ENDOCRINE DISRUPTING CHEMICALS: EFFECTS AND MECHANISM OF ACTION IN THE VTG GENE EXPRESSION IN THE TROPICAL LIZARD *Anolis pulchellus*

by

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Abstract

The effects of several Endocrine Disrupting Chemicals (EDCs) from three main groups (pesticides, plasticizers and natural and synthetic estrogens), in the expression of the vitellogenin (Vtg) gene were studied in male individuals from the tropical lizard *Anolis pulchellus.* A new method of transdermal administration was developed. The interaction of the EDCs with estrogen binding activities in the blood plasma (sex steroid binding protein, SSBP) and a liver cytoplasmic extract (estrogen receptor, ER) was assessed in competitive binding assays. Conditions for the EMSA of this anoline ER were optimized as a tool for the testing ER activators.

A 60% of the [^{3}H]E_2-17β TD administered was found to be internalized after 15 minutes of administration and when compared with the alternate method (intramuscular injections, IM) the TD technique was only 10 time less effective. Using this same technique op-DDT, op-DDE, Mtx, DES, Estriol, and Estrone, (μg amounts) and of BPA, DEHP (mg amounts) were identified as Vtg synthesis inductors at transcriptional and translational levels as shown by RT-PCR and Western Blots analysis. No effect on Vtg synthesis was obtained for Tamoxifen and Gluphosate Isopropyl. These experiments also showed evidence of individual variability in responses to the treatment in wild life populations.

The presence of an inducible putative ER in cytoplasmic liver extract from males treated with E_2-17β as well as a basal similar activity in non-treated males was shown by competitive binding assays. Five (BPA-78%, Estrone-77%, Estriol-73%, DDT-67%, DES-55%) of the eight XEs that tested positive for Vtg induction also binds to the ER with more than 50% of the relative binding for $E_2$17β,

(100%). Methoxychlor (11%) and DEHP (15%) were both Vtg inductors but do not show interaction with this ER.

An E_2-17β binding activity was also shown in the blood plasma of male lizards treated with E_2-17β. High relatives binding were obtained for DDE (84%), DDT (80%), Estrone (76%) and BPA (67%) all of them Vtg synthesis inductors while Estriol (42%), and DES (33%), also Vtg synthesis inductors are classified as poor competitors.

A new consensus estrogen response element (ERE), was designed and used in EMSA with the liver cytoplasmic protein extract for the E_2-ER-ERE complex identification. Apparently the presence of divalent cations like Mg2+ is important for the complex formation since the absence of EDTA in two of the three binding buffer tested improves the complex identification. The presence, in the liver cytoplasmic protein extracts of active general transcription factors (SP1, TFII) and of an ER was demonstrated.

In conclusion; 1) the TD method of administration is a suitable one for the testing of EDCs applied in low doses 2) the induction of Vtg in males is suitable to test for estrogenicity in a laboratory scenario, 3) the presence of a SSBP that binds E_2 in plasma and of the liver intracellular ER was demonstrated for the first time in this system. This research culminates a series of studies on the estrogen regulation of the Vtg synthesis in this tropical lizard and is the first one that test EDCs/Xes in a totally terrestrial organism.

Dedication

To:

Lourdes, my lovely wife and support during the years.

Alicia Moradillos, always looking after me during this hard road… Thanks.

My students, those in the past, those in the future…

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List of Abbreviations

EDCs – Endocrine disrupting chemicals

ER – Estrogen receptor

XEs – Xenoestrogens

o'p'-DDT – ortho-para-dichlorodiphenyltrichloroethane

DDD – dichlorodiphenyldichloroethane

DDE – dichlorodiphenyldichloroethylene

Mtx – Methxychlor

DES – Diethylstilbestrol

BBP – Butyl benzyl phthalate

DBP – Dibutyl benzyl phthalate

DEHP – Di(2-ethylhexyl) phthalate, (DOP – di-octyl phthalate)

BPA – Bisphenol A
.

PCBs – Polychlorinated biphenyls

PVC – Polyvinyl chloride

YES – Yeast Estrogenicity Assay

E_2 17β– Estradiol 17β

E_3- Estrone

E_1 - Estriol

PSD – Premature sexual development

Vtg – Vitellogenin

QSAR – Quantitative Structure Activity Relationship

HRE – Hormone response element

ERE – Estrogen response element

AF – Activation Function

TIF – Intermediary Transcription Factors

Tam – Tamoxifen

mER – membrane locate Estrogen Receptor

COMFA/QSAR – Comparative Molecular Field Analysis/Quantitative Structure Activity Relationship

HPTE – 2, 2-bis (p-hydroxy-phenyl)-1, 1, 1,-trichloroethane

ERKO –Estrogen Receptor Knock Out

ICI-182,780

LF – Lactoferrin

EGF – Epidermal Growth Factor

PPI – Phosphatidyl-inositol

EMSA – Electrophoretic Mobility Shift Assay

TD – Transdermal

IM – Intramuscular

$[^3H]E_2$-17β – Tritiated Estradiol 17β

EDTA – ethylenediaminetetraacetate

SDS – Sodium Dodecyl Sulfate

PAGE – Polyacrylamide Gel Electrophoresis

mRNA – messenger RNA

RT-PCR – Reverse Transcription –Polymerase Chain Reaction

$MgCl_2$ – Magnesium Chloride

ATP – Adenosine Tri-phosphate

WB – Western Blot

Cpm – counts per minute

Alb – Albumin

Gly –Glyphosate

P Buffer – Binding Buffer from Promega EMSA Kit

KC Buffer – Binding Buffer from Kumar and Chambon, 1988.

RE Buffer – Binding Buffer from Ruiz and Echevarría, 2000.

Introduction

A – Endocrine Disrupting Chemicals: General Problem

A major concern has been raised in the environmentalist community with the increased number of chemicals compounds that have been associated with endocrine disorders on wildlife and humans. Endocrine disrupting chemicals (EDCs) are not necessarily environmental pollutants, but have been related to effects that disrupt the normal function of the endocrine system of all kind of vertebrates including humans (Thomas and Colborn 1992; Colborn and Clement, 1992; Colborn et al., 1993, Guillete et al. 1996; Crain et al., 1998, Crews et al., 2000, Bevan et al, 2002). EDCs whose effects have been related to the disruption of functions mediated by the estrogen receptor (ER), and other related steroid hormones receptors are known as xenoestrogens (XEs). The effects of the XEs include developmental disorders, problems with the reproductive system and capacity, and disruptions of sexual determination and differentiation in animals and humans (Colborn et al., 1993; Guo et al., 1995; Jensen et al., 1995, Lind, et al., 2004, Sone et, al., 2004). Examples of described XEs and their effects are summarized on Table I, and some examples of their molecular structures are shown on Figure 1.

Pesticides from the dichlorophenyletanes group (DDT, DDE, DDD), designed for general use, were the first compounds identified as EDCs for their ability to cause feminization disorders in male gull embryos (Fry and

Table 1 - **Examples of EDCs**. A variety of man made chemicals and some natural compounds have been related to endocrine disrupting effects that affect a wide number of wildlife species, humans included. The following table summarize some of them relevant to this study.

COMPOUND	EFFECTS
Pesticides	
Dicofol, Dieldrin/Aldrin	Avian reproduction impaired (Guillete, et al, 1994)
DDT, DDE, Mtx	Avian reproduction impaired, eggshell thinning, gulls and reptiles embryo feminization (Fry, 1981, Guillete et al, 1994,1995) Alterations in early development in amphibians (Bevan et al, 2002).
Synthetic Estrogen and Hormones	
DES Estrone Estriol	Uterine tumors on daughters of females treated with it, developmental defects on male offspring reproductive system, (vom Saal et al. 1995,Newbold 1995; McLachlan et al., 1980). Induction of female specific proteins in male fishes exposed to water sewages. Sumpter and Jobling, 1993, Soto et al, 2004)
PCBs	Sexual determination disrupted and decreases egg hatching in turtles (Bergeron et al, 1994).
Plasticizers	
Phthalates (BBP, DEHP, DBP)	Decrease in testicular size and sperm production on rats (Sharpe et al., 1995). Sterility in second generation males on rats (Wine et al., 1997)
BPA	Developmental abnormalities in amphibians (Sone et al., 2004),
Phytoestrogens	Decrease sheep fertility (Hughes, 1988)

Toone, 1981, Burlington and Lindenman, 1950). They also have been incriminated in the Apopka Lake case (Florida, USA), one of the most well documented cases of ecological problems due to

EDCs spills. These compounds were related to demasculinization problems in male, superfeminization and hatching problems in alligators that resulted in a

drastic decrease of the population (Guillete et al.1994). Although the use of these pesticides has been banned in USA, they are still used in other places of the world. It is important to mention the existence of other pesticides with suspected estrogenic activity like

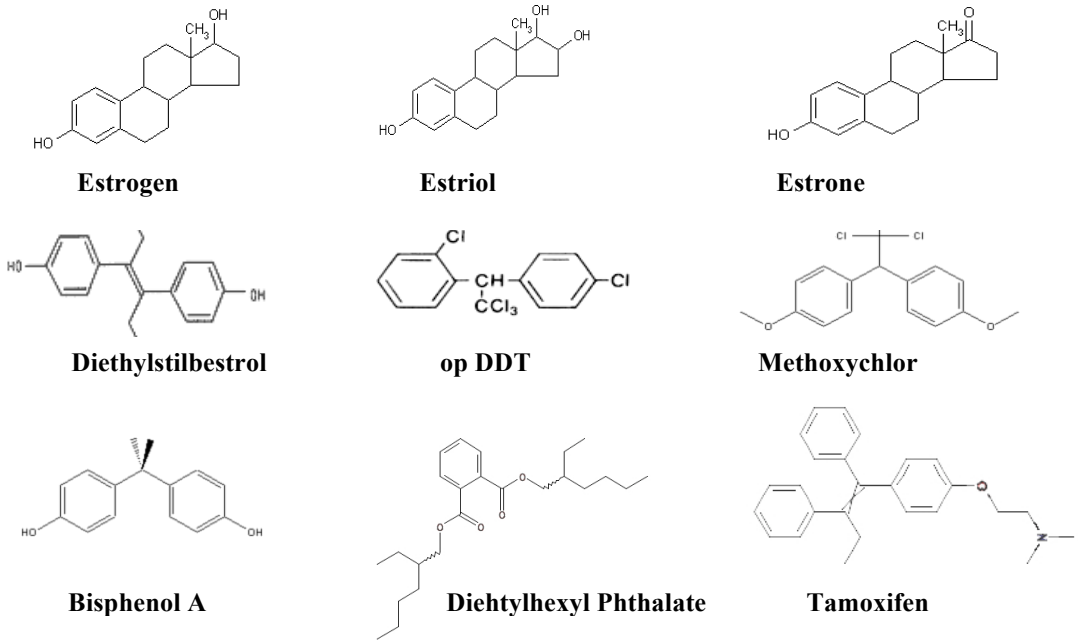

Figure 1- Structures of Endocrine Disrupting Chemicals**.** Variety of structures represented by the EDCs tested in this project.

Methoxychlor (Mtx), endosulfan, toxafene, and dieldrin. At this moment these are still legally used in the USA (Solomon and Schettler, 2000; Gosh et al. 1999, Soto et al., 1994).

Some synthetic compounds of even more wide-spread human use have also been associated to estrogenic effects. For example, chemical resins, used in canning procedures contain Bisphenol A (BPA). This compound has been shown to increase by 58% the proliferation of cells using the E-screen test (MCF-7 cells)

(Brotons et al., 1995). Similar results were reported with the same test for a BPA based dental sealant (Olea et al., 1996). Also BPA, has been purified from water autoclaved in plastic flasks and its estrogenic activity also showed using the MCF-7 cells assay (Krishan, et. al., 1993).

The Polychlorinated Biphenyl's (PCBs) is another group of compounds that is also suspected of having estrogenic activity is PCBs were originally used in lubricants, electric transformers and in microscope immersion oil. Their lipophilic nature made them highly persistent in the environment and suggests that they might be stored in the food chain. The most probable route for exposition to PCBs is through food like fresh water fish and meat. Bergeron et al., (1994) demonstrated estrogenic activity for some PCBs that were capable of inducing sex reversion in turtles (*Trachemys scripta*). Normally in this group male individuals hatch from eggs at lower temperature, but exogenous exposition of the eggs to some PCBs resulted in a sex-ratio deviation toward the female side.

Another chemical group of compounds that have been identified as EDCs/XEs is the phthalates (Moore, et al. 2001, Jobling, et al, 1995). They are used in plastic manufacturing processes used to give flexibility and manageability to polymers such as polyvinyl chloride (PVC). The phthalates are not chemically bonded to the final product and so they can be easily released to the environment (Jaeger et al., 1972; and Autian, 1973). An *in vivo* study in rats reported effects of butyl benzyl phthalate (BBP), such as decrease in testicles size, and sperm production (Sharpe et al., 1995). In other study, Wine et al.,

(1997) found that the exposition to dibuthyl phthalate (DBP) adversely affected some reproductive parameters in rats. They report effects even more adverse in the second generation, including F1 male infertility. However in *in vitro* studies, Harris et al., (1997) found that only 5 out of 35 tested phthalate compounds showed what they defined as a weak estrogenic activity. When assayed by the MCF-7 cells and YES Screen tests, the most potent phthalate (DBP) was approximately 1 million times less potent than Estradiol-17ß (E_2-17β).

Although contradictory evidence has been gathered about the phthalates they have some relevance in Puerto Rico because a relationship between premature sexual development (PSD) in children and the phthalates have been suggested. Significant high levels of dimetyl, diethyl, dibutyl and Di (2-ethyl hexyl) phthalate (DEHP) were observed in 68% of blood samples from girls with PSD (Colón, et al., 2000). Cases of PSD have been documented in Puerto Rico since early 1979 (Pérez-Comas 1982, Bongiovanni, 1983). From 1969 until 1998, Puerto Rico has registered 6580 cases of idiopathic premature sexual development, with 71% (4674) of the cases presenting premature breast development (thelarche) in girls 6 mo to 2 yrs old (Pérez-Comas, 1982, Haddock et al., 1985; Freni-Titulaer et al., 1986; Bourdony, 1998). The reported incidence of thelarche in Puerto Rico is 8 cases per 1000 live female birth, 18.5 times higher than in USA (Van Winter et, al; 1990). Although obviously a direct relationship can't be established at the moment between the presence of these phthalates and the PSD condition on these patients, the evidence presented by

Colón et al (2000) display an interesting point to be further studied about the putative EDC activity of these agents.

A group of compounds that could be considered as natural XEs include products of some plants (coumestrol and genistein) or fungi (vinclozolin). An example of the effect of the so called phytoestrogens is that sheep that grazed on clover leafs show decreased serum levels of progesterone (P) and luteinizant hormone (LH) (Hughes, 1988). Also, water effluents from sewage treatment plants from pulp and paper mills (Davis and Bartone, 1992) and from cattle feedlot (Soto et al. 2004, Orlando et al. 2003) have been associated with endocrine disrupting effects on fishes found downstream from the effluents origins. Some examples of plants with estrogenic substances are soybeans, wheat, alfalfa, grapes and apples (Bowers et al. 2000, Kuhnau 1976). It is believed that the estrogenic agents on these plants and their products could interfere with the endocrine systems of organisms that include them in their diet (Suetsugi et al. 2003).

Endocrine disrupting effects have also been attributed to the synthetic estrogens. The most notorious example is diethylstilbestrol (DES). This is a potent synthetic estrogen that was used during the 70's to help women with pregnancy miscarriage problems. Scientists have related some detrimental effects observed on the progeny of these women to *in uteri* the exposition to DES. For example, daughters of these women present structural anomalies on their uterus, as well as uterine neoplastic lesions and vaginal adenocarcinomas. Similarly, increase incidence of genital malformations and testicular anomalies

has been observed in their sons. Related manifestations have been observed in laboratory animals exposed to DES (McLachlan 1993; Newbold, 1995).

Evidence of estrogenic effects observed in fishes living in areas where water sewages that receive water sewages from highly densed populated and agricultural (cattle and chicken) worked areas. A wide group of pharmaceutical agents have been reported in the sewage and even in the open water (Shannon et al, 2003). These agents included drugs used for treatment of heart diseases, anti-inflamatories, antibiotics, and birth control, as well as growth promoters and other compounds from veterinary use. (Daugthon and Ternes, 1999; Kolpin, et al, 2002)

In summary due to 1) the existence of a great variety of suspected environmental estrogenic substances, and 2) the fact that a great number of suspected EDCs have been already identified in several products intended for general use; the problem of the exposition of human and wildlife to EDCs is one of significant importance to wild life and human health. Consequently this issue have captured the attention of the scientific community in general. Interestingly, these putative effects of the environmental estrogens are continuously challenged (Safe 1994; Safe 1995; Wolf 1995) mainly because a direct cause-effect relationship has been difficult to establish. A great amount of efforts and resources have been directed toward research to describe and document the disrupting effects of EDCs on organisms found in the wild and some *in vivo* experimental approaches. In these latter tests using developmental or physiological effects like malformations, reproductive alterations, and time

scheduled events like first oestrus or pseudo-pregnancy as endpoints have been commonly applied.

More recently, efforts have been addressed to develop assays and techniques for the detection and identification of estrogenic activity in new compounds and the understanding of their mechanism of action. They closely follow different aspects of the mechanism of action of steroid hormones, (i.e. estrogen). Table II presents a summary of some developed assays or applications for estrogenicity. A diversity of approaches are used in these assays including hormone receptor competition experiments, *in vitro* cell proliferation, induction of biomarker proteins in vertebrates and induction of the expression of recombinant genes.

The receptor competitive assay is based on the observations that some EDCs actually bind the ER. This assay gives insight on the relative affinity for the ER of the after 6 days of culture in the presence of the suspected XE to the yield induced by XE compared with Estradiol 17ß (E_2-17β) to the ER (Klotz et al., 1996). In the cell proliferation assay, the growth response of an estrogen-sensitive cell line (human breast MCF-7 cells) to the presence of a XE is measured. The assay compares the cell yield after 6 days of culture in the presence of the suspected XE to the yield induced by estradiol (Brotons et al. 1995; Soto et al., 1995; Villalobos et al., 1995). Vitellogenin (Vtg) is an example of a biomarker protein that has been determined as useful in assessing estrogenic effects (Heppell et al., 1995, Irwin et, al., 2001). Vtg is an estrogen inducible sex-specific phosphoglycoprotein that

Table II – Experimental assays or applications developed to assess estrogenic activity.

ASSAY	TESTED SUBSTANCE	REFERENCE
ER competitivity test	Estradiol-17ß o'p'-DDD o'p'-DDT Kepone Endosulfan	Vonier et al., 1996; Klotz, et al., 1996; Matheews et al, 2000.
Cell proliferation E-screen - MCF7 cells	Estradiol-17ß o'p'-DDT Endosulfan Toxafen Dieldrin BPA DES Coumestrol Nonylphenol	Soto et al., 1994 Krishnan et al., 1993; Olea et al., 1996; Soto et al., 1995;
Biomarker protein Vtg	Estradiol-17ß DES Coumestrol Genistein DDE Nonylphenol	Heppell et al., 1995; Pellisero et al., 1993 Ren et al., 1996
Recombinant systems YES screen - Yeast	Estradiol-17ß Tamoxifen Coumestrol Genistein Nonylphenol Metoxychlor BPA Octylphenol o'p'-DDT DES Estradiol-17ß Diethyl phthalate Dibutyl phthalate Butyl Benzyl phthalate	Coldham et al., 1997 Arnold et al., 1996 Harris, et al., 1997

is naturally synthesized in the liver of mature females of oviparous vertebrates and induced by E_2-17β in males. The assay basically consists in the exposure of the males or juveniles individuals to the suspected XE and the

presence of Vtg on blood serum is determined (Purdom et al., 1994, Ren et al., 1996). On the *in vitro* application of this assay, hepatocytes cultures are treated with the EDC and the levels of Vtg secreted to the medium are determined (Pellissero et al., 1993).

In the YES-screen test the response of *Sacharomyces cerevisiae* cells transformed with an expression vector containing a human ER cDNA, and with another recombinant plasmid containing two copies of the frog Vtg ERE upstream of the ß galactosidase gene are used. The estrogenicity of the suspected XE is determined by the induction of the ß-galactosidase activity compared to the induction of this gene by $E_2$17ß (Arnold et al., 1996; Shelby et al., 1996; Coldham et al., 1997). In addition this type of assay indicates that the estrogenic activity of the agent is mediated by the ER.

The uterotrophic assay is an *in vivo* assay developed to determine the XEs effects on a normal estrogen responsive tissue within a whole animal. In this assay naive female mice are treated with XEs and then analysed to determine if uterine wet weight gain result from the exposure to the XEs as compared with the control animals (Shelby et al., 1996).

B- Steroid Hormones: General Mechanism of Action

The cellular effects of the steroid hormones are mediated by their interaction with specific intracellular receptors. It has been postulated that the EDCs that have reproductive system associated effects (XEs) might be expressing their effects by interfering with the normal response to steroids hormones. The XEs effects could be due to a direct action as an estrogen

receptor agonist or as antagonist. However, the possibility of the interaction of the XEs with other steroid hormone receptors as well as their effects in other steps of the hormonal pathway (hormone synthesis, storage, releasing, transport or degradation) should be taken into account. (Special Report on Environmental Endocrine Disruption, EPA/630/R-96/012, 1997).

The steroid hormone receptors belong to the intracellular receptor super family that acts as ligand-dependent transcription factors. Among others, this family include the receptors for estrogen (ER), progesterone (PR), glucocorticoids (GR), thyroid hormone (TR), retinoic acid (RXR), and the group known as orphan receptors (OR) for which an specific ligand have not been established (Reichel and Jacob, 1993, Mangelsdorf et al., 1996). Five function-associated domains have been identified in a typical molecule of a ER. It has been proposed that these domains are evolutionary conserved among the members of this super family (Figure 2). The E domain is mainly associated with the ligand-receptor interaction. It is also related to an activation function (AF2) and to a gene transcriptional modulation function as it interacts with other coactivator molecules. The A/B domains located at the amino terminal share a constitutive activation function (AF1). The E domain has been also associated to the interaction with the Hsp proteins (70 and 90), to the homodimerization of the receptor molecules and to spatial (nuclear) localization of the receptor. The C domain is a highly conserved region that is associated to the interaction of the receptor with the specific DNA region (response element) where it binds to induce gene expression (Reichel and Jacob, 1993).

