# Reproductive and endocrine effects of o,p'-DDT, an environmental estrogen, and p,p'-DDE, an antiandrogen, in male and female Atlantic croaker during critical periods of their reproductive life history cycles

## Project Scope

The aim of this research was to understand the nature, extent, and mechanisms of reproductive and endocrine toxicity of a representative xenobiotic estrogen, o,p'-DDT, and a putative xenobiotic antiandrogen, p,p'-DDE, in an established vertebrate model, the Atlantic croaker.

The following specific hypotheses were tested:

- Embryological development, gonadal differentiation, puberty, and gonadal growth are stages of the Atlantic croaker reproductive life history cycle that are sensitive to disruption by estrogenic and antiandrogenic chemicals.
- The reproductive toxicities of o,p'-DDT and p,p'-DDE in both male and female croaker are primarily due to their estrogenic and antiandrogenic activities, respectively.
- Endocrine disruption by xenoestrogens in males is mediated by their binding to the testicular estrogen receptor (ER), and disruption by antiandrogens in females is caused by their binding to the ovarian androgen receptor (AR), in addition to the more traditional sites of estrogen and androgen action on the hypothalamus-pituitary-gonadal-liver (HPGL) axis.
- Males, in general, are more sensitive than females to the reproductive effects of estrogenic chemicals.

The objectives of this research project were to determine:

- the potential roles of the ER and the AR in mediating the actions of endocrine disrupting chemicals;
- which stages of the croaker reproductive life history cycle are disrupted by o,p'-DDT and p,p'-DDE;

## Grant Title and Principal Investigator

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## Key Findings

- Separate genes for three estrogen receptors (ERs), ER alpha, ER beta and ER gamma, were found in Atlantic croaker, the first vertebrate species in which three distinct nuclear ERs have been identified.
- Brain androgen receptor (AR)1 levels in both male and female Atlantic croaker are greatly influenced by gonadal status and circulating levels of sex steroids.
- Plasma membrane receptors for estrogens and androgens and nonclassical, nongenomic actions of these steroids have been identified in croaker gonads.
- O,p'-DDT (a xenbiotic estrogen) and p,p'-DDE (a putative xenobiotic antiandrogen) bind to a variety of steroid receptors in fish reproductive tissues. Greater reproductive impairment was observed with o,p'-DDT compared to p,p-DDE. The differing patterns and degrees of impairment of reproductive and endocrine function observed after exposure to these two DDT derivatives is probably largely a reflection of their different mechanisms of endocrine disruption.

**Project Period: October 1997 to October 2001**

- what endocrine and reproductive effects of o,p'-DDT and p,p'-DDE are related to their estrogenicity and antiandrogenicity, respectively;
- the sites of o,p'-DDT and p,p'-DDE action on the HPGL axis; and
- whether there are sex differences in the susceptibility to o,p'-DDT and p,p'-DDE.

## Project Results and Implications

Two major receptor-mediated mechanisms of steroid action have been identified in vertebrates: (1) classic, relatively slow mechanism of steroid action via binding to intracellular nuclear steroid receptors, translocation to the nucleus and binding to hormone response elements of genes resulting in alterations in their rates of transcription (genomic mechanism), and (2) alternative rapid mechanism of action via binding to receptors on the cell surface, called membrane receptors, resulting in activation of ion channels or intracellular secondary messengers (typically nongenomic). There is also the possibility of crosstalk between these two signaling pathways (see Figure 1).

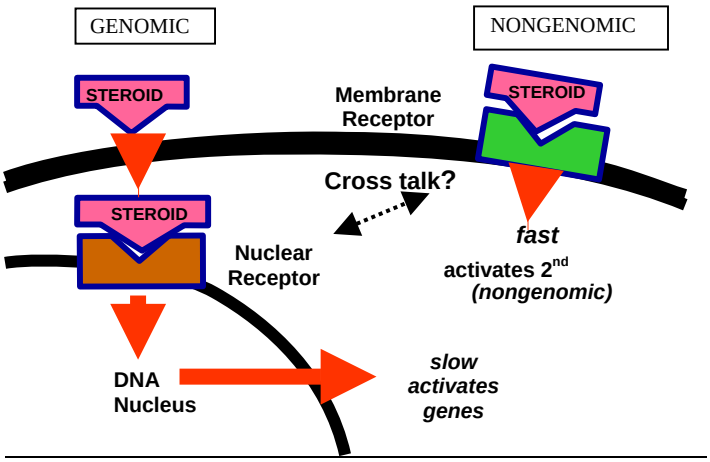


Figure 1. Visual representation of receptor-mediated mechanisms of steroid action

These steroid receptor classes and the genomic and nongenomic actions of steroids and o,p'-DDE and p,p'-DDD in reproductive tissues of the Atlantic croaker were examined to determine potential targets for interference by endocrine disrupting chemicals.

