia magna* has been used extensively to evaluate the
organism- and population-based responses of toxicity or
reproductive toxicity tests. These tests, however, provide no
information about the mode of action of the tested
compounds. Therefore, we applied an ecotoxicogenomic
assessment of *D. magna*. We established a *Daphnia* EST
database and developed an oligonucleotide-based DNA
microarray with high reproducibility and demonstrated the
usefulness of the array for the classification of toxic
chemicals as well as for the molecular understanding of
chemical toxicity in a common freshwater organism. *D. magna*
reproduce asexually (parthenogenesis) when they are
in an optimal environment for food, photoperiod and
population density. Once environmental conditions become
sub-optimal, they alter their reproductive strategy from
asexual to sexual reproduction. Chemicals are able to affect
the sex determination of *D. magna* and we found that
juvenile hormone (JH) agonists (insect growth regulators),
for example, induce the production of male offspring. The
molecular basis of environmental sex determination is
largely unknown in *D. magna*. To understand the molecular
mechanisms of this phenomenon we isolated sex
determination-related genes. Also, we have developed a
method to inject genes into *D. magna* embryos which will
allow us to study gain- and loss-of-function analyses in more

![Figure 3. Evolutionary relationships of androgen receptor sequences.](image)

![Figure 4. Life cycle of *Daphnia*.](image)
detail in this species. Using these techniques, we demonstrated that DSX1 (double sex 1), one of the DM-domain genes, is essential for male differentiation in *D. magna*. To further explore the signaling cascade of sexual differentiation in *D. magna*, a gene expression profile of JH-responsive genes is essential. Thus, DNA microarray analysis has been performed in the gonads of *D. magna* exposed to fenoxycarb (synthesized JH agonist widely used as an insect growth regulator) and methyl farnesoate (JH identified in decapods) at the critical timing of JH-induced sex determination in *D. magna*. We are currently identifying JH-responsive genes in the ovary.

**Publication List**


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