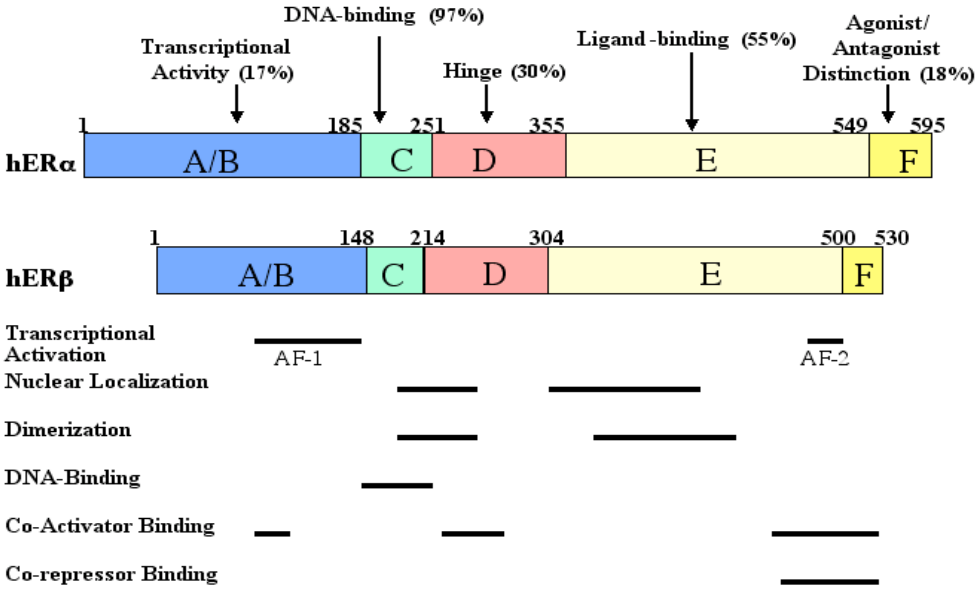

Figure 2 – Estrogen Receptors α and β: Functional Domains and Homology. (Klinge C. 2000).

Once the hormone traverses the cell membrane the first step of its mechanism of action is to bind to its specific intracellular receptor. After that, a conformational change occurs that results in the dissociation of Hsp proteins from the receptor molecules and dimerization of two receptor molecules. This homodimer then recognize and binds with great affinity to a specific DNA sequence known as the Hormone Response Element (HRE) (Figure 3). The minimal consensus sequence for E_2 is: 5'-GGTCAnnnTGACC-3' (n=any nucleotide), (Klock et al, 1987). This sequence is located within the regulatory region of the E_2 responsive genes (Kumar and Chambon, 1988).

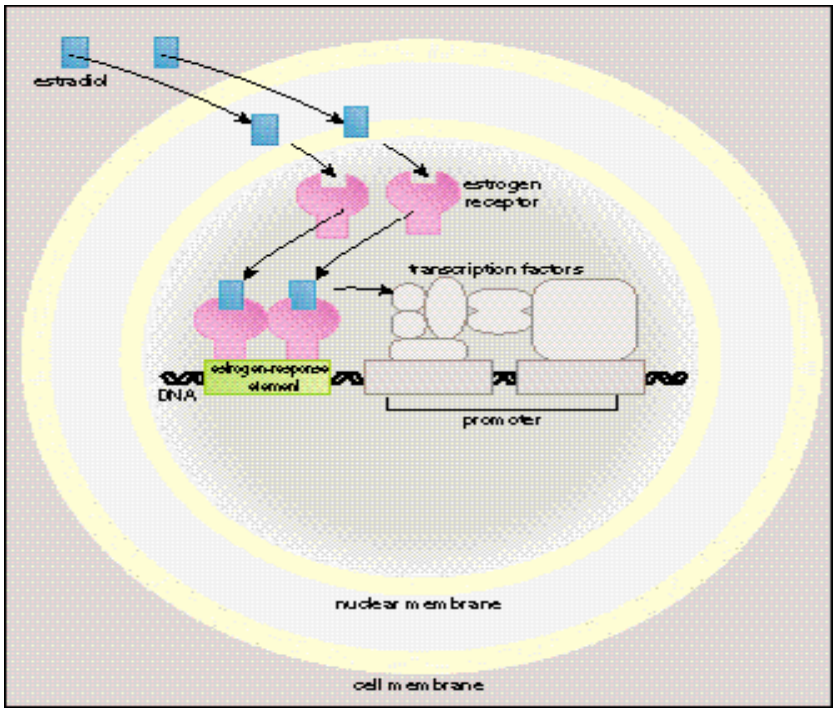

Figure 3 – Mechanism of action of Estrogen. (Arnold and McLachlan 1996).

After the ligand-receptor-HRE complex is formed, recruitment of general transcription factors, intermediate factors and coactivators for the RNA polymerase II,

ensues. This results in the transcriptional activation of the hormone-regulated genes. For example, LFB1/HNF1 and C/EBP are among the transcription factors that interact with regulatory regions for the *Xenopus* Vtg genes (Kalling et al., 1991). The intermediary transcription factors (TIF I y TIF II) and coactivators seem to be responsible for the activator functions (AF-1 and AF-2) of the steroids receptors. Example of other coactivator molecules are Sp1 (Porter et al., 1997); GRIP 1 (Hong et al., 1996), RAC 3 (Li et al., 1997), SRC 1 (Onate et al., 1995)

Table III – Examples of transcription factors associated to estrogen regulated gene expressions.

FUNCTION/MOLECULE	INTERACTION	REFERENCES
General Transcription Factors TBP TAFIIB TAFIID	SHR	Beato, et al, 1996, Tansey and Her 1997.
Intermediate Transcription Factors TIF-1α, TIF-1ß SUG-1 RIP140 PDI, HSP70, p48, p5 HMG1 GRIP170, Grip1 SRC-1 RAC3	ER ER-DBD GR SHR	Le Dourain et al., 1996. von Baur et al., 1996 Cavailles et al., 1995. Landel et al., 1994. Romine et al., 1998. Hong et al., 1996. Onate et al., 1995. Li et al., 1997.
Sequence Dependent Transcription Factors C/EBP LFB1/HNF1	ER-ERE	Kaling, et al., 1991

and RIP 140 (Cavailles et al., 1995) (reviewed in Beato et al., 1996; Tansey and Herr, 1997) (see Table III). The existence of another form (ß) of the estrogen receptor was identified by Kuiper et al., (1996) it in rat prostate and ovaries. In addition Mosselman et al., (1996) reported high levels of the ER-ß mRNA in testicles. Tong et al., (1997) made a comparative study of the interaction of the ER alternative forms (α and ß) with estrogenic (synthetic and naturals) compounds using the Quantitative Structure Activity Relationship (QSAR) technique. Their results suggest that both receptors forms are similar in terms of their ligand structure requirements (see Figure 2). Experiments with ER antagonists showed that for both ER forms the interaction with estrogen was

blocked (Das, et al., 1997). A report from Paech et al., (1997) indicates that the ER-α antagonist (Tamoxifen, Tam) have opposite effects depending on the ER. From the fact that Tam have been previously reported having estrogenic activity in some tissues and ant estrogenic activity in others leads to question if different mechanisms are used by the forms α and ß of the ER for Hsp dissociation, ER molecules dimerization and/or ER-E_2-17β complex hormone response element (HRE) recognition.

The existence of a membrane located ER (mER) has been also suggested also as an alternate pathway to the classic ER mechanism (Watson et al, 1995, Loomis and Thomas 2000). Watson et al., (1995) used an inmunocytochemical approach to show the interaction of E_2-17β with a cell membrane receptor in a pituitary cell tumour. This kind of interaction has been suggested as inducing delayed effects because they require some other steps including protein synthesis. Steroid effects such as fast ion mobilization and generation of action potentials in neurons receptor (Lieberherr et al., 1993) as well as rapid secretion of prolactin on pituitary tumour cells (Norfleet et al., 1999) has been proposed to be mediated by a cell membrane receptor. Estradiol and Raloxifene (a non-steroid estrogen) are both agents that prevent the bone density loss induced by estrogen deficiency (Yang et al., 1996). Bone density loss in ovariectomized rats is associated to a decrease in Tumour Growth Factor B3 (TGB3) expression. Moreover, in this study the TGB3 gene was activated by both estradiol 17ß and raloxifene although this gene does not have an ERE in its promoter. Treatment with E_2-17ß or raloxifene restored bone density. The authors of the study suggest

that both agents have their effect on bone structure by a different mechanism because in uterus raloxifene has an antagonist effect while estrogen has agonist behaviour. However, raloxifene inhibit the Vtg-ERE-CAT gene activation by E_2-17β suggesting a competitive interaction for the ER.

C- Mechanism of action of Xenoestrogens: considerations.

Assays described previously have been helpful to obtain a general insight of the interactions between the XE and the receptor, the expected first step in their mechanism of action.

The great diversity of chemical structures of putative estrogenic compounds that apparently are acting through the same (ER) receptor suggests the existence of alternative mechanisms of action. Waller et al., (1996), used the Comparative Molecular Field Analysis/Quantitative Structure Activity Relationship (COMFA/QSAR) technique to determine in terms of affinity the contribution that the steric and electrostatic factors of a molecule like estrogen may contribute to its interaction with the receptor. Their results suggest that for a molecule to interact with the estrogen receptor its structure should include an OH substituted aromatic ring. They analyzed the affinity towards ER of different putative XEs (phthalates, pesticides, DES and phytoestrogens) to make predictions about the estrogenic potential of the compound based on its molecular structure. As illustrated in Figure 1 not all of the suspected estrogenic compounds fulfilled this steric requirement. An explanation for this apparent contradiction is that some non-phenolic aromatics compounds could be metabolically degraded or bio

activated into phenolic compounds with a more appropriate structure. For example, Mtx was shown to have estrogenic activity *in vivo* but not *in vitro*; however its metabolite HPTE was estrogenic *in vitro* suggesting that Mtx is metabolically activated prior to its interaction with the ER *in vivo* (Shelby et al., 1996). The structural promiscuity on the estrogen like compounds has been explained by Katzenellenbogen (1995) based at least in two structural aspects: the presence of phenolic groups or their structural equivalents, and the lipophilic and hydrophobic nature of the molecule. This later aspect will allow the molecule to gain access to the inside of the cell, provide resistance against metabolic degradation and therefore, facilitate its bio concentration until appropriate active levels are reached. Interestingly, other lipophilic and hydrophobic compounds that do not posses phenolic groups (i.e. endosulfan, kepone and toxafene, and the PCBs) showed a similar behaviour (Soto et al., 1994; Feldman and Krishnan, 1995). However other functionally equivalent groups like cyclic sulphite or ketonic groups may allow them to interact with the ER.

The probability that some estrogenic compounds could be using alternative pathways instead of interacting with the intracellular ER should also be considered. For example, DDT and its derivatives compounds with no phenol substitutions in their structures are reported to have feminizing effects on birds and reptiles (Fry and Toone, 1980; Guillette et al., 1994). In this case, an antiandrogenic activity (aromatase induction) has been suggested as the cause of the mentioned effects (Special Report on Environmental Endocrine Disruption,

EPA/630/R-96/012, 1997). In summary, is clear that suspected EDCs should not be discarded as functional XEs based only on structural criteria.

In order to determine if the estrogenic activity of the suspected compounds is associated *in vivo* and *in vitro* with an interaction with the ER different experimental approaches have been applied. For example Shelby et al., (1996) demonstrated that diethylstilbestrol (DES), HPTE (the metabolically activated form of Mtx), Tam, and o'p'-DDT compete with E_2-17ß for the mouse ER, activate the ERE-CAT reporter gene transcription in HeLa cells, and have a uterotrophic activity in mice. This evidence suggested that at least some XEs follow the steps of the typical pathway of estrogen to produce their effects. Using the competitive assay approach Vonier et al., (1996) showed interaction of EDCs with the crocodile ER in an oviduct protein extract. In the same study additive, synergistic, and competitive effects were observed when EDCs combinations (DDD and DDE; and DDD, DDE, toxafene, dieldrin, arochlor, trans-nonachlor, cis-nonachlor, and chlordane) were tested.

The probability that XEs do not interact with any of the ER forms could also be considered. Das et al., (1997) report estrogenic effects on an ER knock out mice line (ERKO). They showed that cathecholestrogens (hidroxyestradiol 17ß and Kepone (a XE) induce the lactoferrin gene (LF, a mammalian estrogen regulated gene) in this ER free system. As expected, E_2-17β did not induce the effects and the ER antagonist ICI-182, 780 did not inhibit the induction of the LF gene. Since both E_2-17β and the antagonist interact with both forms of ER and since these are ERKO mice for both ER forms the results suggest that in this

mice line the agents produced their effects in an ER (α or ß) independent way. An alternative explanation given by the researchers was that the XEs are interacting with other receptors, which mediate for similar estrogen controlled effects.

In amphibians and birds the most used consensus sequence for the estrogen response element (ERE) is a 13 bp sequence (GGTCANNNTGACC) located on the promoter region of the Vtg gene. Birds and amphibian sequences differ in the 6-8 central base pairs. Little difference are presented in these sequences for different estrogen regulated genes (see Stancel et al., 1995). Table IV includes the sequences from amphibians to humans that not only differ on the middle nucleotides of the sequence but in the ends too.

Table IV. Estrogen response elements sequences for various estrogen regulated genes.

GENE	ORGANISM	SEQUENCE
Vtg A2	*Xenopus*	GGTCANNNTGACC[a]
Vtg II	Chicken	GGTCANNNTGACC[a]
Ovalbumin	Chicken	GGTCA
Prolactin	Rat	GGTCANNNTGTCC
Progesterone Receptor	Human	GGTCGNNNTGACT
Lactoferrin	Human	GGTCANNNTAACC

[a] These are consensus sequences. [b] Underlined bases represent deviation from the consensus.

Based on the existence of these variations the authors suggest the possibility of differential interactions between the hormone-receptor complexes and the EREs resulting in a selective activation of genes. In other words, small changes on these ERE sequences could change the hormone-receptor complex affinity towards these regulatory regions affecting the expression of the accompanying genes.

Similarly, if the conformation of the ER-hormone complex is different to the conformation of the ER-XE complex, differences in the recognition of EREs sequences and hence activation of genes should be. Moreover, depending on the XE that binds to the ER and on the ERE that the XE-ER complex recognizes, the co activators and transcription factors that bind to the XE-ER-ERE complex may also be different. This will then determine what genes are or aren't expressed resulting in an altered gene expression pattern under the XE influence.

Evidence of cross communication between intracellular signal transduction systems and alternative types of estrogen receptor (intracellular as well as membranous) present another possible mechanism for the effects of XEs. The studies of Ignar-Trowbridge et al., (1992) provide results that imply the coupling of the ER and the EGF signal transduction pathways. Interestingly, administration of DES was shown to activate the phosphatidyl-inositol (PPI) mechanism in mouse uterus in an apparently ER- dependent way. This group also reported that some effects of EGF such as DNA and PPI synthesis and the localization of the ER at the nucleus are ER-dependent (Ignar-Trowbridge et al., 1995).

More recently, with the ERKO mice Curtis et al., (1996) showed that although the ER presence is not required for some typical EGF effects (i.e. EGF receptor level increases and autophosphorylation), it is required for other effects like DNA and progesterone mRNA synthesis. Based on these observations they suggest that the signal transduction pathways of EGF and E_2-17β could be in some way coupled in the mice reproductive tract. Then XEs with that may interact with the ER could be also modifying the effects of other regulatory molecules like EGF.

At the moment it has been demonstrated that the following general aspects of the steroid hormone mechanism are common to the XEs mode of action: 1) some of the biochemical and structural requirements necessary for a ligand to interact with the ER, or the need of metabolic activation for some XEs previous to their interaction with the ER; 2) the interaction of some XEs with both ER forms (α, ß); 3) the consensus sequence of the DNA binding site for the ER-Estrogen complex its also recognized by some XE-ER complex, 4) some XEs have effects by means of the cross communication that occurs between ER and other signal transduction pathways.

Despite the existing evidence previously mentioned many steps of the XEs mechanism of action are still poorly understood. From the structural viewpoint, questions still remain about how structurally different compounds can bind or interact with the same receptor. Also, conflicting information exists about the synergistic relationships between XEs. In addition, the interaction that exist between the XEs and other known proteins participants on the ER mechanism

like the Hsp is still unknown. Not much is know about the possible involvement of the differences on the HRE sequences of the ER regulated genes on the mechanism of the XEs.

D - *In vivo* experimental model for EDC estrogenicity

The most common tests for estrogenic activity assessment are *in vitro.* However, these tests with cellular lines do not aid the study of accumulating effects or metabolic activation. Therefore, an aspect that need to be explored is the utilization of well known *in vivo* estrogen dependent processes to verify the effects of *in vitro* tested agents. To these effects the synthesis of Vitellogenin (Vtg) represents a well developed research model to study estrogen regulation of gene expression *in vivo*. This system in the tropical lizard *Anolis pulchellus* have been characterized in our laboratory (Baerga-Santini and Morales 1991; Morales et al., 1991, Morales et. al 1996, Morales and Sanchez, 1996). The Vtg genes are expressed under the strict control of estrogen during vitellogenesis in which the oocytes grow in the female lizard. Vitellogenins are large lipophosphoglycoproteins that function as precursor molecules for the egg yolk proteins. Although they are sex-specific proteins only found in sexually mature females; the treatment of males with E_2-17ß results in the production of detectable quantities of Vtg in their blood plasma. Another related observation made in our laboratory is that anole females in captivity showed a drastic regression of the reproductive system where all the physiological characteristics under estrogen control are deteriorated including a shutdown of Vtg synthesis

after 3-4 days of captivity. These effects have been reverted with the intramuscular administration of E_2-17ß during or after the captivity period.

There is abundant information about the estrogen regulation of the Vtg genes at the molecular level (Shapiro, 1982; Shapiro et al., 1989; Beato, 1989; Callard et al., 1990), and the key role played by ER in the recognition of the ERE and the Vtg gene's activation is well known (Beato 1989, Beato et al., 1995). Based on the previously mentioned Vtg properties and the known information about the molecular mechanism of vitellogenesis we considered the expression of Vtg in male anoline lizards as a confident biomarker of the exposition to estrogen-like activity. Hence, the analysis of Vtg synthesis in this lizard can be used as suitable experimental approach to explore the estrogenic potential of suspected EDCs compounds that may be found in the soils and waters of Puerto Rico.

Consequently in this study we have tested the estrogenic potential of suspected EDCs *in vivo* in *Anolis pulchellus*. At the same time we studied in parallel both the E_2-17β and XEs mechanism of action in term of the formation of the ligand-receptor complex and further interaction of this complex with the ERE of the vertebrate Vtg gene *in vitro*.

Materials and Methods

A. Equipment and Chemicals

General chemical reagents and as well as EDCs were obtained from Sigma-Aldrich, Co., St. Louis, MO. Radioactive isotopes and detection kit for Western Blots were obtained from Amersham, Arlington Heights, IL, and Ultraviolet Products (UVP) San Gabriel, CA, respectively. Membranes and micro-concentration kits were purchased from Millipore, Corp. Materials for the EMSA analysis were obtained from Promega Co., Madison WI. Electrophoresis equipment and materials were obtained from Bio-Rad Laboratories (Richmond, CA) and the autoradiography materials from Eastman Kodak, Co., Rochester, NY.

B. Animals

Anolis pulchellus lizards (Figure 5) used in this study were collected in pastures around the island. Animals of this small grass species are easily captured with bare hands during evening hours while sleeping.

Figure 5 – *Anolis pulchellus,* one of the anoline tropical lizards found in Puerto Rico. It has sexual dimorphism, male (left) and female (right). It is found in coastal humid grassy areas of the island. It has been studied in our laboratory since 1980.

Groups of 5 to 7 animals were maintained in 5 gallon tanks with live plants and branches for climbing, placed at the Biology Department Animal House facilities under high humidity conditions and a photoperiod of 12 h light

and 12 h darkness at ambience temperature (28°-30°C). They were fed with *Drosophila* flies *ad libitum* and provided with water daily.

C. Hormones and Xenoestrogens preparation for treatment

Previous studies from our laboratory have shown that intra-muscular injections of 50 µg Estradiol-17ß (E_2-17β) induce Vtg synthesis in male lizards (Soto-Caraballo, 1990). Therefore, E_2-17β was used as the control treatment for the induction of Vtg gene expression E_2-17β was dissolved in absolute ethanol at a concentration 5 µg/µl and stored at -20°C. In this study, a new method consisting of extra-dermal application of the hormone has been developed. For this application, the hormone was spread over the ventral surface of the animal, and rubbed with the side of the micropipette tip. Once the surface was dry, the animal was returned to its cage. This trans-dermal (TD) mode of treatment was preferred over intra-muscular injection because it presented less stress and reduces mortality and better simulate the external casual type of contact with the agent that probably occurs in the free environment. Xenoestrogens (XEs) compounds were dissolved or diluted in absolute ethanol at the required concentration, stored at –20°C and administered in the same trans-dermal mode as the hormone. The vehicle (ethanol 95%) applied alone was the negative control for the treatments.

D. Determination of Trans-dermal treatment efficiency

To determine how efficient the trans-dermal method of treatment was, recently captured animals were measured and weighed. Five (5) µl, of [^{3}H]-E_2

($5X10^6$ cpm) were applied on their ventral skin. After the skin was visually dry, animals were returned to their cages and were sacrificed at 15, 30 and 45 minutes after treatment. Then blood was drained from the neck veins and lungs, and heart and carcasses were homogenized in a Polytron (Brinkman, Corp.) homogenizer. Aliquot of these solutions were diluted in scintillation liquid and counts per-minute were determined (LS Analyser, Beckman Instruments, Irvine, CA). The skin area where the [^{3}H]-E_2-17β was applied was also minced and counted, so that the most accurate amount of total counts applied could be obtained. Results were expressed as percent (%) of total applied that was assimilated.

E. Blood Plasma Preparation

Blood was collected after decapitation and plasma was prepared as previously described (Baerga-Santini and Morales, 1991) to minimize protein degradation. Micro tubes were rinsed with 1.2x standard saline citrate (SSC)/heparin stock solution (0.18 M NaCl, 0.018 M sodium citrate, pH 7.0,15 µg/ml heparin). Plasma was obtained by addition of an equal volume of SSC/heparin stock solution to the blood for a final concentration of 0.6x SSC and samples were centrifuged at 3,000 rpm for 30 sec at room temperature. One-tenth ($1/10^{th}$) volume of anticoagulant/heparin stock solution (2 M ε-amino-caproic acid, 1 M NaCl, 0.2 M ethylenediaminetetraacetate [EDTA], 0.1 M sodium phosphate, pH 7.3 and 15 µg/ml heparin) was added to the supernatant. Samples were stored at -20°C until assayed. Total protein

concentration was determined with the Bio Rad Protein assay using BSA as standard. Ten (10) to twenty (20) µg of proteins were analysed on SDS-PAGE.

F. Polyacrylamide Gel Electrophoresis (PAGE)

Protein samples were analyzed by non-denaturing or denaturing SDS-PAGE. Non-denaturing PAGE was prepared with Tris-HCl 1.5 M, pH 8.8 and run in Tris Base-Glycine buffer pH 8.2. Samples were mixed with 1/5th volume of Bromophenol Blue-Glycerol (3:1) loading buffer. Gels were run at 100 V for the first 15 min and then at 150 V. Proteins were visualized by staining the gel for 30 min in 1% Coomassie Brilliant Blue R-250 solution. After distaining with 18.6% Methanol, 7% Acetic Acid, the gel was washed 30 min in 1% glycerol and dried at 80°C on a vacuum dryer. Denaturing PAGE (SDS-DTT) was prepared with Tris-HCl-SDS, pH 8.0 and run in Tris HCl-Glycine-SDS buffer, pH 8.2. Samples were mixed with 1/5th volume of Bromophenol Blue-Glycerol (3:1), (1%) SDS-DTT (10 mM) loading buffer and heated 5 min at 65°C before applied to the gel. The running and visualization of proteins were performed as described above for non-denaturing gels.