Studies on Three Nuclear (Genomic) Estrogen Receptors

Estrogens are considered to be primarily female reproductive hormones; however, recent evidence suggests they may also have important roles in the regulation of gonadal function in males. Estrogens have been detected in the testes and male plasma of several vertebrate species including the Atlantic croaker. The ER in the testis of the Atlantic croaker has a high affinity for estrogens and xenoestrogens and is a potential target for endocrine disruption by xenoestrogens.

As part of this study, researchers demonstrated the presence of three distinct nuclear estrogen receptor subtypes in the Atlantic croaker: ER alpha, ER beta, and a previously unrecognized form, ER gamma. This was the first evidence for three estrogen receptor subtypes in any vertebrate species. The three nuclear ER subtypes are genetically distinct and have different distribution patterns in Atlantic croaker tissues. The distribution of the three ER subtypes was investigated in croaker brain tissues by *in situ* mRNA hybridization. ER alpha is abundant in the liver, less abundant in the testes, and barely detectable in the ovary or brain. Differences were observed in the distribution of ER beta and ER gamma in several brain regions, including those involved in the regulation of the reproductive endocrine system. For example, ER beta but not ER gamma was detected in the magnocellular neurons of the preoptic area of the anterior hypothalamus. Their unique distributions within the brain suggest that the ER subtypes may

serve distinct neuroendocrine roles. The three ER subtypes are differentially expressed in reproductive tissues that are known targets of estrogen action and exhibit differences in their binding affinities for estrogens. ER beta and ER gamma fusion proteins can each bind estradiol-17β (the most potent estrogen) with high affinity. ER beta is abundant in the testes, but less abundant in the ovaries. ER gamma is abundant in the ovary; expression in the testes is also high. The high levels of ER gamma in both male and female reproductive tissues suggest that the nuclear ER gamma may mediate some of the endocrine disrupting effects of o,p-DDT, o,p-DDE, or other endocrine disrupting chemicals.

## Relevance to ORD's Multi-Year Research Plan

This project contributes to the first long-term goal of the ORD's MYP (LTG-1): to provide a better understanding of the science underlying the effects, exposure, assessment, and management of endocrine disruptors.

Researchers demonstrated the presence of three distinct nuclear estrogen receptor subtypes in the Atlantic croaker: ER alpha, ER beta, and a previously unrecognized form, ER gamma. Two distinct nuclear androgen receptor subtypes (AR1 and AR2) were also characterized in the Atlantic croaker. The presence of different receptor subtypes in target tissues with different binding affinities for steroid hormones and endocrine disrupting chemicals (EDCs) has important implications for the regulation of physiological processes by steroid hormones and their disruption by EDCs.

Researchers also demonstrated that in addition to the classic genomic mechanism of steroid action mediated by nuclear ER and AR receptors, steroids also can act at the cell surface of both testes and ovaries to initiate rapid, nongenomic responses, and that these actions are mediated by ER and AR membrane receptors. The affinities of the membrane and nuclear ERs for the tested xenostrogens are similar, which suggests that nongenomic steroid actions may be as sensitive as genomic ones to endocrine disruption. The affinities of the membrane ARs for androgens or antiandrogens appear to be lower than the nuclear ARs, suggesting that nongenomic steroid actions may not be as sensitive as genomic ones to endocrine disruption.

Studies on Two Nuclear (Genomic) Androgen Receptors

Two distinct nuclear androgen receptor subtypes (AR1 and AR2) have been characterized in the Atlantic croaker. AR1 is present at high concentrations in the brains of males and females; AR2 is found predominantly in the gonads. The two ARs demonstrate widely different binding affinities for certain androgens, AR1 binding being more specific for testosterone. A study was conducted to investigate the role of gonadal steroids in the regulation of AR1 in the brain of the Atlantic croaker.