G. Western Blot Analysis

For the analysis of the plasma Vtg, 12 µg of plasma proteins were separated on 5% SDS-PAGE (Mini Protean II, Bio Rad) and transferred to an Immobilon (PVDF) membranes (Millipore) using the Electro-blotter system (Bio Rad). The transfer reaction was performed at 35 volts, 4°C in an electro-blotting buffer containing 25 mM Tris Base, 100 mM glycine and methanol 20%. The membrane was incubated in blocking buffer (1x TBS pH 7.4, 150 mM NaCl,

0.05% Tween 20 and 5% non-fat dry milk) for 1 h at room temperature. For immuno-detection, membranes were incubated 1h at room temperature with Anti S1 Vtg antibody diluted 1:20000 in blocking buffer (or in both, Anti S1 and Anti Albumin [1:10000] for double labeling immuno-detection). After three 5 minutes washes (1x TBS pH 7.4, 0.05% Tween 20) at room temperature, the membrane was incubated with horseradish-peroxidase conjugate goat anti rabbit IgG in a dilution of 1:10000 on blocking buffer for 1 h at room temperature, and washed as described above. Immuno-reactive bands were detected by enhanced chemi-luminescence according to the Amersham's ECL-Western -Blot protocol or UVP Bio-Imaging Systems.

H. Total RNA Isolation.

All plastic, as well as glassware was made RNAse free by treatment with 0.1% with Diethyl-pyrocarbonate solution (DEPC, Sigma Co). Glassware was treated for 5 minutes and then baked at 275^{o}C for 4 hrs. Plastic ware was treated for 30 min and left overnight at 37^{o}C. Electrophoresis equipment, surfaces, dissection equipment was cleaned with RNAse Zap (Ambion Inc., Austin, TX). Solutions were prepared with RNA grade reagents, DEPC treated, autoclaved, or filtered (0.2 µm, Millipore) ddH_2O. Total RNA Isolation was done using the RNAeasy Kit (Qiagen Co., Chatswoth, CA). After dissection the livers were weighed and immediately incubated in RNA-later (Ambion, Inc) following manufacturer instructions until ready for RNA isolation. Livers were homogenized in ice with a Polytron homogenizer with burst of 20-40s and

processed following manufacturer's instructions for the RNA-Easy kit (Ambion, Inc). RNA concentration was determined with an UV spectrophotometer (Beckman Instrument Inc.) at 260/280 nm. Samples were stored at –80^{o}C.

I. Formaldehyde agarose gel electrophoresis of RNA

Agarose gels (1% agarose) were prepared by boiling 0.8g agarose, 7.5 ml 10x MOPS buffer (10x Stock: 0.2 M MOPS, 0.01 M EDTA, 0.05 Sodium Acetate, pH 7) and 63 ml ddH2O in the microwave oven for 3 min. After cooling to approximately 55°C, 4.05 ml of 37% formaldehyde and 4 µl of 1% ethidium bromide were added. RNA (5 µg) was dried out in the Savant evaporator and dissolved in 10 µl of RNA loading buffer (1.44 ml formamide, 320 µl 10X MOPS, 520 µl 37% formaldehyde, 360 µl H_2O, 200 µl glycerol, 0.0030 g xylene cyanol and 0.0030 g bromophenol blue). Samples were heated at 95°C for 5 min. Electrophoresis was carried out in 1X MOPS buffer at 100 V for 40 min. After separation, the gel was placed in a UV transiluminator (Fotodyne Inc.) to be photographed and RNA integrity estimated by rRNA (28S and 18S) proportion.

J. Semi quantitative Vtg RT-PCR

1. Reverse Transcription (cDNA Synthesis)

In a PCR (250 µl) micro tube 3 µg of total RNA were mixed with 500 ng of oligo-dT primer and water up to 13µl. This mixture was heated at 70^{o}C for 10 min and immediately incubated in ice. One (1) µl of 500 µM dNTPs mix was added followed by 4 µl of first strand 5x buffer for Super Script II (Gibco BRL Co., Gaithersburg, MD) and water up to 19 µl. The mixture was incubated 5 min

at 42°C and 200 U of Superscript II (1 µl) was added and re-incubated 50 min at 42°C and 15 min at 70°C. One (1) µl (2 U) of RNAse was added and the mix incubated for 20 min at 37°C. Samples were stored at –80°C until ready for PCR amplification.

2. Semi quantitative PCR

In a 250 µl PCR reaction vial, 2 µl cDNA were incubated in the following reaction mix: 5 µl 10XPCR buffer, 200 µM dNTPs, 0.2 µM of each primer, and 2.5 U of Taq Polymerase for each 50 µl (Perkin Elmer Co., Norwalk, CT.).

Amplification was performed in a Thermocycler (Perkin Elmer) at 1 min 94°C - 2 min 52°C - 1 min 72°C, for 30 cycles. A pre- and post holds (4 min at 94°C and 7 min at 72°C respectively) were applied to the program. Amplified DNA was analyzed in a 0.8% agarose TAE gel stained with 0.1% ethidium bromide and visualized in a Gel Doc System (Fotodyne Inc). Figure 5 shows a flowchart diagram summarizing the previously described experiments.

K. Protein extraction

The intracellular protein extraction procedure was basically as in Riley and Callard (1988). All biochemical procedures were carried out at 0-4°C and all solutions were supplemented with 1.45 µl/ml of protease inhibitor cocktail (AEBSE 1000µM, Aprotinine 80 µM, Best 5 mM, E64 1.5 mM, Leupeptine2 mM, Pepstatine A 1 mM; EMD-Calbiochem, Richmond, CA). Control and treated lizards were weighed, sacrificed and their liver

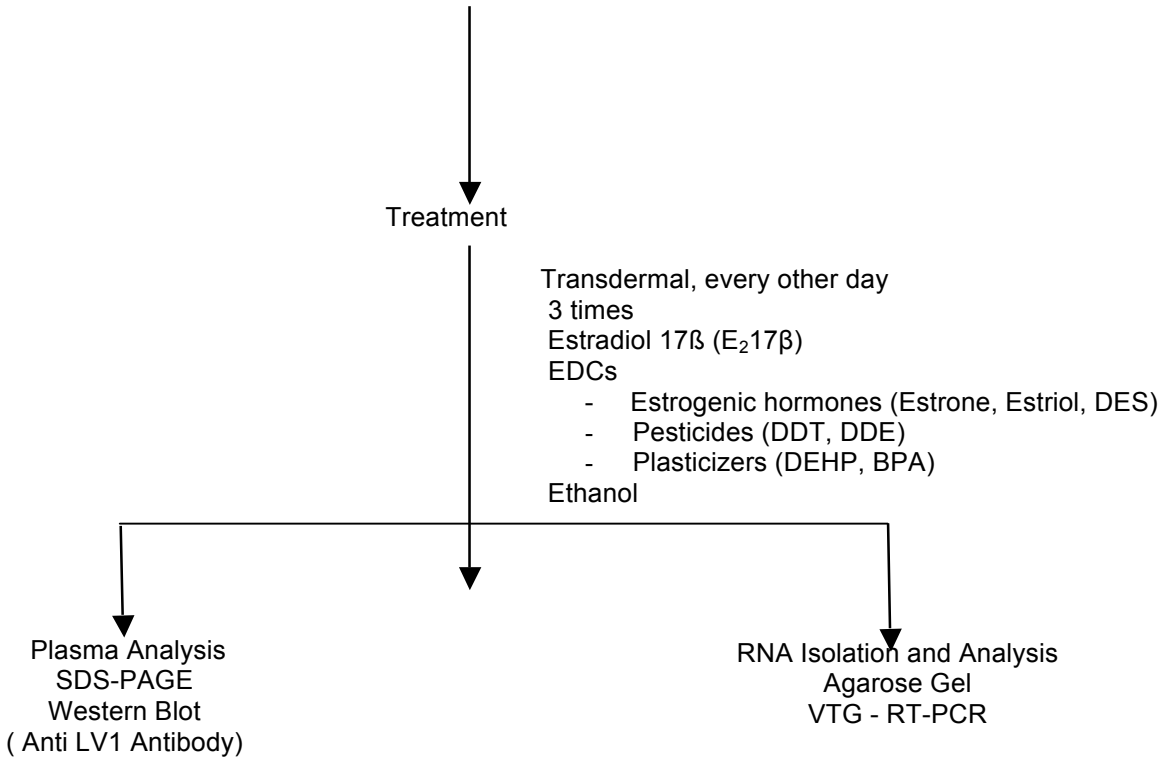

Figure 5 - Flowchart summarizing the experimental design for estrogenic assessment of suspected compounds. Male animals were treated within 12 h after capture with suspected estrogenic compounds (EDCs) every other day, for three days. Plasma was analyzed for Vtg by Western Blot using Anti LV1 antibody. Total RNA was isolated and analyzed by RT-PCR for VTG mRNA. The vehicle (ethanol) and E_2-17ß were used as negative and positive controls, respectively.

excised and incubated in ice cold 0.65% NaCl with 1.45 µl/ml of protease inhibitor cocktail. Tissue was minced in 0.65% NaCl containing the protease inhibitor cocktail; washed two times, weighed and homogenized in 3.0 volumes of homogenization buffer (50 mM Tris-HCl, 1 mM EDTA, 12 mM monothioglycerol, 30% glycerol). The homogenate was centrifuged at 14,000 g for 20 min. The surface lipid layer was discarded and the supernatant centrifuged at 100,000 g for 1 h to obtain the cytosolic fraction. The remaining nuclear pellet was washed three times with 30 volume of nuclear washing buffer (10 mM Tris-HCl, 3 mM $MgCl_2$, 12 mM monothioglycerol, 0.25 sucrose,

pH 7.5), and incubated with 0.7 M KCl extraction buffer (0.7 M in homogenizing buffer, pH 7.5) for 1 h at 4°C with mixing every 15 min. The final nuclear suspension was centrifuged at 100,000 g for 1 h and the supernatant was saved as the nuclear extract.

Cytosolic and nuclear extracts were frozen at -70°C until assayed. Total protein concentration was determined by spectrophotometric analysis using the Bio-Rad protein assay at visible light (595 nm) and BSA for the standard curve. Since the nuclear extract consistently resulted in a more diluted fraction, the cytosolic extract was used for the experiments.

L. Estrogen Receptor and Plasma Steroid Binding Protein Assays

To assess the presence of the estrogens receptor in the liver protein fractions and of steroid binding proteins in blood plasma a binding assay was performed as follows: 3 µg/µl of proteins (as determined by Bio-Rad Protein assay) were incubated 2-4 hr in ice-cold TEMG buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM 2-Mercaptoethanol, 10% glycerol, pH 7.5) containing 7nM [^{3}H]-E_2-17β and or 700 nm of E_2-17β (or the EDCs for competition) at 4^{o}C over a rocking platform. After this incubation, the reaction was applied to micro-concentration columns covered with a nitrocellulose membrane with a 30,000 Da cut-off range (Amicon, Millipore Corp) and centrifuged at 4^{o}C by 15 minutes at 3000 rpm (Beckmann JL-21). The membrane was washed 3 times with 3 ml of ice-cold TEMG buffer each by centrifugation as above. The [^{3}H]-E_2-17β counts per minute retained in the membrane were determined in a LS Analyser (Beckman Instruments, Inc.) scintillation counter.

M. DNA probe labelling for Electrophoretic Mobility Shifts Assays (EMSA)

1. Oligonucleotide design

The sequences of the estrogen response element of five (5) estrogen-regulated genes were obtained from the Internet via Gene-Bank (http://www.ncbi.nlm.nih.gov/). Using the Box-Shade and Text-Shade Algorithm from Work-Bench (San Diego Super Computer Center, University of California, San Diego, http://workbench.sdsc.edu/) the sequences were aligned and the following consensus sequence was obtained: **5'-GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT-3'**. This 35 bp oligonucleotide containing the ERE consensus sequence was commercially synthesized (Integrated DNA Technologies, Inc.) and used as a probe.

In preparation for the labelling reaction and further experiments, the synthesized single stranded oligonucleotide was made double stranded as follows: the lyophilized oligonucleotides were dissolved in ddH_2O at 1µg/µl. From this stock solution, 10 µl of each strand were diluted in STE 1x (100 Mm NaCl, 200 mM Tris-Cl pH7.5, 10 mM EDTA), to a working solution of 1.87 pM, mixed, boiled for 3 minutes and then let to cool at room temperature over night to allow for hybridization.

After this, labelling was performed by the 5' end phosphorylation reaction using the T4 kinase enzyme as follows: In a 1.5 ml micro-tube 1 µl (200 ng) of the double stranded oligonucleotide, 1 µl of the 10X T4 kinase buffer, 1 µl of [^{32}P] -ATP (3,000 Ci/mmol at a 10 µCi/ml concentration), 7µl of dH_2O, 1µl of the T4 kinase (5-10 U) were mixed and incubated for 10 minutes at 37ºC.

Reaction was stopped with 1µl of 0.5M EDTA. The probe was purified using the Nuc-Trap columns (Stratagene, Co., La Jolla, CA.) following manufacturers instructions. Counts per minutes (cpm) were determined diluting 1µl of labelled probe in 5 ml of scintillation liquid in a LS Analyser (Beckman Instruments, Inc).

N. Electrophoretic Mobility Shift Assay (EMSA)

These assays were performed following the instructions of the literature provided by the EMSA kit manufacturer (Promega, Co., Madison, WI) with some modifications to optimize for complex formation. In a 500 µl tube, 50 µg of cytoplasmic proteins, and binding buffer (10-20 µl total volume) were incubated on ice for 30 min with E_2-17β (10^{-7} M) to activate the ER at 0^oC.

The labeled probe was added (20,000 cpm) followed by another 20 min incubation at room temperature. Gel loading buffer (250 mM Tris HCl pH 7.5, 0.2% Bromophenol Blue, 40% Glycerol) was added to the negative control only. Three different binding buffers (from Promega EMSA Kit, 2004, Ruiz-Echevarria, et al. 2000, and Kumar and Chambom, 1988) were tested during the course of these experiments. Samples were separated in a non-denaturing 4% 0.75 mm 0.5x TBE PAGE (Mini-protean III, Bio Rad Laboratories) at 350 volts for 10-12 minutes. The gel was dry-out, wrapped in a thin plastic sheet and exposed in a Molecular Imager Fx Pro Plus (Bio Rad Laboratories) cassette for 1-2 h, and analyzed in Quantity One software.

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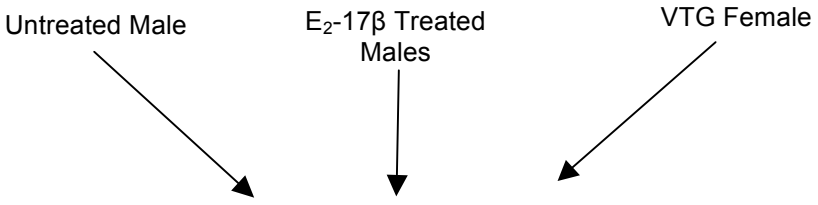

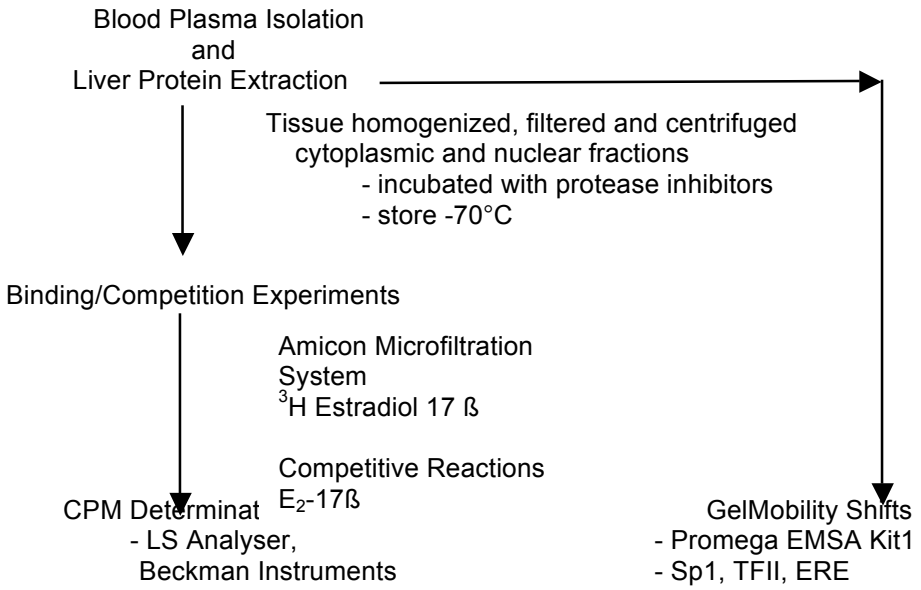

Figure 6. Flowchart summarizing the experimental design of objectives 2, 3 and 4.
Liver protein extract from recently captured VTG females, E_2 treated males, and non-treated males was prepared, aliquot and stored at -70C. It was incubated with ^3H-E_2 to demonstrate the presence of the ER in the extract. A competition experiment with non-radioactive E_2-17ß and with the suspected EDCs was done to show the specificity of the interaction. The same kind of experiment was done with blood plasma from treated animals, to show the presence of steroid binding proteins and their interaction with EDCs. Gel Mobility Shifts were also performed to demonstrate the quality of the liver extract and to confirm the presence of the ER.

To determine the integrity of the protein extract Gel Shift Assays were performed first for two general transcription factors (SP1, TFII) using oligonucleotide probes from the Promega- EMSA Kit for these two proteins. In some experiments, the binding reaction included unlabeled probe (specific, and a non-specific unlabeled probe) to compete the binding of the labeled probe as an indication of the specificity of the complex formed. Figure 6 shows a flowchart diagram for these experiments.

Statistical analysis

Statistical data analysis (descriptive statistics as well as ANOVA) was performed with SPSS software, (SPSS Inc, Chicago, Il). If the ANOVA test showed a significant difference ($P < 0.05$), the Tukey's Multiple Comparisons Test was used to identify differences between means.

Results

A. Trans-dermal method of treatment

In our laboratory the regulation of Vtg synthesis by estrogen in lizards have been studied since 1980. Prior to this present study the method used for administration of hormones was intra-muscular injection (IM) at the tail base. This technique of IM administration proved to be an effective and secure route for the hormone to get access to the animal bloodstream. However, like all invasive systems, this method implies a considerable amount of pain, stress and high mortality rates, especially in multiple applications experiments. Since one premise of this research was to consider anoline lizards as a potential sentinel species for environmental contamination, an important objective was to develop a non-invasive and less stressful mode of chemical administration. In the trans-dermal method (TD) minute quantities of the test compound (hormone or EDC) dissolved in ethanol 95% are applied directly over the ventral surface of the lizard body. In this way there is a fast uptake of the dissolved chemical via the skin into the animal, taking only seconds to dry off the animal surface. After the application of the test compounds, animals are returned to their cages in a short time and with much less injury and stress. This technique resulted in a large reduction in the mortality of experimental animals exposed to many treatments. It is important also to point out that this method more closely resembles the external, casual, way of exposure to the pollutant that may occur in the environment.

The effectiveness of the trans-dermal treatment was assessed by administering doses of 50μg of E_2-17β to male lizards every other day. Figure 7

illustrates the Western Blot (WB) analysis of blood plasma samples pooled from several animals tested for Vtg protein synthesis. An electrophoretic profile of three main bands was observed in the plasma from the treated animals. In contrast, no bands were visible in lane #1 (C-) corresponding to the blood plasma from animals treated with the vehicle (ethanol 95%). The protein band profile observed in the animals that tested positive included the Vtg proteins Vtg-169, Vtg-153 and Vtg-116 typically observed in female lizards during active reproductive stages but, normally absent in males (Morales et al. 1991).

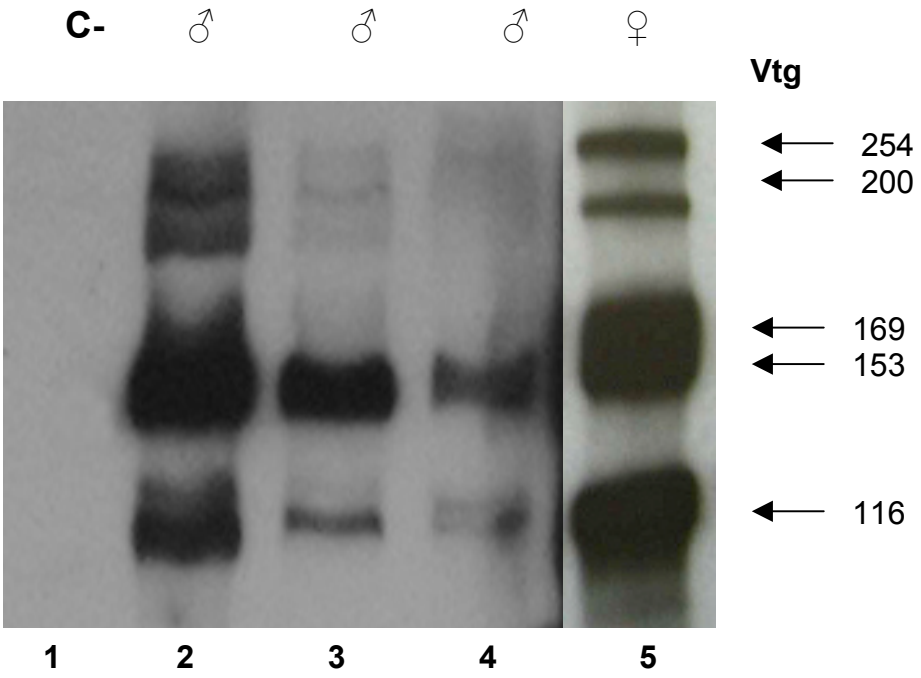

Figure 7- Effects of trans-dermal administration of Estradiol-17β on males of *Anolis pulchellus*
Male individuals of *Anolis pulchellus* were trans-dermal (TM) treated every other day for 3 times (lanes 2-4) with 50 µg of $E_2$17β dissolved in Ethanol 95% as vehicle (see Experimental Procedures). After sacrificed, blood plasma from each group (3 animals per group) was pooled. Fifteen (15) µg of blood plasma proteins were analyzed per lane by Western Blood with an Anti S1 (Vtg) antibody developed in our lab. Lane 1, males treated with Ethanol 95%, lane 5, vitellogenic female.

An important question to be answered was in terms of the delivery efficiency of the trans-dermal method. How much of the original amount of hormone intended for the animal treatment, had access to the interior of the lizard body? For this purpose, an experiment was designed in which ^{3}H-$E_2$17β was administered TD and after different time intervals the amount of cpm incorporated into different parts of the lizard body were determined. The results show that after 15 min, about 60% of the $5x10^{6}$ cpm administered was located inside the body of the animal. Approximately 20% remain in the outside of the skin and 17% in the plastic tip used for the administration (Figure 8). No differences were observed between 15 and 30 minutes after the administration (Data not shown).

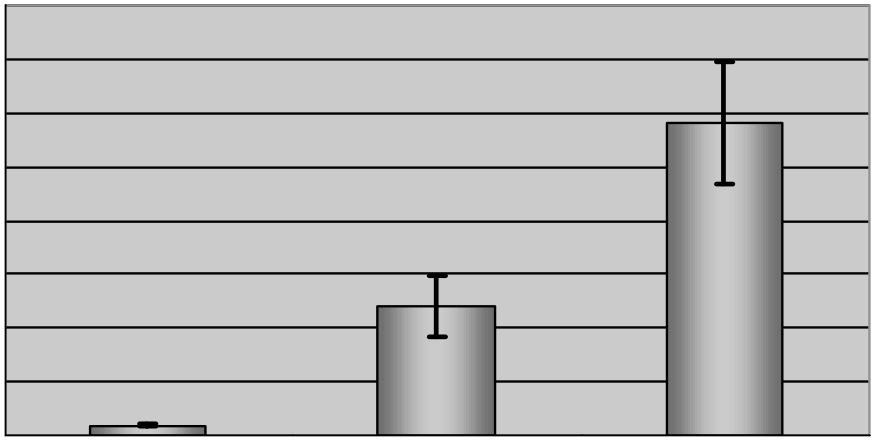

Figure 8- Effectiveness of the transdermal approach developed in our laboratory.
Five (5) ul ($5x10^{6}$ cpm) of ^{3}[H]-E_2-17β were applied TD. Animals were sacrificed after 15 minutes. CPM was determined for the skin area where the radio-labeled hormone was applied and for the body carcass. Data represents two independent triplicate experiments (6 individuals for each bar). Standard deviation bars are shown. Asterisk (*) denotes significant differences between the groups ($p < 0.05$).

To compare the effectiveness of the TD method with the previously used method (IM) for E_2-17β administration, we performed an experiment to establish the lowest dose of the administered hormone able to induce detectable levels by Western Blots (WB) of Vtg in male plasma. The results illustrated in Figure 9 show that the E_2-17β delivered by the IM technique was capable to induce Vtg with a dose 10 times lower than when applied by the TD (lanes 2 in both panels). However, the TD method also allows the effective application of hormone at very low concentrations (0.5-0.005 µg). **Thus, considering all the mentioned positive factors for the TM, this was the method of choice for this study.**

B. Effects of Endocrine Disruptors on the synthesis of Vtg in male lizards

The main hypothesis of this project was that **as the synthesis of Vtg in the tropical lizard *Anolis pulchellus* is induced by $E_2$17β, likewise Endocrine Disrupting Chemicals (EDCs) with estrogenic activity (Xenoestrogens (XEs)) should also induce Vtg.** Therefore, the presence in wild male plasma of Vtg is a positive indicator of XE contamination in the environment.

To test this hypothesis, three main types of EDCs (pesticides, plasticizers and hormones), were tested in male individuals. They were administered using the TD method (the more casual approach) and their effects on Vtg synthesis was assessed, by analyzing blood plasma samples for Vtg by WB.

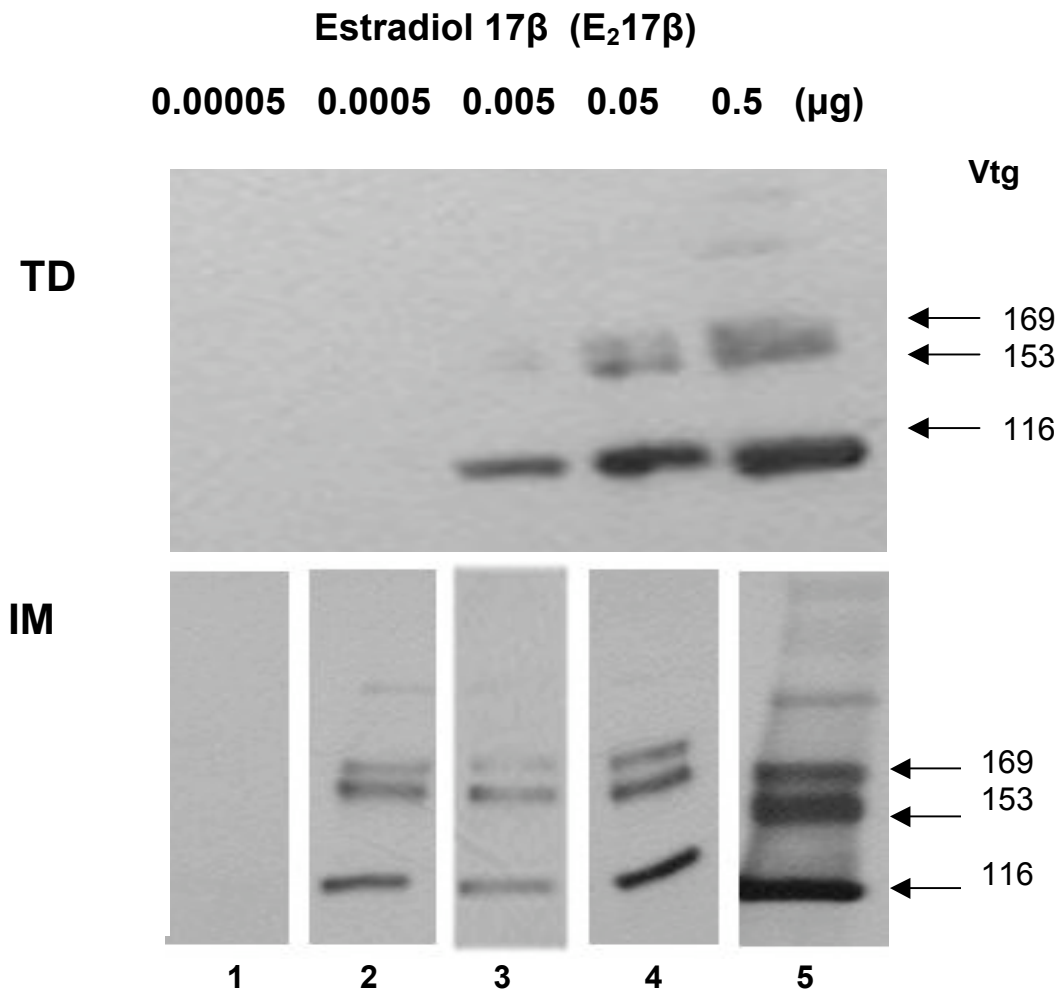

Figure 9- Determination of the Minimal Dose for $E_2$17B: Trans-dermal (TD) vs Intramuscular (IM) Male individuals of *Anolis pulchellus* were treated **TD** or **IM,** with lowering doses (0.5–0.00005µg) of $E_2$17β every other day for 3 times. After sacrificed, blood from each group was pooled. Fifteen (15) µg of blood plasma proteins were analyzed per lane by WB.

1. Pesticides

The results of these experiments show that three of the tested pesticides were capable to induce Vtg synthesis in detectable levels (Figure 10). The tested pesticides belong to the family of the dichlorophenyletanes, (DTT, DDE and

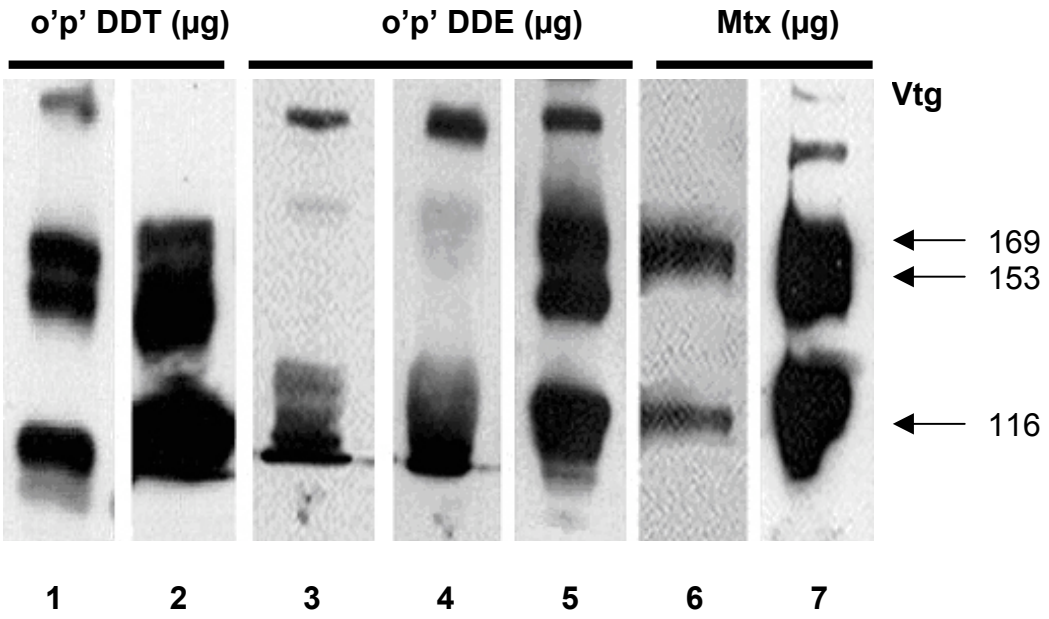

Figure 10- Effects of pesticides on Vtg synthesis.
Males individuals were treated TD with different pesticides (DDT, DDE and Mtx) every other day for 3 doses to determine if estrogenic effects were observed. After sacrificed, blood plasma from each group was pooled and 15 µg of proteins were analyzed for VTG by Western Blot.

Methoxychlor (Mtx). Bands corresponding to Vtg-169, Vtg-153 and Vtg-116 were observed after the three treatments. This band profile is consistent with the one reported by Morales, et al (1996) after the treatment of male individuals with $E_2$17β. Of the three pesticides tested op DDT give the stronger induction of Vtg, even at the lower doses of 60µg.Other pesticides tested such as lindane, and toxafene did not induce the synthesis of Vtg (data not shown).

2. Plasticizers.

To test if an estrogenic activity can be associated to plasticizers in the lizard system; four different phthalates were applied to male individuals. Figure

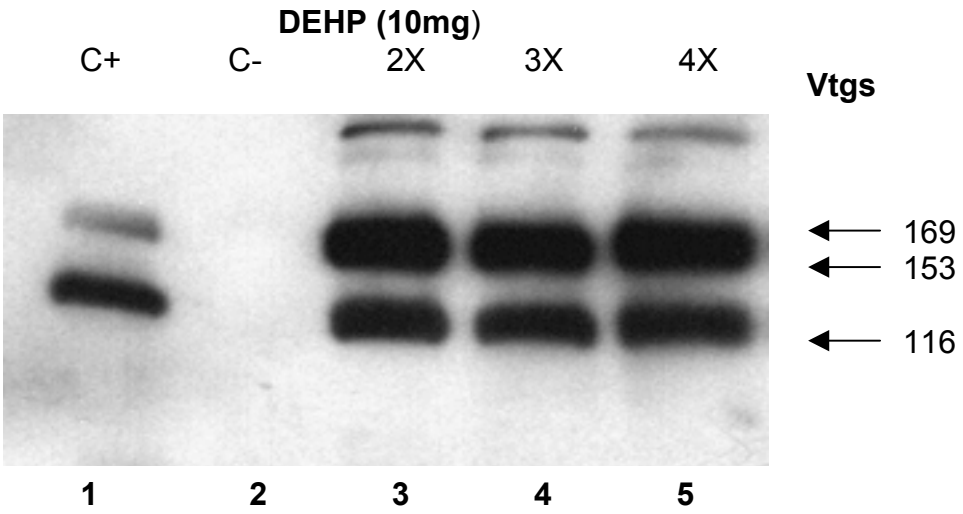

Figure 11 – Induction of VTG by DEHP.
Animals were treated every day with 10 mg (10000 µg) of DEHP every other day and sacrificed after 2, 3 or 4 treatments. Fifteen (15) µg of proteins from pooled blood plasma were analyzed for VTG by WB.

11 shows that Diehtylhexylphthalate (DEHP) was able to induce the Vtg proteins at very high dose doses (10mg). Other phthalates tested; DOP (Dioctylphthalate), Dibutylphthalate (DBP) (and Dibutylbenzylphthalate (DBBP) failed to induce the Vtg synthesis (data not shown).

Another plasticizer tested in these animals was BPA. BPA was able to induce Vtg in males with doses from 500 µg to 1000 µg (Figure 12) while 250 µg was not enough to induce a detectable level of Vtg. To demonstrate that similar quantities of blood plasma samples were applied in each lane, an antibody against the lizard Albumin (Alb) was also included in the WB assay as an internal

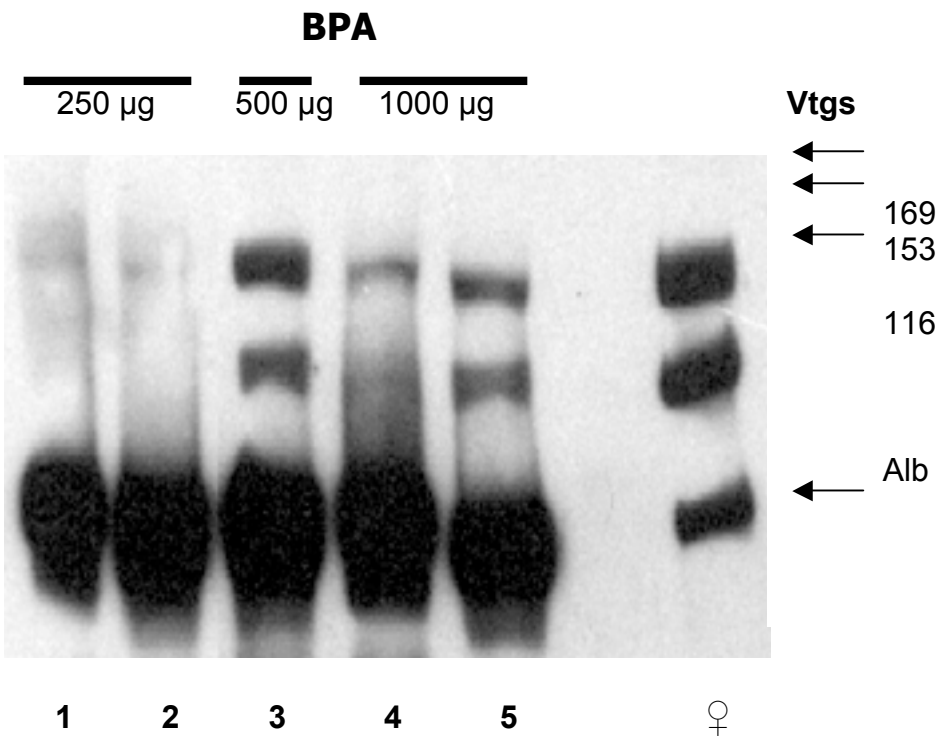

Figure 12- BPA induce VTG synthesis in *Anolis* males.
Animals (3 per group) were treated with BPA every other day for 3 treatments. Fifteen (15) µg of blood plasma proteins were analyzed individually for VTG by WB. Albumin was included as an internal control.

control. This allows to monitor the Alb band (that should be stable under the E_2 treatment) at the same time that the modulation of the inducible Vtg bands.

3. Hormones.

The third group of EDCs tested included female hormones, Estriol (E_1) and Estrone (E_3), and a synthetic non-steroidal estrogen Diethylstilbestrol (DES). These three compounds were capable to induce Vtg synthesis at lower doses (0.5µg for E1, 0.5µg for E_3 and 0.005 µg for DES) than the other EDCs tested (Figure 13). As expected given their close chemical similarity to $E_2$17β, these EDCs were more effective as Vtg expression inductors.

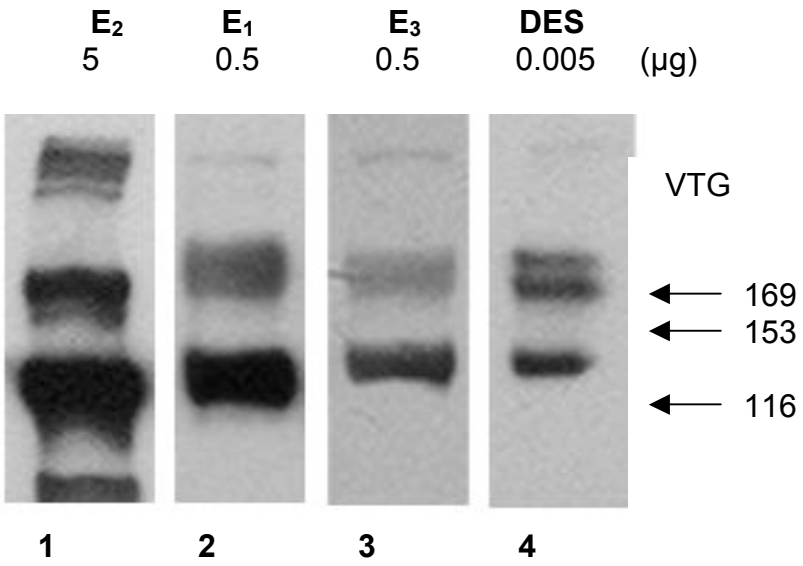

Figure 13- VTG induction by natural and synthetic hormonal compounds.
Animals were TD treated with Estriol (E_1), Estrone (E_3) and Diethylstilbestrol (DES) every other day for 3 times to determine the lower Vtg synthesis inducing dose in males. After sacrificed, blood plasma from 3 animals was pooled and 15 µg of proteins were analyzed for VTG by WB. $E_2$17β (E_2), was included as a positive control.

4. Effects of Antiestrogen – Tamoxifen

Some studies have demonstrated that Tamoxifen (Tam) is an antiestrogen (Jordan et al, 1987). However, additional reports have suggested that Tam agonistic effects on the estrogen receptor (Kleinsmith et al. 2002, Rivera-Gonzalez et al. 1998) Thus, we decided to test the capability of Tam to induce Vtg in male lizards.

Figure 14 demonstrate that none of the Vtg proteins were detected in plasma samples from animals treated with increasing doses of Tam, from 500 µg to 2000 µg (4 applications in 6 days). Albumin was used as an internal control for this analysis. This experiment was performed 5 independent times with minor modifications but similar negative results were obtained in all of them.

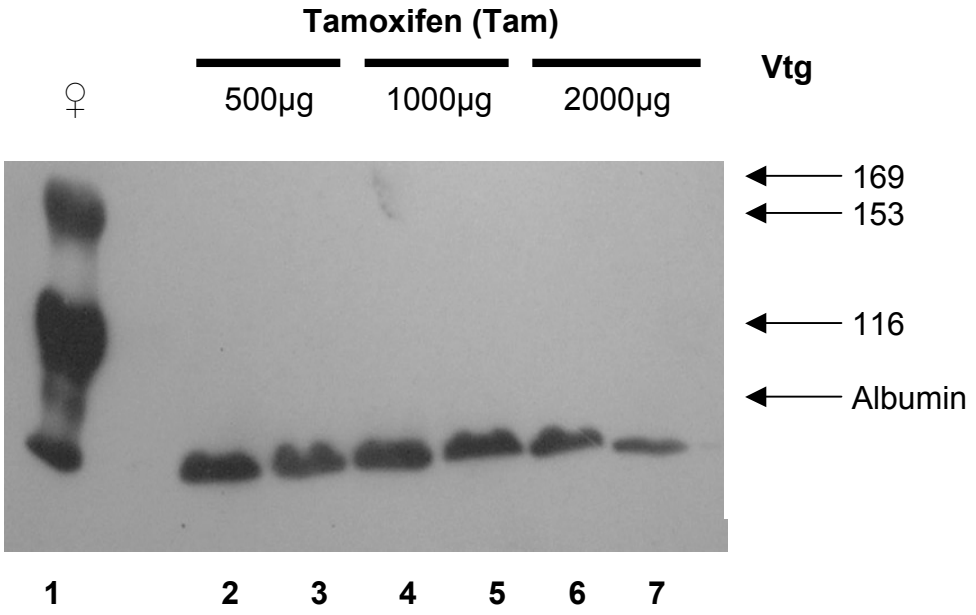

Figure 14– Tamoxifen do not induce VTG in Anolis males.
Animals were treated with increasing doses of Tamoxifen (Tam) every other day for a week. After sacrificed 15 µg of blood plasma proteins were individually analyzed for VTG by WB. Albumin was included as an internal control. This experiment was performed 5 times.

5- Reversion of the negative effects on Vtg synthesis in captive female lizards

Previous studies from our laboratory have shown that $E_2$17β treatment revert the detrimental effects of captivity in Vtg synthesis observed on female anoline lizard (Morales and Sanchez, 1996). Thus, a second approach taken in this study to demonstrate estrogenic activity of EDCs was to test if they were capable to reverse the inhibition of Vtg synthesis. Female lizards were kept under captivity for 15 days and afterwards they were treated with the three pesticides, Mtx, DDT, and DDE (500 µg). This dose was higher than the one shown to be estrogenic in males (see Figure 10). Figure 15a shows that residual Vtg protein is still circulating in the blood after 15 days of captivity. These basal levels are increased by the treatment with all three pesticides demonstrating the *novo* protein synthesis. RT-PCR analysis of liver RNA

extracted from these females confirmed an effect of pesticides at the transcriptional level. As shown in figure 15b, no Vtg mRNA was present at 15 days of captivity in untreated females. In contrast the presence of Vtg mRNA was strongly indicated by the synthesis of the complementary 300 bp oligonucleotide detected in RNA from females treated with DDT, DDE and Mtx after captivity. In this experiment we also tested the common garden the herbicide, Glyphosate (Glf) Isopropyl amine salt, (Round Up). The results demonstrate that Glf does not have estrogenic effects since no Vtg RNA or protein was induced.

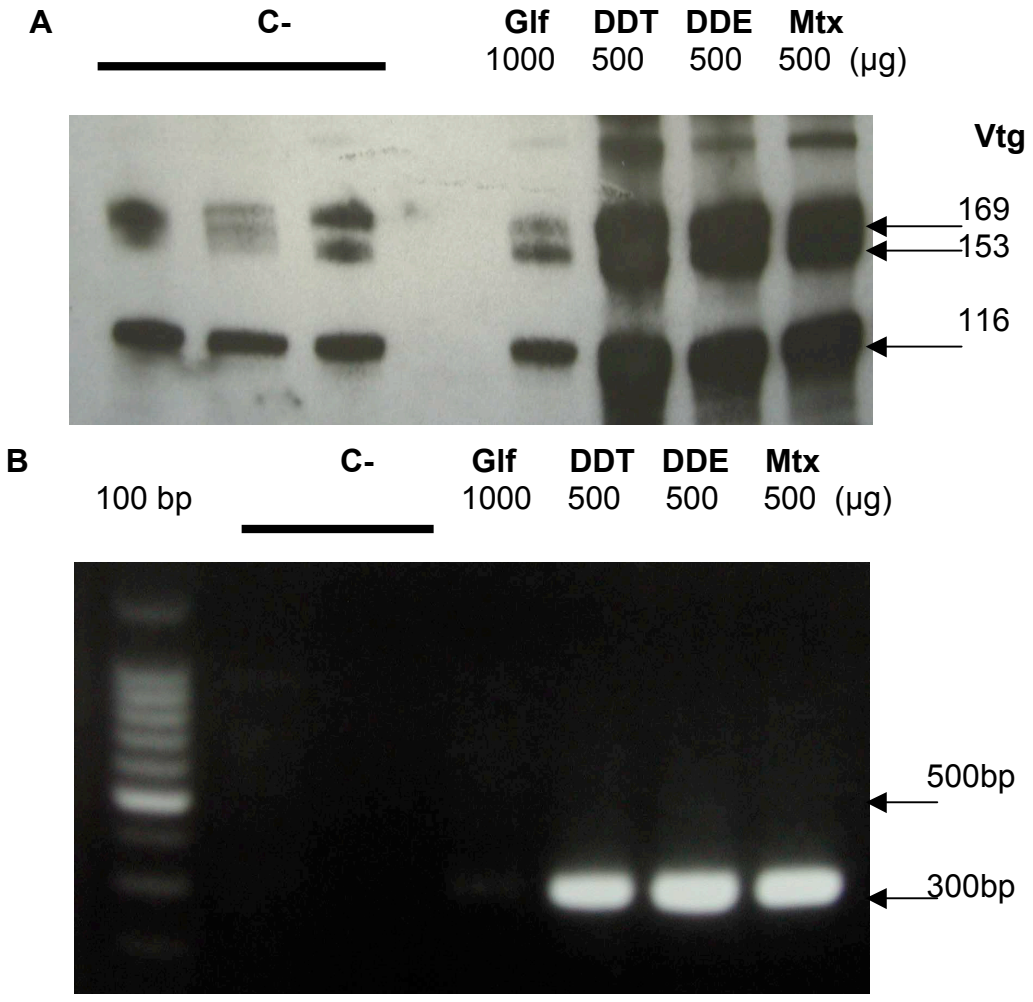

Figure 15- Vtg synthesis reverted by pesticides in anoline females.