The regulation of the brain androgen receptor by gonadal steroids was examined in gonadectomized male and female croakers. The males were divided into two groups (n=12/group): (1) sham controls (i.e., gonads not removed) and (2) gonadectomized. The females were divided into five groups (n=18/group) as follows: (1) sham controls; (2) gonadectomized controls; (3) gonadectomized and injected with 3 mg 5'-dihydrotestosterone; (4) gonadectomized and injected with 3 mg testosterone; and (5) gonadectomized and injected with 3 mg estradiol-17β. Brain tissue of fish in all groups were analyzed for AR1, and circulating steroid levels were measured in blood plasma

In males, AR1 concentrations in nuclear fractions of brain tissue were determined in sham-control and gonadectomized test animals. Compared with sham-controls, gonadectomy in males induced significant declines in plasma testosterone concentrations and concentrations of AR1 in the brain tissue. Gonadectomy in females caused significant decreases in brain AR1 levels, as wells as significant decreases in circulating estradiol-17β and testosterone levels. AR1 levels were partially restored in gonadectomized females by treatment with testosterone or estradiol-17β; however, 5'-dihydrotestosterone (derived from testosterone) treatment of gonadectomized females did not increase AR1 levels significantly above those in gonadectomized controls.

The results of this study show that brain AR1 levels in both male and female Atlantic croaker are greatly influenced by gonadal status and circulating levels of sex steroids. The gonadectomized croaker model appears to be useful for investigating the influence of endocrine disrupting chemicals on androgen receptor abundance and hence androgen action.

Androgen Down-regulation of Ovarian Steroidogenesis by a Nongenomic Mechanism

The presence of androgen nuclear receptors in the ovaries of several vertebrate species, including the Atlantic croaker, suggests that androgens have an important role in ovarian function. However, this functional role remains unclear. The purposes of this study were to determine the effects of androgens on ovarian steroidogenesis in the Atlantic croaker and to investigate the possible involvement of genomic and nongenomic mechanisms in mediating the androgen effects *in vitro.*

Low physiological concentrations of a variety of androgens were added to ovarian tissue incubations as follows: 17β-hydroxy-5α-androstan-3-one [DHT], 34 nM to 34 μM; 11-ketotestosterone [11-KT], 1 nM to 10 μM; or the synthetic androgen Mibolerone, 33 nM to 33 μM. The effects of co-exposure of androgens and p,p-DDE (625 μM) or cyproterone acetate (1.2 μM), antiandrogens that act on nuclear androgen receptors, also were investigated. Androgen treatment consistently inhibited gonadotropin-stimulated estradiol production in a concentration-dependent manner. These actions were not reversed by co-incubation with either of the nuclear androgen receptor antagonists (p,p'-DDE or cyproterone acetate), which suggests that this androgen action is not mediated by binding to the nuclear androgen receptor. Androgen effects in the ovarian tissues were observed to be rapid, with a trend of decreased estradiol production after five minutes (although the effect was not significant until after 30 minutes of androgen exposure). Androgen binding studies also were performed that showed the presence of a high-affinity androgen binding site in a plasma membrane preparation of croaker ovarian tissue.

The series of experiments described above demonstrate that androgen action on the ovary is rapid, nongenomic, and at the cell surface. This study also characterized a high-affinity androgen-binding moiety on croaker ovarian membranes, thus fulfilling all of the criteria for its designation as an androgen membrane receptor. This is the first clear evidence for the presence of androgen membrane receptors in vertebrate ovaries. Preliminary evidence from additional experiments indicates that various xenobiotic chemicals that display relatively high binding affinities for the ovarian *nuclear* AR display little or no binding to the ovarian *membrane* AR, suggesting that the cell surface may not be an important site of endocrine disruption for those chemicals.

Estrogen Down-regulation of Testicular Steroidogenesis by a Nongenomic Mechanism

The possible role of the testicular ER in the regulation of steroidogenesis also was investigated to identify potential biomarkers of xenoestrogen effects in the testes. Estrogens and xenoestrogens were shown *in vitro* to down regulate testicular androgen production, but similar to the effects of androgens in the ovary, this action was cell surface-mediated, rapid and nongenomic, and therefore not mediated by a nuclear ER. A high-affinity, plasma membrane receptor for estrogens was identified in croaker testis which binds a variety of xenoestrogens and is the likely mediator of the steroidogenic actions of estrogens and xenoestrogens in this tissue. This study provides the first clear evidence that xenoestrogens can mimic the actions of estrogens by a nongenomic, receptor-mediated mechanism.