A) Females were kept in captivity for 15 days and then treated with pesticides every other day for 3 treatments. After sacrificed, blood plasma (15 µg) was analyzed for Vtg by WB.

B) RT-PCR from total RNA from liver of females kept in captivity (C-) and treated with pesticides. Synthesis of Vtg mRNA (300bp) was observed after the treatments with the pesticides. C-, females in captivity non-treated. Glf for gluphosate isopropyl amine salt (active ingredient of Round UP).

6- Studies on Dose/Response Relationship of EDCs Treatments

In order to demonstrate that the effects observed over the Vtg synthesis are a direct result of the treatment with the EDCs, we performed experiments to analyze the dose/effect relationship. First of all, a lineal cause/effect relationship on the induction of Vtg synthesis with E_2 has been observed. As shown in figure 9 increasing levels of Vtg were obtained during E_2-17β IM treatments as dose increase from 0.0005ug to 0.5ug and from 0.005 to 0.5 in TD administration. However, no clear-cut direct relationships were observed for the EDCs treatments (for example see Figure 10 DDE, Figure 12 BPA). To clarify this behavior of the EDCs/XEs estrogenic activity, one representative of each EDCs/XEs group (Mtx, BPA, and DES) was used in a different experiment. Three groups of seven animal each, was treated with an increasing dose for several days and then the blood drawn from the animals was analyzed individually on a WB assay.

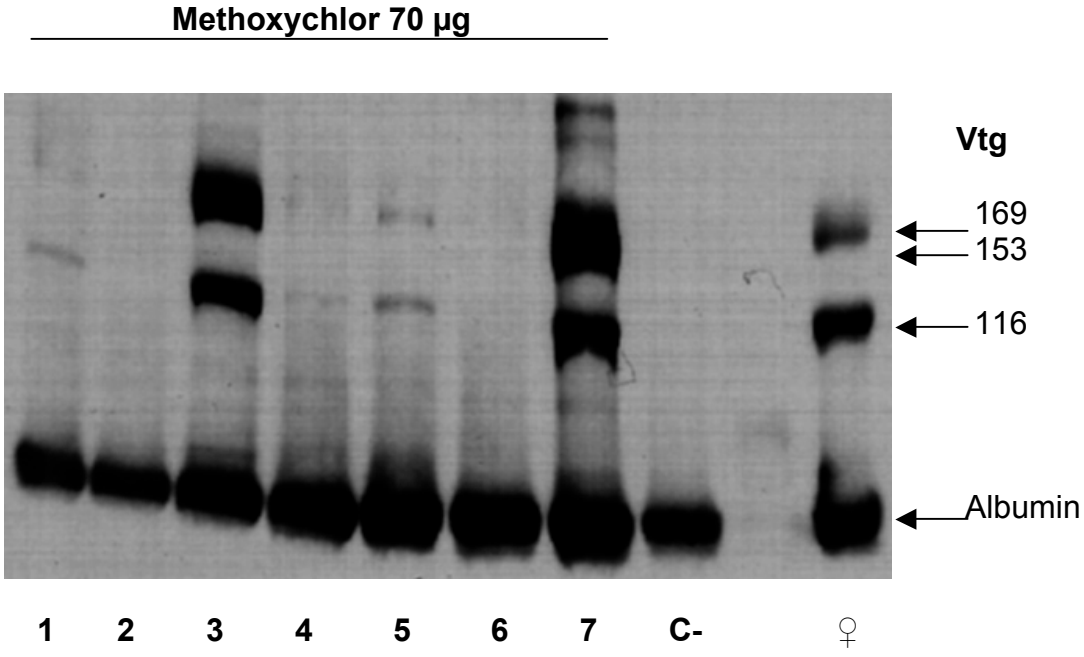

Figure 16 – Effects of Mtx in the synthesis of Vtg
Seven individuals were treated with 70 µg of Mtx every other day for 3 treatments. After sacrificed, blood plasma was individually analyzed for VTG in WB. Table V shows a summary of the results of three treatments with Mtx (70 µg, 140 µg and 280 µg).

For Mtx, three groups of 7 males each were treated with 70 µg, 140 µg or 280 µg, every other day for 3 times. The results from Mtx are summarized in Table V. As the dose increases 2, 5 and 6 individuals out of seven show a positive Vtg reaction. Figure 16 shows the results of a WB analysis of the plasma from animals treated with 70 µg of Mtx. Vtg is only present at high levels in the blood of two animals out of seven animals that were treated with Mtx. Some animals show traces of Vtg indicating low level of induction. These results suggest that the level of induction on all the treated animals in the group is not the same.

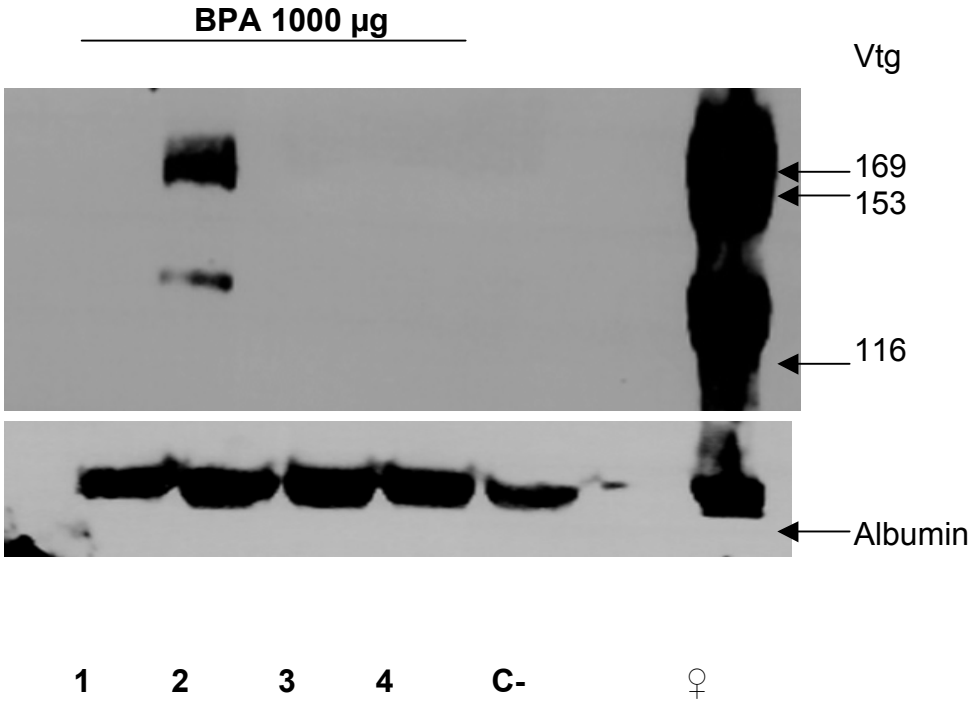

Figure 17 – Effects of BPA in the synthesis of VTG in Anolis males.
Seven individuals were treated with 1000ug of BPA every other day for 3 treatments. Three of them died before 3rd day of treatment. After sacrificed, blood plasma was analyzed individually for VTG in WB.

A very different response was observed when the effect of BPA was analyzed in a similar experiment. In this case 375 µg, 500 µg to 1000 µg were applied three times every other day to each group. Results are presented in Figure 17and Table V.

Induction of Vtg on 1 or 2 individuals per group of 7 was observed for the three concentrations of BPA tested. Therefore an increase in the amount of BPA applied does not resulted in more animals showing Vtg in the group. These experiments were repeated 3 times with very similar results.

As expected, the more powerful and consistent response was observed in animals treated with DES. With the exception of one animal at the highest concentration (30µg) all treated animals show Vtg in the plasma. Six, seven and five individuals (out of 7/6) tested positive for Vtg, as result of treatment with 0.3, 3, 30 µg of DES, respectively (Figure 18, Table V).

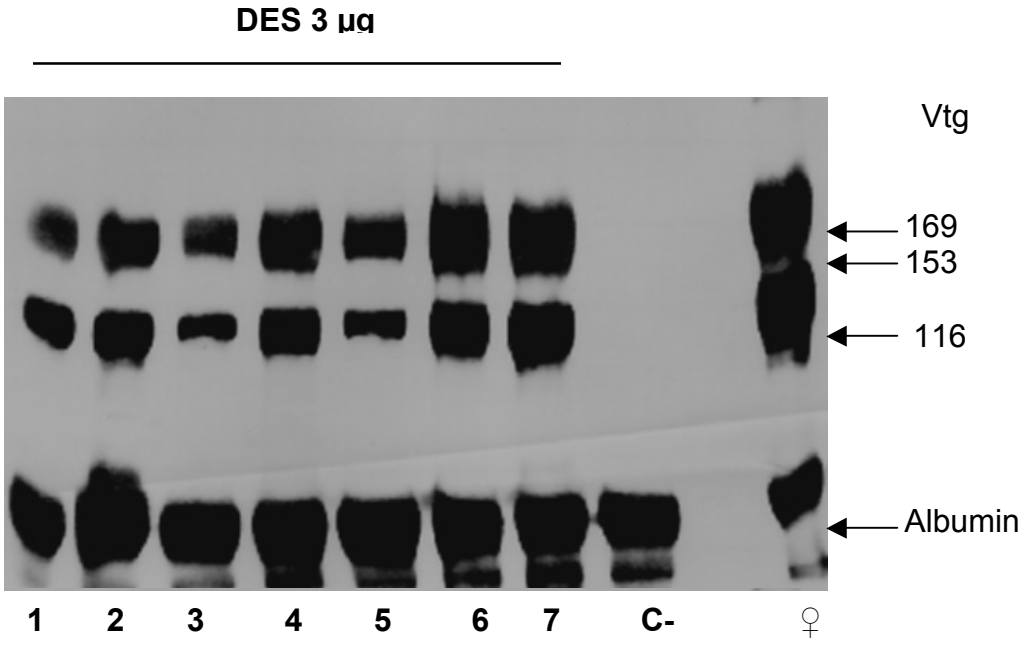

Figure 18 – Effects of DES in the synthesis of VTG in *Anolis* males.
Seven individuals were treated with of 3µg of DES every other day for 3 treatments. After sacrificed, blood plasma was individually analyzed for VTG in WB.

C. Interaction between the EDCs/XEs and Estrogen Binding Proteins

Once some EDCs were corroborated as XEs by their ability inducing Vtg *in vivo* in this lizard system, the next step of this study was to determine if this effect involved an interaction with the estrogen receptor by means of an *in vitro* binding assay.

1. Inhibition of [^{3}H]E_2-17β binding to intracellular liver proteins by E_2-17β and EDCs/XEs

To demonstrate the presence of an specific E_2-17β binding activity in the lizard liver, a cytosolic protein extract from livers of E_2-17β treated and non- treated male animals were incubated with [^{3}H]-$E_2$17β. This binding was performed in the presence and absence of radio-inert $E_2$17β to show specificity.

Table V– Individual WB analysis of blood plasma. Five to seven individuals were treated with Mtx, BPA or DES as explained in Experimental Procedures. Blood samples from these experiments were analyzed individually. Numbers under Vtg+ and Vtg- denote the number of animals producing Vtg in each treatment

Endocrine Disrupting Chemicals								
Metoxychlor (Mtx)			Bisphenyl A (BPA)			Diethylstilbestrol (DES)		
Dose (µg)	Vtg +	Vtg -	Dose (µg)	Vtg +	Vtg -	Dose (µg)	Vtg +	Vtg -
70	4	3	375	2	5	0.3[a]	6	0
140	5	2	500	1	6	3.0	7	0
280	5	2	1000[a]	1	3	30[a]	5	1

a). Dead animals: 3 with BPA 1000 µg;1 with DES 0.3µg and 30µg

.

As expected, binding of [^{3}H]-$E_2$17β to the liver protein extract was achieved and E_2-17β (100 fold, $p < 0.05$) was able to inhibit this binding (Figure 19). Interestingly, binding was observed in the extract from non-treated animals indicating the presence of E_2-17β binding activity in naïve males. However, more than twice the amount of binding was obtained with the liver protein extract from treated males. In addition, in the treated males approximately sixty percent (60%) of the radio labeled E_2-17β was displaced by the radio-inert E_2-17β while only 42% was displaced in the non-treated animals. Altogether these studies demonstrate that the E_2-17β treatment to male lizards induced the expression of the liver protein responsible for hormone binding a putative ER.

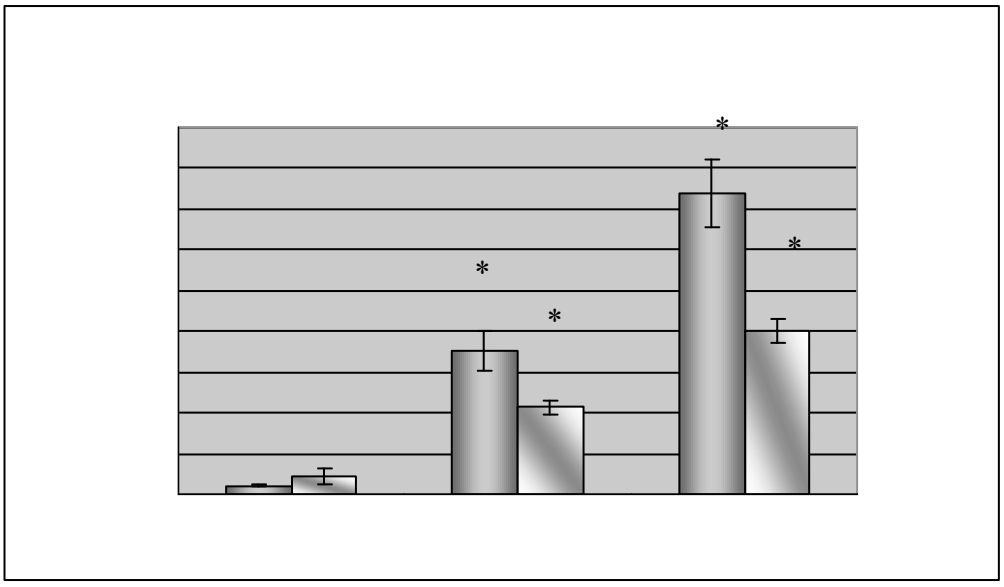

Figure 19 – Estrogen receptor binding analysis with $E_2$17β.
Eight hundred (800) µg of a crude liver protein extract from E_2 treated and non-treated males were incubated with 7nm [^{3}H]-E_2 (gray) in the absence or presence of 700 nm radio inert E_2 (black). Counts per minute retained by the extract were determined. Data is from 2 triplicate experiments. Standard deviation bars are shown. Asterisk (*) denotes significant differences between groups ($p < 0.05$). Counts per minute (cpm) retained by the nitrocellulose filter membrane are used as background control

Following the membrane-binding approach described above we tested whether the EDCs/XEs under study could displace the radioactive $E_2$17β retained by the membrane (bound). To test this possibility, the liver cytosolic protein extract from $E_2$17β treated males was incubated with [^{3}H]-$E_2$17β in the presence of 700nm of radio-inert form of the EDCs/XEs under study. These results are shown in figure 20 and summarized in Table VI. As expected, $E_2$17β showed the higher inhibition of radioactivity binding (60%, p <0.05). Taking this inhibition as a 100% of binding, the relative binding calculated for the pesticides was around 66% for DDT and 45% for DDE while Mtx basically shows no binding (22%). Among the two plasticizers tested, BPA shows approximately an 80% of

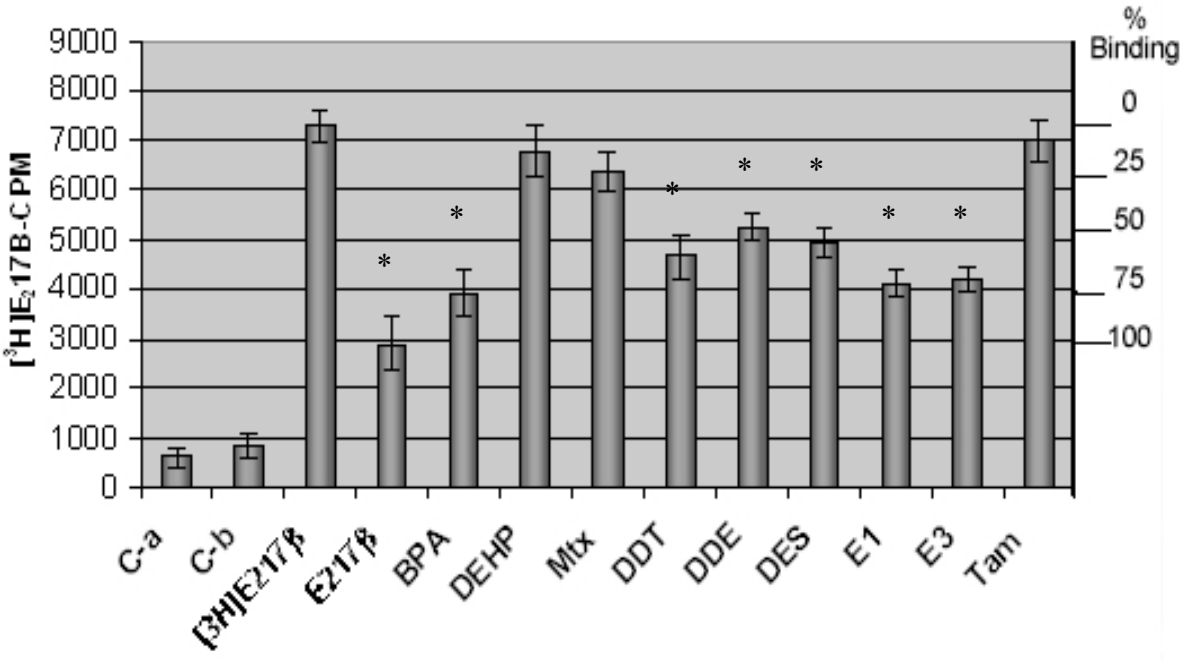

Figure 20- Analysis of ER binding with EDCs.
Eight hundred (800) µg of crude liver protein extract obtained from $E_2$17β treated males were incubated with 7nm [^{3}H]-$E_2$17β in the presence or absence of 700 nm radio inert E_2-17β and EDCs/XEs separately. CPM retained by the extract was determined. Data is from 3 triplicate experiments.. Two backgrounds control are included in these experiments: the nitrocellulose filter membrane (Ca) and 800 µg of casein protein as a non-specific binding control (Cb). Standard deviation bars are shown. ANOVA was performed followed by Tukey test to identify differences between groups. Asterisk (*) denotes significant differences between groups ($p < 0.05$).

binding but DEHP shows no interaction. Within the hormonal compounds tested, Estriol (E_1) and Estrone (E_3) show the same level of binding (about 75%) while DES only shows a 55% of binding. Lastly, Tamoxifen (Tam) show no inhibition of the $E_2$17β binding. In summary, our results demonstrate that the EDCs showing estrogenic activity in the Vtg induction experiments with the exception of Mtx and

Table VI – Relative binding of EDC with the ER from *Anolis* lizard.

Competing compound	% inhibition	% Relative Binding[(a)]
E_2-17β	60	100
BPA	47	78
DEHP	9	15
Mtx	13	22
DDT	40	67
DDE	27	45
DES	33	55
Estriol	44	73
Estrone	46	77
Tamoxifen	7	11

(a)- determined comparing the percent of inhibition of E_2-17β with the percent of inhibition of the XEs

DHEP, interact with the relative ER as shown by is ability to displace the [^{3}H]-$E_2$17β from the lizard liver $E_2$17β-binding protein.

2. Inhibition of [^{3}H]-17β-estradiol binding to blood plasma proteins by EDCs/XEs

It has been well documented that in vertebrates there are blood plasma proteins that have the ability to bind steroid molecules. These proteins may play a role as plasma steroid hormone carriers (Sallhanick and Callard, 1980, Martin and Xavier, 1981, Ho, et al., 1987 and Riley et al., 1987). Thus we performed an experiment to examine the steroid binding activities present in the lizard blood plasma and the ability of the EDCs to disrupt E_2-17β binding. This effect may cause a faulty steroid transport and balance *in vivo*. *The* results from this experiment are illustrated in Figure 21 and summarized in Table VII. The

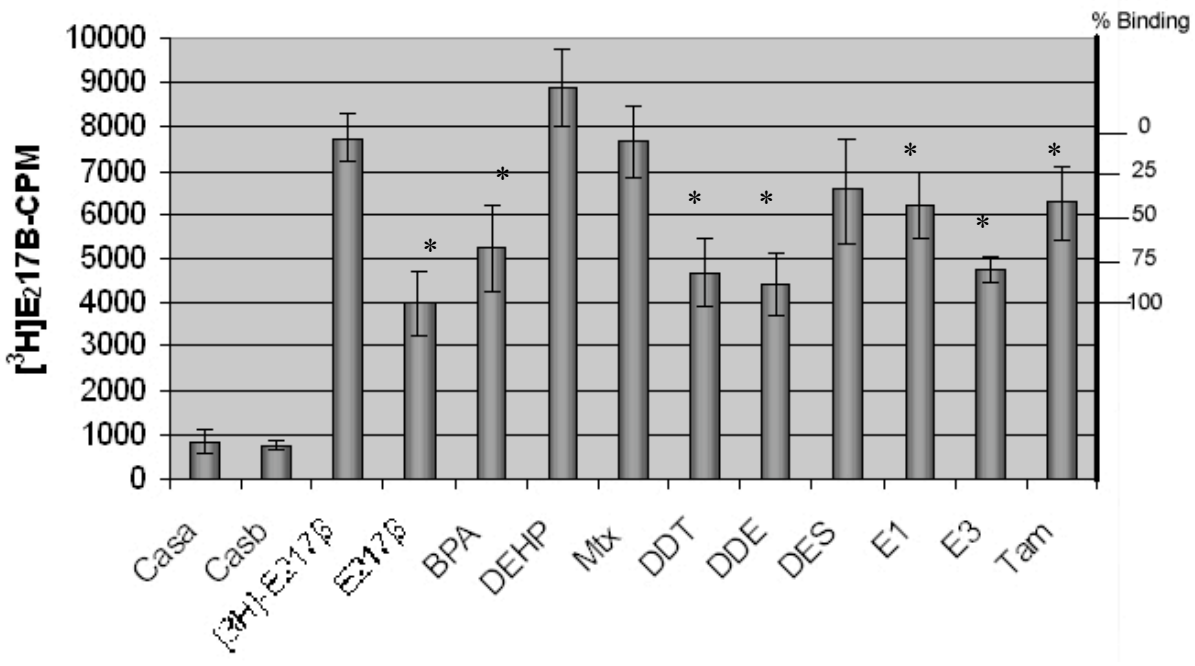

Figure 21- Analysis of blood plasma proteins binding with EDCs
Blood plasma proteins (200µg) were incubated with 7nm ^{3}[H]-$E_2$17β in the presence or absence of 700 nm radio inert $E_2$17β and EDCs/XEs. CPM retained by the membrane was determined. Data is from 3 triplicate experiments. Standard deviation bars are shown. ANOVA was performed followed by a Tukey test to determine differences between groups. Asterisk (*) denotes significant differences ($p < 0.05$). Both controls (Cas a and Cas b) are the cpm retained in the filter membrane in presence of casein.

presence of $E_2$17β binding proteins in male lizard plasma was confirmed ([^{3}H]E_2-17β) and $E_2$17β has the higher order of inhibition of radioactivity with a approximately 50% displacement ($p < 0.05$).

Taking the inhibition by $E_2$17β as a 100%, the following relative percent binding were calculated. Of the plasticizers, BPA showed 67% while DEHP shows no binding. Among the pesticides, again non-inhibitory effect was observed for Mtx while DDT show 80% and DDE show 86% of binding. In terms of the hormonal compounds, surprisingly a low effect was observed for DES (33%) or Estriol (E_1), but a better displacement of almost 80% was observed for Estrone (E_3) ($p < 0.05$).

Table VII – Relative binding of EDCs with the plasma SSBP.

Competing compound	% inhibition	% Relative Binding[(a)]
E2-17β	49	100
BPA	33	67
DEHP	0	0
Mtx	1	2
DDT	40	80
DDE	42	84
DES	16	33
Estriol	21	42
Estrone	38	76
Tamoxifen	20	40

(a)- determined comparing the percent of inhibition of $E_2$17β with the percent of inhibition of the EDC.

In summary, these results are comparable to the results obtained for the estrogen binding activity in the cytoplasmic liver protein extract. It is important to

point out that in the lizard blood plasma there is estrogen binding activity that also interacts in a detectable level with 4 of the EDCs that tested positive for Vtg inductors.

D. Activation of the liver ER and subsequent complex formation with the Vtg gene ERE

According to the accepted mechanism of action for steroid hormones, following activation by the hormone, the receptor form a homodimer, and then it is able to recognize a DNA stretch known as the hormone response element (HRE). This DNA protein interaction promotes of the recruitment of the transcriptional machinery (Jensen, et al; 1982, O'Malley and Tsai, 1992, see also Fannon et al, 2001 for a review).

To demonstrate that the E_2-17β-binding protein in the anoline liver protein extract, shown in this study to be able to interact with EDCs/XEs, could mediate a similar mechanism as described above that results in the induction of gene expression, electrophoretic mobility assays (EMSA) were performed. In a first step, to assess the integrity and activity of proteins in a cytosolic liver protein extract prepared from E_2-17β treated males, EMSAs for two general transcription factors, (SP1 and TFII), were performed. The oligonucleotides used for the identification of these factors as well as a HeLa cells nuclear protein preparation used as a positive control were provided in a Promega EMSA kit. Figure 22 A and B show, that while no mobility shift was observed in the protein extracted from the lizard livers, defined bands were obtained with the HeLa cells nuclear proteins corresponding to these two transcription factors.

These results indicate that the EMSA was functioning well but the lizard protein extract appears to be inactive. After a new anoline liver protein extract was prepared, EMSA experiments were performed but looking for ER induced shifts.

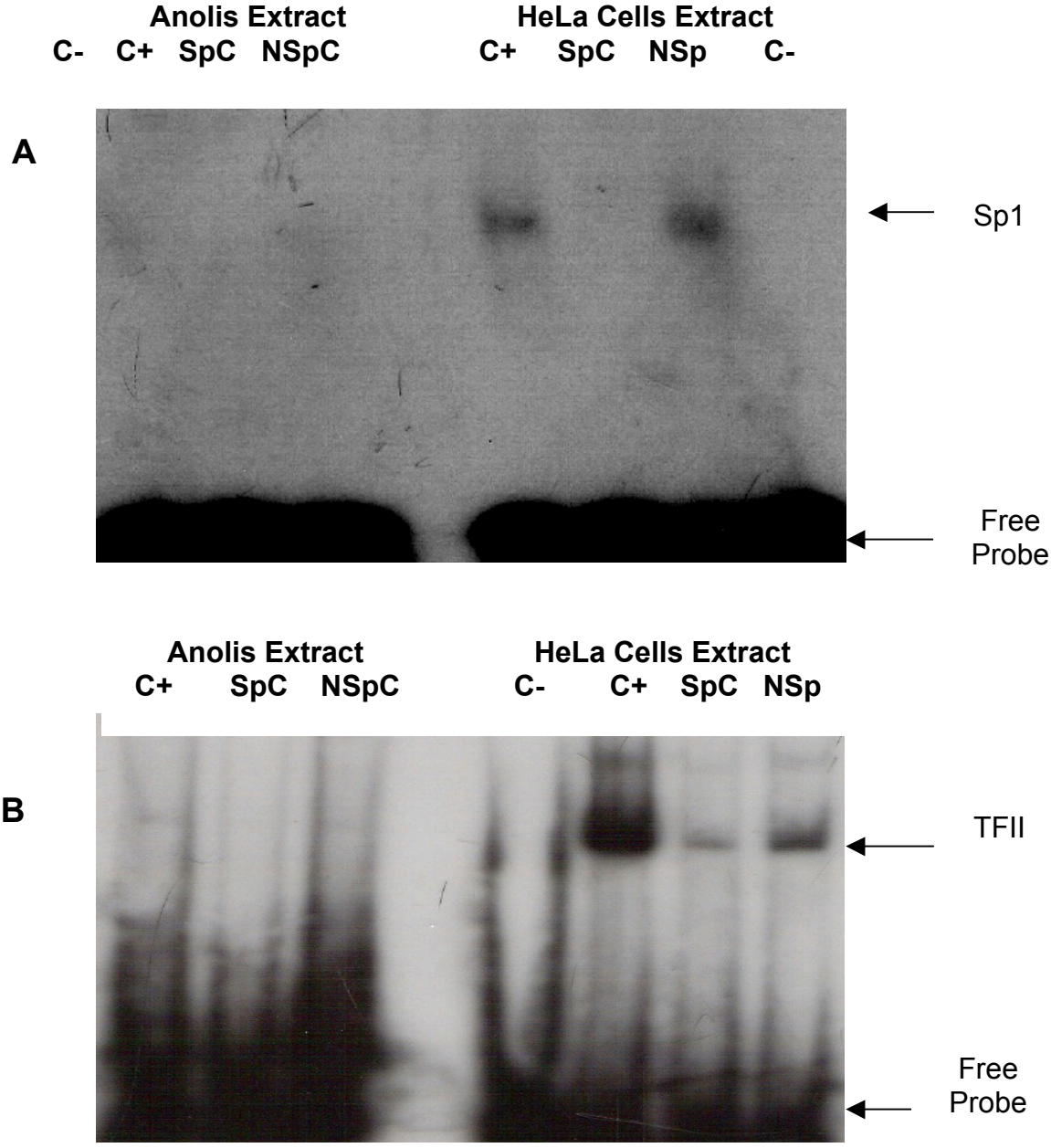

Figure 22 – Localization of general transcription factors in *Anolis* liver cell extract vs HeLa cells extract. Fifty (50) µg of Anolis cell extract (10 µg from HeLa) was incubated 15 minutes with the binding buffer in the presence and absence of competitors at room temperature (RT). After that 1 ul of ^{32}[P] labeled oligouncleotide for the Sp1 transcription factor **(A)** or the TFII transcription factor **(B)** was added and let at RT for 30 min. Then it was analyzed in Non-denaturant PAGE at 350V for 15 minutes. SpC, (Specific competitor), NSpC, (Non-specific competitor). Negative control (C-) has no protein in the incubation reaction.

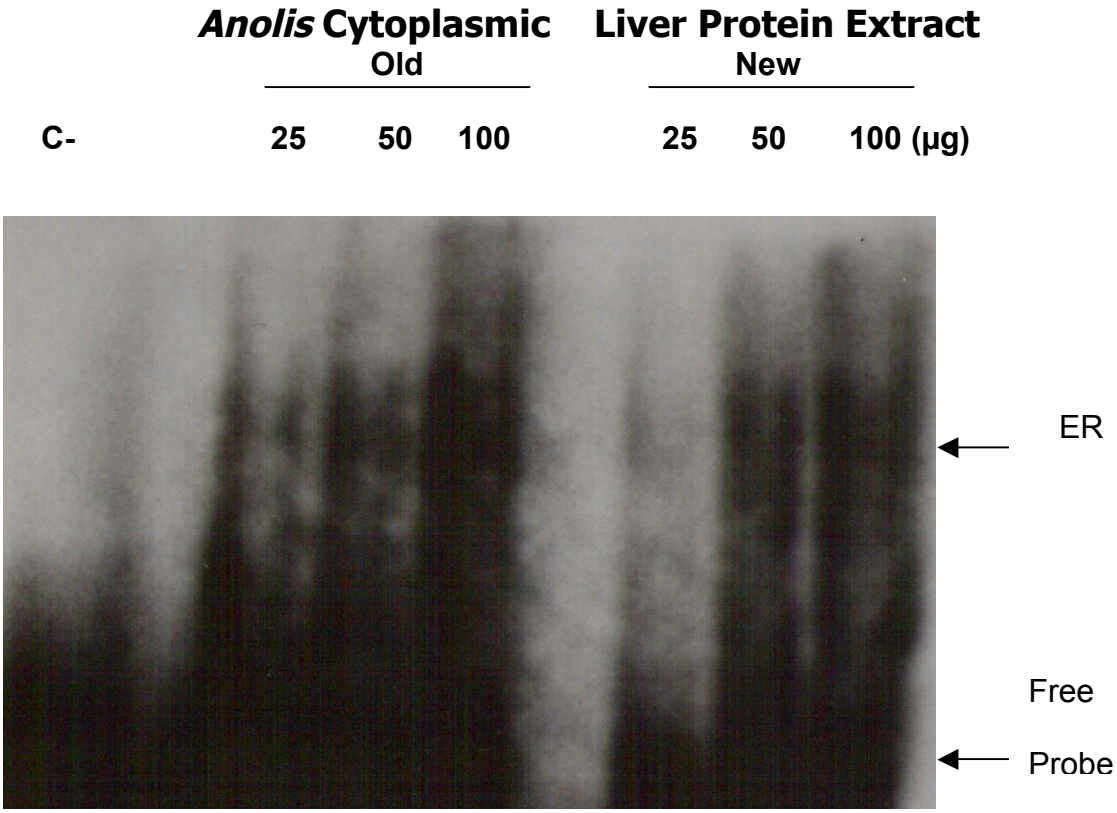

Figure 23 – ER in liver protein extract of *Anolis pulchellus*.
Increasing amounts of intracellular liver protein liver intracellular extract from males treated with $E_2$17β was incubated with $E_2$17β for 12 hr at 4°C for ER activation. After this the reaction was incubated with binding buffer 15 min at RT and 30 min on ice with a ^{32}[P]radio-labeled ERE for ER. Reactions were analyzed by EMSA in Non-denaturing PAGE as in Experimental Procedures.

Figure 23 illustrates the results obtained using increasing amounts of proteins from the old and the new batch of lizard proteins. Different incubation times were also tried. Although a mobility shift is suggested by the increase of the band as the amount of protein is increased, due the excessive background in the gel the experiment was not considered conclusive. To increase the sensitivity of the assay, a new ERE oligonucleotide was designed using ERE's from 5 different estrogen regulated genes, including vitellogenin from *Xenopus*, chick and *Gallus*, lactoferrin

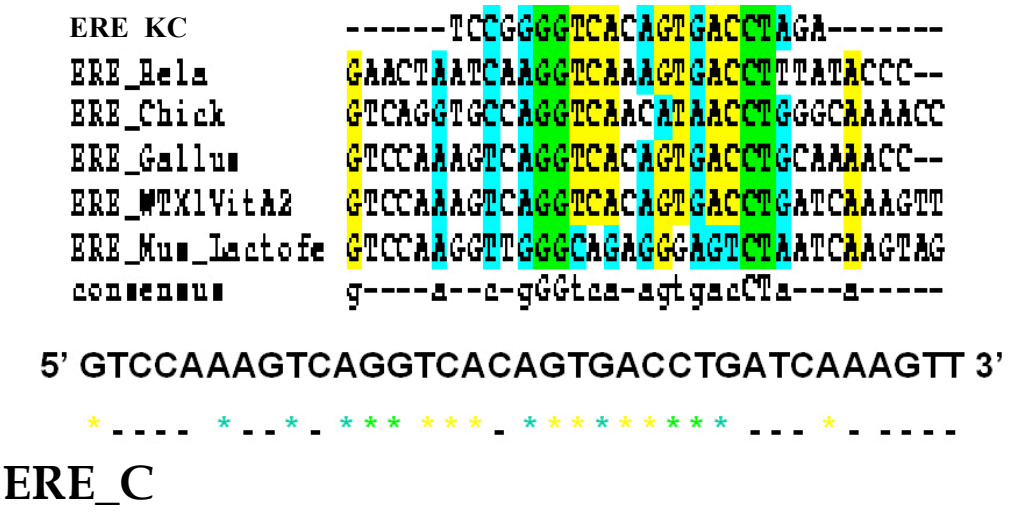

Figure 24. Bioinformatics analysis for an anoline consensus oligonucleotide for the ERE. Reported EREs for some estrogen regulated genes were used in the Workbench bioinformatic tool from the University of San Diego Supercomputer Center available at the web. The original consensus oligonucleotide used was ERE_KC from Kumar and Chambon 1988. (ERE_CS, ERE-Cardé/Santiago)

from mouse, the reported ERE from HeLa cells and the 22 bp ERE (KC) we have been using (reported in Kumar and Chambom, 1998). The sequences found in the literature search were analyzed with the Molecular Biology Software tool Benchmark from University of San Diego Supercomputer Center and a new consensus oligo-nucleotide sequence was generated. As shown in Figure 24, this new consensus ERE (ERE_CS) oligonucleotide was 13 bp longer (6 bp toward 5' end and 7 bp toward the 3' end) than the ERE (KC) used in our earlier experiments The new ERE_CS was tested with the HeLa cells protein extract and with the lizard liver protein extract using the binding conditions of the Promega kit instructions (Figure 25). A mobility shift is shown in the panel with the HeLa cells extract; demonstrating that the new designed ERE was recognized by the ER protein from the HeLa-Cells extract. However, no shift was observed with the anoline protein extract. After this

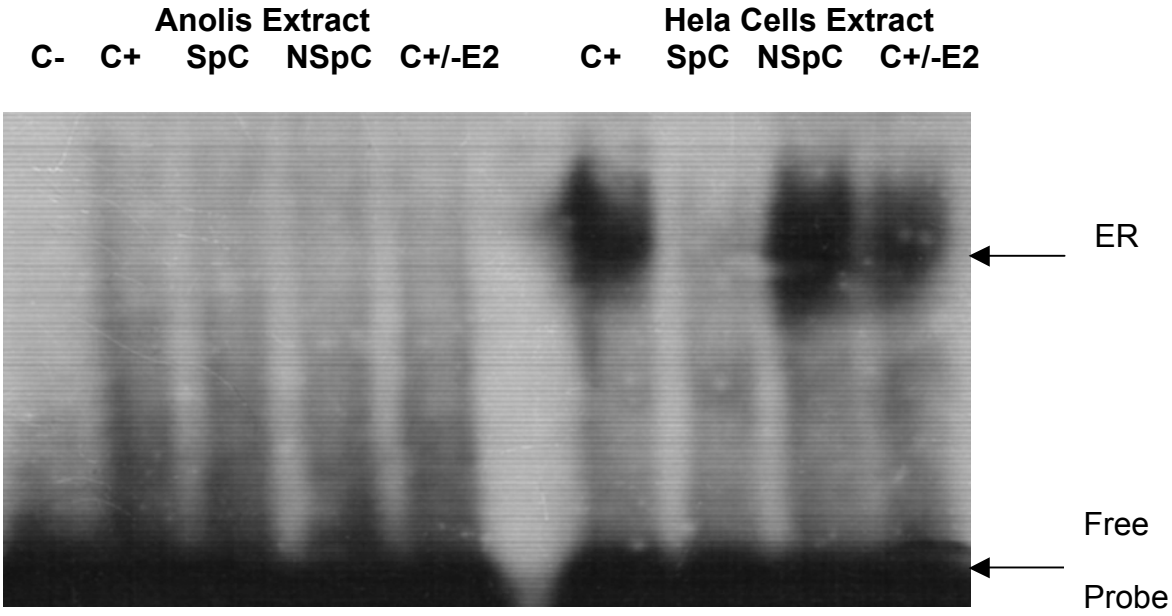

Figure 25 – ER in Hela Cells Extract localized with a new consensus oligonucleotide.
Fifty (50) µg of cell extract from *Anolis* males treated with $E_2$17β and 10 µg from HeLa cells extract) were incubated 4hr and analyzed as described above. SpC, (specific competitor), NSpC (Non-specific competitor). C+/E2, Binding reactions without E_2 added for activation (see Experimental Procedures).

negative result, we performed a literature search for binding-reaction buffers used in reported EMSA experiments. Table VIII shows a comparison between the components of two different binding buffers (Ruiz and Echevarria, 2000 and Kumar and Chambon, 1988) and the Promega Kit buffer what we have been using in our experiments. We used these two buffer conditions (RC and KC) in an experiment to locate Sp1 and TFII transcription factors in the HeLa Cells extract obtaining excellent results (data not shown). Then, we performed similar experiments to demonstrate these two general transcription factors in the anoline protein extract. The shifts caused by the binding of both SP1 and TFII present in the anoline liver protein extract are shown in Figure 26. Note that the shift in the lane in which the Promega Kit's buffer (P lane) was used is almost imperceptible while the other two binding

Table VIII – Comparison of Binding Buffers for the EMSA.
A literature review of binding buffers for EMSA reported was performed and two of them (RE, KC) were selected for a test with the anoline protein extract. (**P** from Promega, **RE** from Ruiz and Echevarria, 2000; and **KC** from Kumar and Chambon, 1988)

Components	**Promega (04 (P)**	**Ruiz-Echevarria (00) (RE)**	**Kumar-Chambon (88) (KC)**
Glycerol	20%	5%	10%
MgCl2	5mM	3mM	-
EDTA	**2.5mM**	-	-
DTT	2.5mM	1mM	1mM
NACl	**250 mM**	-	-
KCl	-	40mM	20-100mM
Mercapto EtOH	-	1.5%	-
Poly dI-dC	**0.25mg/ml**	-	-
Tris HCl	50mM	-	10mM
Hepes	-	10mM	-

conditions result in sharp strong bands corresponding to the complexes retarded in the gel. These experiments demonstrated the presence of active transcription proteins in the lizard liver protein extract.

We then tested the binding of the new consensus oligonucleotide for the Estrogen Receptor (ERE) in the anoline liver protein extract using the RC and KC binding buffers. Fifty (50) µg of protein extract were incubated with $E_2$17β (10^{-7} M) at room temperature and after 1hr, 20,000 cpm of the [^{32}P] labeled ERE were added and incubated for 30 minutes on ice followed by analyses in a 5% non-denaturing PAGE. As Figure 27 shows a prominent gel shift caused by the lizard ER was observed in both of the new binding conditions. Similar results were obtained with incubation times from 1 hr to 12 hr.

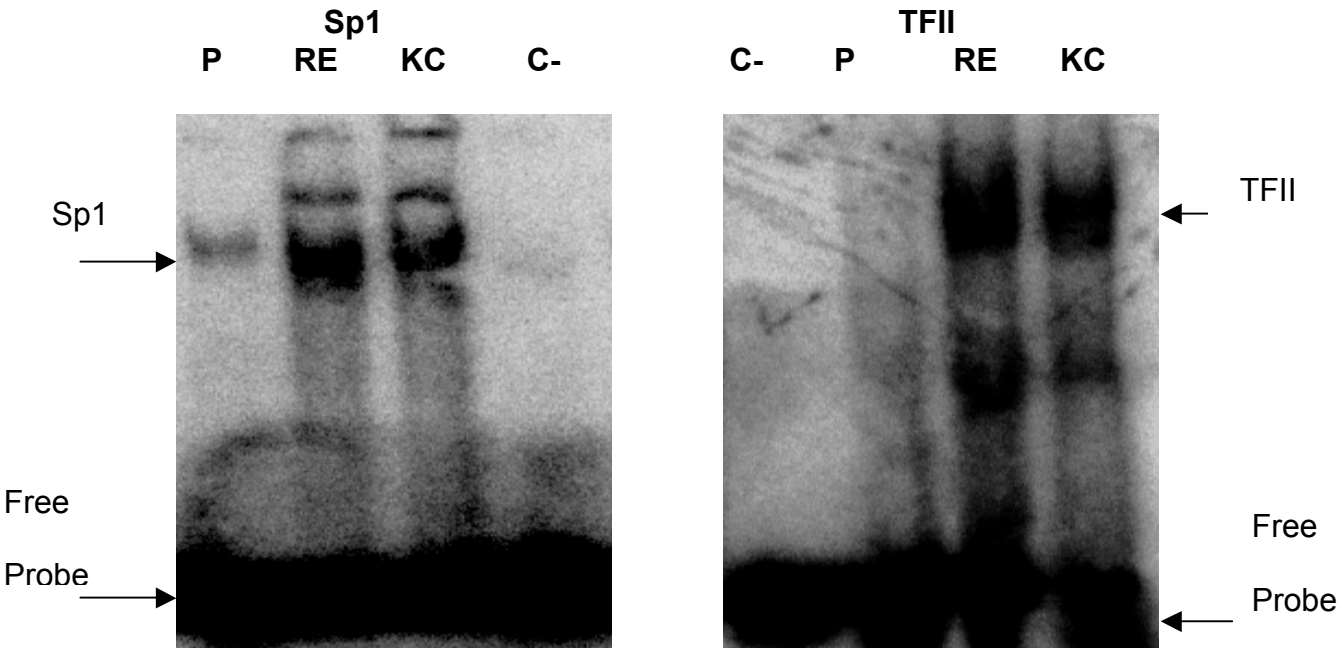

Figure 26– Detection of Sp1 and TFII transcription factors in intracellular protein extract from *Anolis pulchellus*.

Fifty (50) µg of protein extract were incubated 15 min at room temperature (RT) with three different binding buffers (See Table VIII) and then 20,000 cpm of radioactive labeled oligonucleotides for Sp1 and TFII in was added and incubated 30 min at RT. Binding reactions were analyzed in Non-denaturing 5% PAGE at 350V for 15 min.