Summary of Studies Evaluating ER and AR Distribution and Function

Both ERs and ARs are found in the brains and in both the testes and ovaries of the Atlantic croaker, indicating that estrogens and androgens have important endocrine functions in both males and females. These studies revealed a greater diversity of nuclear ERs and ARs than previously identified and demonstrated the presence of membrane ERs and ARs in both the testes and ovaries of the croaker. The different binding affinities of nuclear ERs and ARs for sex steroids and EDCs in the various target tissues is likely to have a major influence on the suite of effects observed at different EDC exposure levels. The presence of multiple nuclear ER and AR subtypes provides a possible mechanistic basis for these differences which warrant further investigation. Plasma membrane receptors for estrogens and androgens as well as nonclassical, nongenomic actions of these steroids have also been identified in croaker gonads. The affinities of the membrane and nuclear ERs for the xenostrogens tested are similar,

which suggests that nongenomic steroid actions may be as sensitive as genomic ones to endocrine disruption. The affinities of the membrane ARs for androgens or antiandrogens appear to be less than the nuclear ARs, suggesting that nongenomic steroid actions may not be as sensitive as genomic ones to endocrine disruption.

Reproductive and Endocrine Effects of o,p'-DDT and p.p'-DDE

Male and female croakers were exposed to o,p'-DDT (xenoestrogen) or p,p'-DDE (antiandrogen) throughout their life cycles. Measures of reproductive function included indices of gametogenesis, sex differentiation, hatching success, and sperm motility, as well as indices of endocrine function such as steroid and gonadotropin secretion and ER or AR concentrations after exposure to the study compounds. The sites of estrogen action were determined by *in situ* hybridization of the ER mRNA; sites of androgen action were determined by AR assays. Estrogenic and antiandrogenic actions of the study compounds were assessed by specific assays. Interactions of the study compounds with the ER and AR were examined in competition studies.

Several major differences in the patterns of the reproductive and endocrine effects of chronic *in vivo* exposure to the two DDT derivatives were observed. No evidence was obtained for an action of p,p'-DDE (antiandrogen) on the neuroendocrine system controlling luteinizing hormone (LH) secretion, whereas LH secretion was reduced in fully recrudesced fish chronically exposed to o,p'-DDT (xenoestrogen) during the later phase of the reproductive cycle. Gonadal growth was impaired and blood steroid levels were decreased in males and females exposed to the higher concentrations of both DDT derivatives, but a decreased capacity of the gonads to synthesize steroids was only observed after o,p'-DDT treatment. One interpretation of these findings is that the sites of action of o,p'-DDT and p,p'-DDE on the hypothalamus-pituitary-gonadal axis controlling the reproductive cycle differ. However, the failure to demonstrate significant impairment at these sites with p,p'-DDE may also be a reflection of its apparent lower potency in disrupting reproductive and endocrine function in croaker compared to o,p'-DDT. Similarly, the lack of significant effects of p,p'-DDE on an earlier stage of gonadal recrudescence, a period when o,p'-DDT showed a profound influence, may be partially attributable to its overall lower potency. Sex differences in the susceptibility of croaker to p,p'-DDE but not o,p'-DDT at certain stages of the reproductive cycle could also be related to their relative potencies. Although the receptor binding studies confirmed that only o,p'-DDT has the potential to interfere with ER-mediated estrogen actions, the AR in croaker gonads recognizes both DDT derivatives. Thus, in addition to its estrogenic action, o,p'-DDT could potentially have an antiandrogenic action, whereas p,p'-DDE probably only has an antiandrogenic action. It is concluded from these studies that the differing patterns and degrees of impairment of reproductive and endocrine function observed after exposure to these two DDT derivatives is probably largely a reflection of their different mechanisms of endocrine disruption.

These studies provide mechanistic explanations of how xenoestrogens and xenobiotic androgens or antiandrogens can interfere with reproductive function and development in the Atlantic croaker, and are expected to apply to other fish species. The study findings provide data useful for assessing risks associated with DDT contamination in fish populations in the Southern California Bight.

## Investigators

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## For More Information

http://www.utmsi.utexas.edu/people/staff/thomas.htm

## NCER Project Abstract and Reports:

http://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/164/report/0