For a qualitative comparison of the estrogen induced ER in males, with the expected ER from reproductive mature females, an EMSA analysis was performed with the liver protein extract from both individuals. Although no sharps bands are detected the presence of diffused E_2-ER-ERE_CS complex is observed. As observed in figure 28, also the specificity of the reaction is demonstrated (Sp lane) The specificity of the ER-ERE complex was also demonstrated in an experiment where the same incubation reaction was performed but increasing amounts of the specific competitor (non-labeled ERE) were added. As shown infigure 29, concentrations on the order of 100 fm are sufficient to displace almost completely the radio-labeled probe from the complex.

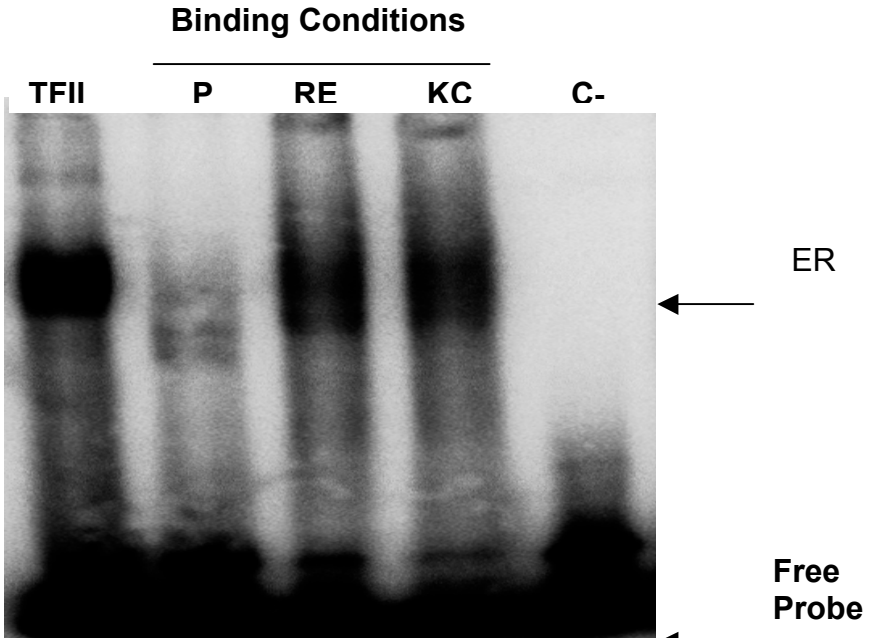

Figure 27 – Estrogen Receptor in intracellular protein extract from liver of *Anolis pulchellus*.. Fifty. (50) µg of intracellular protein extract from $E_2$17β treated animals were incubated with 7nM $E_2$17β and different binding conditions 1 hr at 4^0C followed by 30 min at RT with 20,000 cpm of radioactive labeled ERE. TFII was used in the experiment as a control of the viability of the extract. See Experimental Procedures for details.

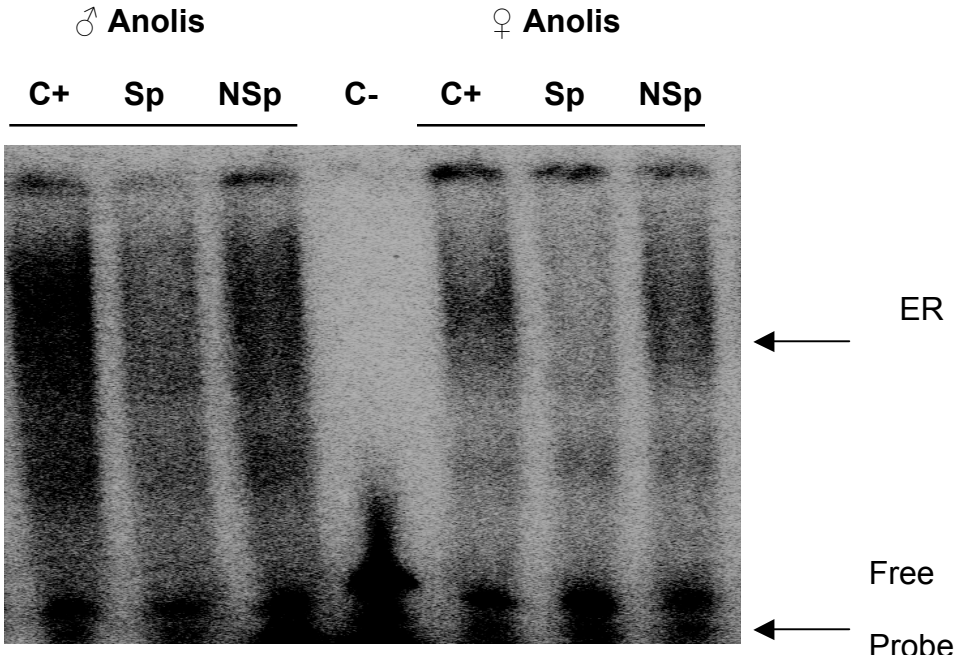

Figure 28 – Estrogen Receptor in intracellular protein extract from liver of treated males and vitellogenic females *Anolis pulchellus*.Fifty (50) µg of intracellular protein extract from Vtg females and treated males were incubated with $E_2$17β on ice. After 1 hr, 20,000 cpm of radioactive labeled ERE was added and incubated for 30 min at RT. See Experimental Procedures for details.

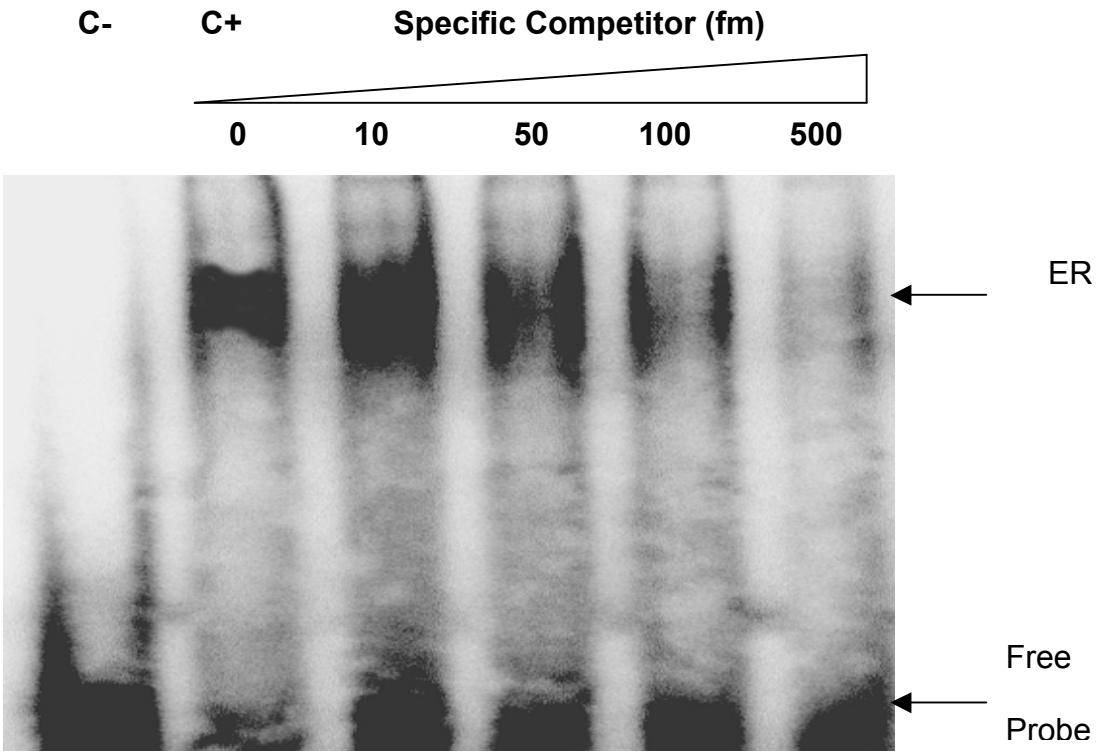

Figure 29 – Dose dependent competition to show the specificity of the E2-17β-ER-ERE complex.
Fifty (50) µg of intracellular protein extract were incubated with $E_2$17β on ice. After 1 hr, 20,000 cpm of radioactive labeled ERE and increasing amounts of the unlabeled ERE were added and incubated for 30 min at Rt. See Experimental Procedures for details.

In summary our results clearly demonstrate the presence of an E_2-17β binding protein in the lizard liver protein extract, which in the presence of the appropriate conditions can recognize a consensus ERE following the activation with E_2-17β, and that is specifically competed in a dose dependent fashion.

Discussion

A major problem in environmental health science that threatens wild life as well as human population is the worldwide exposure to chemicals in the environment. This has been related to endocrine and reproductive systems malfunctioning (McLachlan and Korach, 1995). These substances known as endocrine disrupting chemicals (EDCs) are commonly used in a diversity of aspects intrinsic to our modern way of life. It has been hypothesized that many of the effects induced by EDCs with estrogenic effects (known as Xenoestrogens; XEs) are mediated by the estrogen receptor (ER) modulation of gene expression; nevertheless other mechanisms of action have not been discarded.

During the last 20 years our laboratory has studied the molecular biology of the hormonal regulation of the Vtg synthesis in the tropical lizard *Anolis pulchellus*. The maturity of this research system allows us to further approach problems that imply risk to our modern life as the one described above.

In this research we have used our knowledge of reproductive biology in this tropical reptile as the base to investigate the possible use of male individuals as sentinel species for estrogenic contamination as well as an experimental model for the *in vivo* evaluation of suspected EDCs/XEs. At the same time, the experimental design applied in this research has provided significant insights on the mechanism of action of estrogen as well as on the interaction of EDCs/XEs with the *Anolis* ER.

Treatment methods

A first aim of this research was **to determine the feasibility of our model system (*in vivo* induction of VTG in *Anolis* male) to assess estrogenic potential**

of putative EDCs. In the past, our laboratory has used intra-muscular injections (IM) as the method used to deliver estrogen to restore the expression of the Vitellogenin (Vtg, a female sex specific protein) gene in captive females. This repression is one of several detrimental effects on the female reproductive system due to captivity. Intra-muscular treatment has also been extensively used to induce vitellogenin synthesis in male lizard (Baerga-Santini and Morales 1991; Morales et al., 1991, Morales et. al 1996, Morales and Sanchez, 1996). However the mortality rate using the IM technique was high, especially in experiments requiring repetitive injections. Therefore, a first task in this work was to find a more natural or casual way to deliver both the E_2-17β and the XEs than the intra-muscular injections. Given the lipophilic nature of the chemicals we started to test a newly devised trans-dermal (TD) method using E_2-17β as a positive control and ethanol, the vehicle for E_2-17β and XEs, as negative control. Although only 60% of the applied compound got access into the treated animals, the typical pattern of female lizard Vtg's was induced in males even at such low doses as 0.5-0.005 μg of E_2-17β (Figure 9). Although the results indicate that the IM method seems to be 10 times more effective for a given intended dose than the TD however it is necessary to take into account that almost all of the applied volume injected with the IM technique enters the animal body. An important consideration is that the reduction in invasiveness, pain, and stress of the TD method resulted in a much better general condition of the animals during the experiment with the consequent reduction in mortality.

In the very early testing of XEs with low solubility in aqueous solution (like DDT) we administered them using the IM technique. Two situations were observed:

first, at the point where the hypodermic needle make contact with the body fluids the XE came out of solution forming a white precipitate that almost blocked the needle gauge; secondly, the animals that received the XE via IM only survive 4 to 8 hours after the injection procedure presumably by the direct effect of the precipitation of the chemical once entering inside the body. In this respect the TD approach also provide a suitable method for external administration of higher doses of these types of XEs dissolved in ethanol. Finally, this more casual and less invasive method was proven effective not only inducing the *de novo* synthesis of Vtg in males but also, in reverting the Vtg gene expression in females kept in captivity as well as the IM method did in previous works (Morales and Sánchez 1996).

In conclusion, this mode of administration is an excellent technique to evaluate and observe the effects of estrogenic substances in lizards. It mimics the external at random contamination with chemicals pollutants that might take place in a spraying procedure or by environmental accumulation of chemical disposals.

Pesticides Inducing Vtg

In this study, using a terrestrial animal it has been shown that DDT and some of its metabolites can induce the Vtg synthesis as result of an external exposition (Figure 10). In an earlier pilot experiment in which DDT- Technical grade was injected, animals die within hours after treatment and even applying it trans-dermally no VTG induction was detected. After a literature search we found that the technical grade DDT is a mixture of three forms, p'p'-DDT (85%), o'p'-DDT (15%), and o'o'-DDT (trace amounts) (Welch et al 1969) and the more estrogenic isoform reported is o'p' DDT (Bitman et al. 1968, Soto et al. 1994). In our hands Vtg induction was

observed with o'p' DDT, and o'p' DDE. As shown in figure 31, the main structural difference between o'p' and p'p' DDT isoforms is the distribution of Cl- substitutes in the aromatic ring. This seems to determine the potential estrogenic effect of the compound, possibly affecting its interaction with the ER receptor. A noticeable difference between the level of Vtg induction resulting from DDT and its metabolite DDE in males was observed (Figure 10). Doses as low as 60 µg of the first one were able to induce Vtg, while approximately the double of that amount were necessary to obtain a comparable level of induction with DDE. Although a detailed comparison on the potency of the XEs in Vtg induction is out of the scope of this study, the observed results suggest a similar trend to what has been shown in other studies that also reported DDT as more potent than its derivative DDE (Andersen et al. 1999, Soto 1994).

In females, where we tested these compounds for their capability to revert the captivity effect, no differences were observed between equal doses of DDE and DDT (Figure 15). This could explain as a saturation effect where the large amount of the XEs given (500 µg) is at the saturation level and slight differences can not be discriminated. Also the levels of ER in the liver cell could be larger in females even in captivity than in naïve males, so the effect of the XE is amplified more in females than in males. The capability of these compounds to revert the inhibition of Vtg synthesis produced by captivity in females similarly as E_2-17β did indicates that these pesticides might have similar mechanisms than estradiol at the gene level.

Our results agree with those reported about the estrogenic potential of these pesticides. DDT and its metabolites have been shown to mimic estrogen and

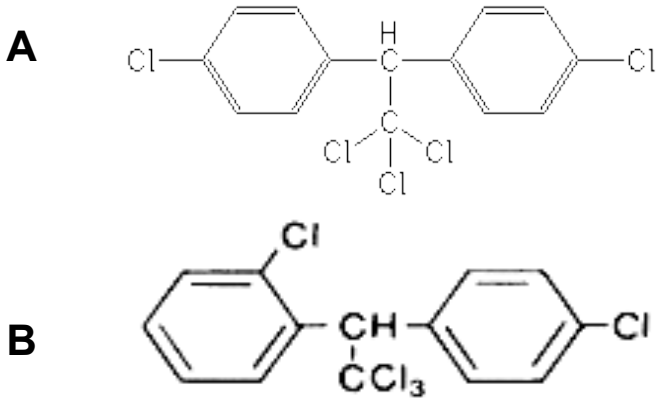

Figure 30- Structural Differences in DDT isoforms. Apparently the differential distribution of Cl-groups has different effects on the induction of VTG synthesis in *Anolis pulchellus* lizards. A –pp DDT, B op DDT

displayed harmful feminizing effects at morphological and reproductive level in, birds, fish and reptiles (Frye and Toone, 1981; Tyler et. al 1998, Guillette el al 1994, 1999 and Willingham and Crews 1999). Our results confirmed the estrogenic potential of the pesticides and in this way also validate the external application to lizards as a confident system for testing of XEs/EDCs. Adding to this, our analyses go beyond morphological or reproductive effects with the reversion of the captivity effects in females, which involve a stress effect, but at the gene level. Using RT-PCR we demonstrated the presence of Vtg mRNA, induced *de novo* in females kept 14 days in captivity and treated with the EDCs/XEs afterwards. This analysis confirms an effect at the transcriptional level that we had already demonstrated at the translational level by Western Blot. These molecular techniques were applied in all the assessment of the estrogenic effects of the EDCs.XEs in males. Our study also

shows the effect at the gene expression level in males by inducing the mRNA and protein synthesis of Vtg, a female-specific protein.

A claim for anti-androgenic effects of DDT and its metabolites has made in other species. In rats DDE was shown to bind to the androgen receptor (AR) and to induce thoracic nipple retention in male exposed *in utero* (Kelce et al. 1995). In alligators diminishing in phallus and testicles size as well as poly-follicular ovaries in females has been explain as results of DDT metabolites interference with the enzyme aromatase (Guillete 1995, 1994). It is possible that some effects of this kind exists in our system but we did not notice any obvious morphological changes and no direct influence in the endpoint analyzed in this study was observed.

Methoxychlor (Mtx) was another member of the DDT family tested in this research work. Its estrogenic potential was evidenced in both the induction of Vtg on males and in the reversion of the inhibition effects of captivity in females (Figures 10 and 15). It is important to mention that while DDT was banned in 1972, Mtx continue to be used in agriculture because of its shorter time of persistence in the atmosphere (a reported representative half-life of approximately 120 days vs. 2-15 years for DDT) and lower toxicity, (Kapoor et al., 1970). Even so, it is a dangerous chemical associated with a decrease in the weight of the testis, seminal vesicles and prostrate in adult rat and testis in rabbits (Tullner and Edgcomb, 1962; Reuber, 1980). Its estrogenic activity has been attributed to demethylated phenolic derivatives that result from its metabolic bio-transformation in the liver and not to the non-metabolized Mtx compound. This will be discussed later in more details.

Dose Effect Experiments

An intriguing characteristic of the effects for XEs/EDCs is the observed lack of lineal correlation between doses and effects contrary to what is so typically observed in hormonal treatments. Originally in our experiments, after sacrificing lizards, the blood collected from individuals in the same experimental group was mixed and analyzed as one sample. For example blood from four (4) animals treated with 250 µg of Mtx together, blood from 4 animals treated with 500 µg of Mtx together and successively. In this way, this sample represented a physical average of the total plasma protein (including Vtg) produced as result of the treatment in 4 animals. With this experimental approach no clear cut relationship between dose/effect were shown since in many cases no differences were produced between different treatments and sometimes no effects were observed with a higher dose while effects were shown with lower doses. For example DDE 125 µg and 250 µg resulted almost in a similar level of Vtg induction, while 375 µg resulted in a noticeable much higher level of induction (Figure 10).

The idea that “individual variation” is a significant influence in the response to treatment in wild populations was addressed. To get insight in how different each animal in a group may respond to same treatment some modifications were introduced in other similar experiment. For example, seven (7) animals per treatment group were tested with the same dose per group, another group was treated with a different dose and so on, and the blood plasma analysis was done individually. Results from these experiments showed that the thought that treated animals were responding differently to the same treatment was correct. In the Mtx experiments, it

was shown that the number of individuals per group that show Vtg levels increases as the dose applied to the group increases, thus resembling a lineal dose/effect relationship at the group level. Even so, all animals within the same treatment group do not responded in the same way (Table IV) showing that there is a high variation in individual response to the treatment as had been suggested.

Plasticizers and Vtg synthesis induction.

Among the group of man-made compounds used in the synthesis of plastic products we tested the estrogenic potential of the phthalates and BPA. The only phthalate (among 5 tested) that show estrogenic activity in our experiments was DEHP, which is the most world-wide used and distributed. Our interest in testing this kind of chemical was due to a relationship between phthalates and Premature Sexual Development (PSD) suggested by the study of Colón et al., (2000) in which abnormal levels of phthalates were reported in serum of PSD patients in Puerto Rico. The fact that doses on the order of 10 mg were needed to induce Vtg with DEHP compared for example with 60 µg of DDT or only 0.005ug of E_2-17β suggest that the estrogenic potential of this kind of compound in this system is extremely low. Similarly, Harris et al., (1997) reports that only 5 out of 35 phthalate compounds tested *in vitro* in MCF-7 and YES Screen tests showed what was defined as a weak estrogenic activity, where the most potent phthalate (DBP) was approximately 1 million time less potent than E_2-17ß.

BPA, another plasticizer tested in our study give positive results in the range of 500 µg to 1000 µg while 250 µg were negative (Figure 12). Again the lack of a lineal

dose/response relationship was evident since treatments with 500 µg and with 1000 µg resulted in similar levels of Vtg induction. In addition animals treated with the same dose (1000 µg) and whose blood plasma was individually analyzed showed different levels of induction. For example just one animal (out of 7) of each treatment group shows Vtg induction as result of the BPA treatment (Table V) independent of the amount applied. It seems that in the population there are few individuals that are sensitive to BPA. Our results demonstrate that Vtg synthesis induction was possible with lower amounts of pesticides (e.g. DDT) than the doses of plasticizers used. However it has to be pointed out that plasticizers are a very viscous liquid at room temperature and have to be diluted in the vehicle. This resulted in very large volumes to be applied on the animal ventral surface (and sometimes all over the animal body) what could produce lost of sample and consequently much less absorption. On the other the pesticides are solid at room temperature and they were dissolved directly in the vehicle what allowed a better control of sample volume. The application of a lower volume may facilitate the absorption process trough the abdomen skin. This may imply that this *in vivo* system is not as sensible for the plasticizers as it is for the pesticides and that possibly the experimental approach of TD application is not that good for this kind of viscous liquid plasticizers.

Estrogenic Hormones inducing VTG

A third group of compounds reported in the literature as estrogenically active was tested in our Vtg induction system: estrogenic hormones. Included in this group were Estrone (E_3), Estriol (E_1) and Diethylstilbestrol (DES). The consideration for the inclusion of the first two was the fact that being both of them estrogen metabolites,

considerable quantities can be present in the water effluents from big cities that eventually reach to water bodies like rivers and lakes (Brody and Rudel, 2003). In the same way water effluents that drain from cattle farms are possible sources of the same kind of contamination since products that contain hormonal compounds are used to induce an accelerated growth of farm animals (Ralof, 2002, Schiffer et al., 2001). Although our animal model is strictly terrestrial it is found in grassy and pasture areas that usually are directly and/or indirectly related, to these water bodies and to agricultural developments.

DES, a synthetic steroid drug developed for treatment of miscarriages in women has also been classified as estrogenic in the environment. Our purpose in testing it, was to show, if it's estrogenic effects shown in mammalian can be confirmed in reptilians.

Even though all three of them show estrogenic effects inducing Vtg in male lizards, the results (Figure 13) indicated clear differences in their effectiveness. It seems that E_1 have a more potent effect than E_3 in the Vtg induction. A similar behavior was also shown for these compounds in their ability to cause deviation from expected sexual ratio in turtles with temperature dependent sexual development (Crews et al., 1996). Comparing the effects of E_3, E_1 and $E_2$17β at an incubation temperature that normally produces only males (26^{o}C), E_1 was the more potent, resulting in a greater number of females at a lower dosage. On the other hand at the male-biased incubation temperature (28.8^{o}C) E_3 had a similar potency than E_1, and both of them were more potent than $E_2$17β. The authors explain that both hormones (E_1, E_3) bind to the ER but unlike $E_2$17β, their cooperative interaction with the ER is

reversible and dependent upon temperature over a range of 0-30°C. They concluded that a synergistic effect occur between the E_3 and $E_2$17β with the incubation temperature that is dependent on the receptors levels (which also varies with the temperature). They also reported that E_1 do not have that kind of synergistic interaction with the incubation temperature. In our experimental design, all individuals were full-grown mature males kept at ambience temperature (28°C-30°C). Since a developmental window or temperature dependent effects have not been shown for this reptilian species this kind of interaction were not expected for hormonal treated individuals. In earlier pilot experiments in which minimal doses for the *in vivo* Vtg induction were determined a minimal dose of 0.05µg for E1 and 0.5µg for E_3 was obtained (Data not shown). Although this project was not designed to systematically establish the differential estrogenic potency of these compounds a general trend was noticeable. Our results may suggest that a more effective interaction occurs between the ER and E_1 than with E_3 *in vivo*.

The fact that when testing DES, estrogenic effects were observed with treatments 10 to 100 fold less concentrated (0.005 µg) than with Estrone and Estriol, but about the same minimal dose for Estradiol, confirmed the higher estrogenic potency expected for DES. Likewise in experiments where blood plasma was individually analyzed, as expected, this synthetic hormone strongly show its estrogenic capability when almost 100% of the animals (treated with 0.3-30 µg) demonstrated Vtg induction (Table V). Probably, a maximal level of induction for DES was reached with the slowest dose tested (0.3 µg). If IM doses of 0.0005 µg of E_2-17β have been capable of Vtg inductions in juvenile anoles (Morales and Sanchez

1996), and if TD doses of 0.005µg of $E_2$17β and DES were able to induce Vtg in detectable levels (Figure 9), then 0.3 µg treatments of DES should be more than enough to induce the maximal levels of Vtg synthesis.

Lack of Dose response relationships.

That the lack of clear dose response relationship in studies with EDCs is a confounding issue was confirmed by the fact that the National Toxicology Program and the NIEHS convened a panel of experts from academia, government and industry to evaluate the evidence on this matter (Schmidt, 2001). One outcome of this panel was the verification that some EDCs exhibit dose response relationship described as non-monotonic, meaning that within a certain dose range, a chemical's effects on a given end point actually become greater as the dose is reduced. The dose response curves can be shaped like a U, with high response at both low and high levels of exposure, or like an inverted U (∩), with the greatest response at intermediate dose levels (Almstrup et al, 2002, Schmidt 2001).

One chemical found to exhibit non-monotonic relationships was BPA. Studies showed that low-dose exposure of mice to BPA produces prostate enlargement with an inverted U shaped dose-response curve (Howdeshell et al 1999). The authors explain that since the hormone receptors are up regulated (stimulated) by low doses and down regulated at high doses this result should no be surprising. Howdeshell results could not be reproduced in different strains of mice and rats a fact used by defenders of the plastic industry to indicate that the results are not robust, nor an universal phenomenon, therefore should have not implication for humans (Schmidt,

2001). One premise that has to be taken in account is the fact that unlike traditional toxicology, in which the dose always starts out from zero, exposure to EDCs adds incrementally to which is already present in the body as the naturally occurring hormonal estrogen. Thus is difficult to define the actual dose of estrogenic substance to which its animal has been exposed.

The intensity with which this issue is being discussed in scientific circles just demonstrated that the absence of dose-effect relationship in toxicological studies is a more common finding and should be more intensively studied. In these terms the absence of a defined dose-effect relationship in our study could be in some way expected for EDCs, even so, it was not a specific aim of this study to establish dose-response relationships. Experiments using a broader range of concentrations to both sides of the range that we tested should give more information about this observed behavior.

From these experiments it can be stated that when recently captured wild animals are used experimentally, several important metabolic as well as physiological factors may be affecting or interacting with the applied treatment. For example sex gender, sexual status, age, endocrine system, and endogenous levels of sex steroids are suggested as important physiological factors influencing the susceptibility of aquatic and terrestrial wildlife species to EDCs (Kawai et al., 2003). Therefore they should be taken into account to understand and explain the obtained results. Over some of this factors the researcher has very little or not control at all, (age, endogenous hormonal levels, and levels of stress response to captivity). Genetic differences and epigenetic outcomes can also be responsible of the differences in

response to EDCs treatments. That sex differences or sexual status affects the accumulation and excretion of PCBs in wild animals has been reported in marine mammals (dolphins) due to the different physiological status between for example males and nursing females as well as young siblings (Tanabe, 1985). In our lizards is obvious that reproductive mature females have higher levels of E_2 in their bodies than the males and they should be naturally better adapted to deal with estrogenic substances than males. But since our experiments were performed in male individuals, the sex of the animals has no effects in the results.

In terms of age, developmental stages and reproductive cycles have been related to the levels of Vtg and E_2-17β in fishes (Ito, 2000). This variation was associated to seasonal states of reproductive maturity in females. Differences in levels of estrogen detected in males for example were associated to different maturity stages. Although we try to collect and use in our experiments only adult individuals it is possible that variations in age were present among the collected individual.

Although in Puerto Rico the absence of yearly reproductive seasonal differences have been established for anoline lizards, the existence of subtle differences have been suggested by other researchers (Guillette 2002, Personal Communication). Differences in hormonal levels, steroid binding protein levels, egg laying timing, and so on are parameters that guide reproductive biology around the planet in all animal groups. However they have been demonstrated for areas where marked seasonal climatic changes occur which is not the case in Puerto Rico as well as in other tropical regions. Although not statistical analysis has been performed in our laboratory, slight variations in the number, size and reproductive stage of animals as

well as in the number of eggs isolated from mature females have been observed in specimens collected during the year. This may indicate that seasonal related variations may exist even though reproduction is still going on during the whole year.

In summary, this first set of experiments let us **to identify a group of EDCs with estrogenic activity demonstrated by their capability to induce *in vivo* Vtg synthesis in *Anolis pulchellus* male lizards.** This is the first, *in vivo* system from a totally terrestrial animal used for this type of study. Of the tested compounds the better induction levels were achieved by the hormonal compounds (DES, Estriol and Estrone) and by the pesticides (o'p'-DDT, o'p'-DDE, and Mtx). Although in a more inconsistent and weaker way two plasticizers, BPA and DEHP were also able to induce Vtg synthesis. Our results also demonstrate that the EDCs/XEs are able to promote changes at the molecular level/gene expression, and not only morphological changes. This study shows that the **Vtg synthesis induction in *Anolis pulchellus* lizard is a useful and powerful model for the *in vivo* assessment of the estrogenic potential of EDCs and their specific site of action.**

Interaction between estrogen and liver estrogen binding proteins

Once we demonstrated the estrogenic capability of some EDCs by inducing Vtg in our model system in a way similar as $E_2$17β, we proceeded to further dissect some steps of this hormonally controlled mechanism. It has been shown that the synthesis of Vtg in all egg-lying vertebrates occurs in the liver cell of female as well as male as a response to estrogen stimulation (Le Guellec et al.1988, Pelissero et al. 1993, Morales et al. 1991 and 1996). Also, in our laboratory, vitellogin-like proteins

were detected in the secretory products of liver explants from anoline lizards cultured under estrogen treatment (Morales et al, 1991). Therefore, we decided to identify the E_2-17β binding activity presumably present in a liver protein extract. This receptor activity had been suggested but not demonstrated in this system, until now. By means of *in vitro* $E_2$17β binding experiments we successfully show this binding activity not only in a protein extract from E_2-17β treated male lizards but a basal level of activity was also observed in extracts from un-treated individuals (Figure 19). The presence of a basal level of ER in males has been also suggested in other groups like mouse (Greco, 1993) human (Smith et al 1994) and fish (Larkin et al 2003). The fact that the ER gene is one of the genes up regulated by $E_2$17β (Larkin 2003) explain the approximately two-fold difference in $E_2$17β binding activity detected between the treated vs. non-treated animals. The approximately 60% of radio-labeled hormone displacement by the non-radioactive $E_2$17β can be considered low for a specific hormone-ER interaction when compared with other binding studies in which higher than 80% is obtained (Andersen et al 1999, Shelby et al, 1996, Klotz et al, 1996). A possible explanation is that the liver protein extract used in our competitive binding assays consisted of a complex cytosolic fraction (Riley and Callard, 1988, see Material and Methods). The large diversity of proteins in the extract (Figure 31) resulted in a very low relative concentration of the specific ER protein among the total protein content in the extract and thus in a low binding percent in the reactions. Thus can not be compared with the relative concentration of cloned ER proteins or ER domains used in some published binding experiments where relative concentration of ER protein present is almost 100% and no other factors that can non-specifically

interfere with the interaction are present. Also since this extract was prepared from liver of E_2-17β treated animals residual E_2-17β remaining in the preparation can compete with the radio labeled ligand during the binding experiments.

Nevertheless the obtained 60% of labeled ligand displacement indicates that most of the binding activity detected in this experiment is specifically competed by E_2-17β and in lesser degree by E_1 and E_3. **This is the first time that the presence of the estrogen receptor (ER) has been demonstrated in our *Anolis* model system**.

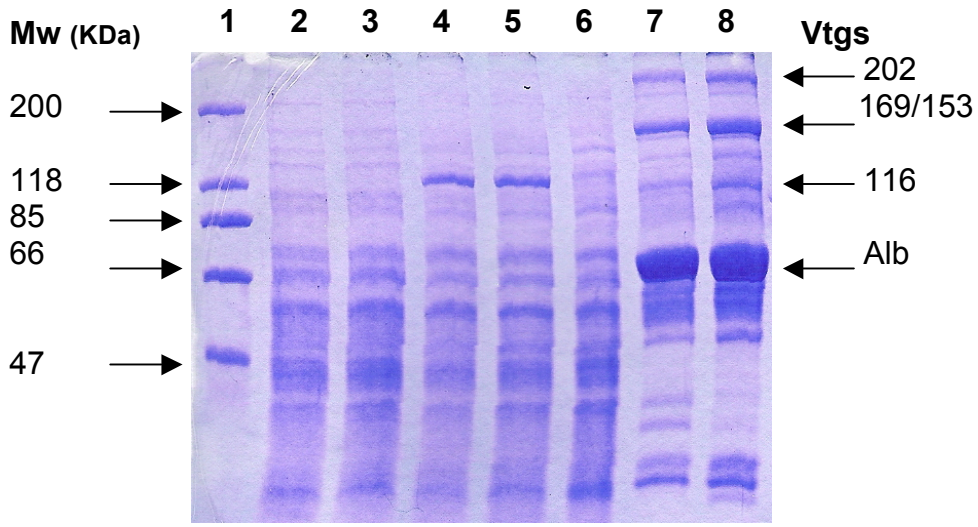

Figure 31 – Polyacrylamide gel electrophoresis analysis of the liver protein extract. Fifteen (15) µg of protein were analyzed per lane in 5% SDS-PAGE. The cytoplasmic extract samples (lanes 2-6) were used in the competitive binding assays and in the EMSA. Lanes 2, 3 and 6 are cytoplasmic extract from males and lanes 4 and 5 from E2-17β treated males showing Vtgs. Note the integrity of the multiple bands. Lanes 7, 8 are blood plasma samples from the same individuals on lanes 4 and 5. The vertebrate ER has a Mw approximately of 70KDa.

Interaction between the anoline ER and XEs

To analyze the mechanism of the estrogenic activity shown by the EDCs/XEs under study, the [^{3}H]E_2-17β/ER binding assay was used. A competitive interaction with the natural ligand (E_2) for the ER was expected for those EDCs/XEs acting

through the ER. Therefore the next step in this research was to test the interaction of the EDCs/XEs with the anoline ER in terms of its ability to compete the radio labeled E_2-17β. The value of EDCs/XEs interaction is expressed as relative binding calculated as a percent of the E_2-17β binding (displacement).

Our purpose with these experiments was specifically to test if any interaction did occurs between the Vtg inducing EDCs/XEs and the E_2-17β binding activity (ER) in the liver protein extract. A detailed comparison of our results with other binding experiments in the literature is beyond the scope of this project. Our experiments were not designed to determine relative potentialities or binding affinities for the EDCs tested neither included increasing dose curves for the displacement of the [^{3}H]E_2-17β by the tested compounds. After a literature research and some optimization a concentration of 7nm of [^{3}H]$E_2$17β vs 100x the concentration for the non-labeled $E_2$17β and the XEs was used in the assay. Thus it is then impossible for us to calculate IC_{50} values (average of the concentrations necessary for 50% inhibition of [^{3}H]E_2-17β binding) for the tested compounds. However, the general trend observed in our results can be qualitatively compared with the results found in the literature.

Taking the 60% of [^{3}H]E_2-17β displacement from the ER by non-labeled E_2-17β as a 100%, (maximum displacement) the EDCs/XEs tested can be distributed in 3 groups by their relative displacement of [^{3}H]$E_2$17β binding (Figure 20, Table VI): 1) BPA, Estriol and Estrone with over 70%, 2) DDT, DDE and DES, with 45-65% and 3) DEHP, Mtx and Tam showing very low level (less than 25%) of displacement.

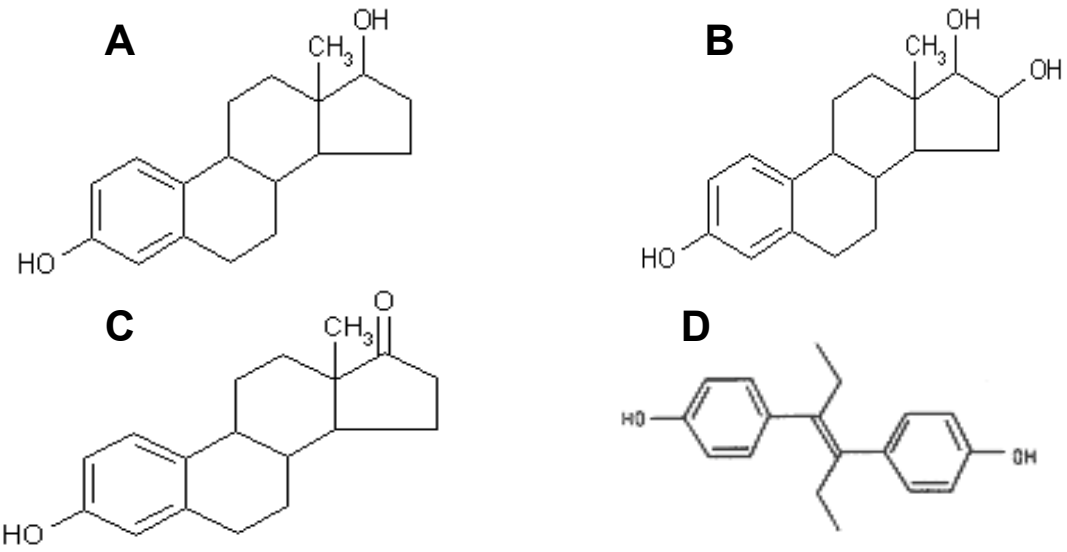

Figure 32 - Structures of A- Estradiol (E_2), B- Estriol (E_1), C- Estrone (E_3) and D- DES. Note that three of them share the basic steroid structural core Subtle differences in their substituted groups could be accountable for their differences estrogenic actions in *Anolis.* Note that all of them share the OH groups.

The fact that E_1 and E_3 are in the group with the higher [^{3}H]E_2-17β percent of binding/receptor interaction was expected because both of them share the same basic steroid structure with E_2-17β (Figure 32). In addition they have been shown to function as natural estrogens, present in organisms like mammals and birds (Moore et al. 1982) and consequently in our Vtg induction experiments both of them induced considerably high levels of Vtg synthesis (Figure 13). In agreement comparably close IC_{50} values for Estriol (10^{-9}M) and Estrone (10^{-8}M) have been reported using several cloned ERs. These values are very close to the IC_{50} for $E_2$17β (10^{-10}M – 10^{-9}M) (Matthews et al. 2000). A surprising result in our experiment is that BPA, which was not as consistent as the two hormonal compounds in Vtg synthesis induction, was the XEs with the higher level of [^{3}H]E_2-17β inhibition (almost 80%). The interaction between BPA and other ERs was also tested by Mathews et al. (2000) who reported that IC_{50} for BPA was around 1000 to 100 fold less than E_2-17β. These values came

from an *in vitro* binding assay where the ligand binding domain (LBD) of several cloned estrogen receptors were used in the assay, including the LBD from the green anole. The difference between the relative higher interaction shown by BPA in our experiments and the reported lower binding results in the cited experiments may indicate that the interaction between this EDC and the anoline ER in a protein extract is less stringent than the interaction between the EDC and cloned receptor portions. It is possible that the presence of the complete receptor as well as other factors present in the protein extract may stabilize a non-perfect EDC/receptor interaction.

Other interesting although unexpected, observation in our study is that pesticides like DDT and DDE showed a similar ER interaction as the powerful synthetic estrogen DES. In our model system DES show a higher level of Vtg induction than the two pesticides (Figures 10, 13, 15); but in the [^{3}H]E_2-17β/ER competitive assays DDT shows a higher level of labeled hormone displacement (67% vs. 55%). This result may indicate that the interaction of DES for this anoline ER although less specific than that of DDT, is more efficient in the recruitment or activation of the transcription machinery resulting in a better interaction with important chemical groups in the receptor necessary for gene expression. On the other hand the interaction between DDT and the ER can be so intimate that the turnover rate for dissociation can be limited and thus a less effective complex is formed. Matthews et al. (2000) reported that different ERs from human, mouse, chicken, green anole and rainbow trout exhibit differential ligand preference and relative binding affinities for different natural and synthetic compounds. For example they reported that DES showed an IC_{50} lower than E_2-17β for the human and mouse ERs, but with chicken,

anole and rainbow trout, DES showed a higher IC_{50} than E_2-17β. This suggests that an universal relationship does not exist between estrogenic potential of a XEs and its interaction with the ER.

Finally, a minimal EDC/ER interaction was observed for DEHP, Mtx and Tam in the [^{3}H]E_2-17β competitive binding assay. The evidence for DEHP in our model system suggests that it has some estrogenic activity, although very weak, since a huge dose (10mg) was required for the induction of Vtg. Thus a very poor interaction, if any, was expected to occur with the ER. Also at the low concentrations (700nm-7000nm) used for the competitive assay the interaction is not promoted between DEHP and the ER in liver extract preparation. After these considerations the effect of DEHP should better be defined as a toxic effect.

However, the relationship suggested by Colón et al (2000) between the presence of higher levels of this phthalate in the blood of patients with premature sexual development than in the normal population, and the evidence found in this study about the DEHP estrogenic effects on the Vtg synthesis in *Anolis* (Figure 11) calls for a more detailed and in deep study of this compound. In other studies the endocrine disrupting activity of DEHP has been also expanded to anti-androgenic effects in male reproductive system (Moore et al. 2001). Exposure to DEHP *In uteri* and during the lactational period resulted in male offspring with undescended testes, areola and nipple retention, epididymis prostate and seminal vesicle weight reduction as well as diminished sexual activity.

Methoxychlor is an interesting XE, showing estrogenic activity *ín vivo* but not *in vitro*. As mentioned earlier several studies in rats indicate that Mtx behave as a

typical estrogen (Eroschenko et al. 1996, Metcalf et al 1996, Walters et al 1993). Mtx stimulates uterine growth, accelerate vaginal opening and increase uterine ER expression. Shelby (1996) using an in vitro binding assay (mouse ER), the ERE-CAT reporter (HeLa cells) and the uterotrophic assay in mice demonstrate that the estrogenic activity of Mtx relies in 2, 2-bis (p-hydroxy-phenyl)-1, 1, 1,-trichloroethane (HPTE, a metabolite in which Mtx is converted in the liver. Note in figure 33 that HPTE has two (2) reactive OH available similar as the estrogens. Also HPTE by far, has a higher IC_{50} and relative binding affinity (RBA) than Mtx and other members of the DDT family in cloned ER binding experiments (Matthews, 2000). These characteristics explain the estrogenic activity of Mtx *in vivo* but not *in vitro*. The lack of interaction shown by Mtx with the ER *in vitro* in our experiments suggests that similar as have been reported (Shelby et al. 1996) Mtx after being internalized by the lizard is quite well metabolized in a more effective estrogenic form (i.e. HPTE), resulting in the robust induction of Vtg synthesis. It would be interesting to determine the ability of HPTE to bind to anoline ER using the $[^3H]E_2$-17β competition assay. Similar as for almost all XEs, other pathways of action cannot be discarded to explain the effects of Mtx. For example, Mtx have been reported stimulating some estrogen-responsive mRNAs (lactoferrin and glucose-6-phosphate dehydrogenase) in mouse uterus. The implication is that this stimulation has to be mediated through a non-estrogen receptor mechanism because the test was carry in mice Knock-out (ERKO) both ERα and ERβ (Ghosh et al.1999). Therefore Mtx could act bypassing the classical ER mediation.

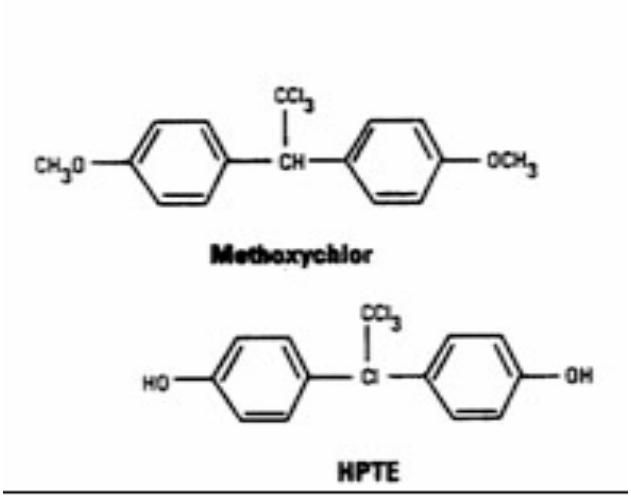

Figure 33 – **Chemical structures of Metoxychlor (Mtx) and HPTE,** an estrogenic metabolite. Note de phenolic substitutions instead of the methyl groups

One unexpected result that came out from our [^{3}H]E_2-17β binding assays experiment, was the apparent inability of Tamoxifen (Tam) to compete for the ER in the protein extract. The fact that Tam in doses as high as 500 µg to 2000 µg, (Figure 14) showed no induction of Vtg, was an expected result, since Tam is known as a non-steroidal anti-estrogen classified as an ER antagonist (Bentrem, et al. 2001, Jordan and Morrow, 1999, Pink and Jordan 1996, Jordan and Godsen 1982). Even so estrogenic effects of Tam have also been reported. For example Tam (as well as E_2-17β) was capable of reversing the sex determination pattern in alligators with a corresponding increase in aromatase activity (Crain et al. 1997). But considering that either way, acting as an agonist or as an antagonist, some kind of interaction has to take place with the ER, a higher level of competition was expected in our *in vitro* binding system. Thus it was interesting to observe that Tam only displaced about 11% of the radio-labeled hormone that E_2-17β displaced. In a reviewed study where

Tam was tested as a competitor for the ER, the IC_{50} reported was in the range of 10^{-9} M (for cloned human ER) to 10^{-8} M (for cloned mouse, chicken, anoline and rainbow trout ERs). Those values are similar to DES, Estriol and Estrone, but lower than for BPA, and DDT (Matthews and Zacharewsky, 2000).

Apparently, our experimental setting for ER binding was not able to provide the optimal conditions for the interaction with Tam. It should be pointed out that in all articles reporting some displacement of radio-labeled ligand by Tam came from experiments using pure recombinant ER proteins or fragments. For example in comparative experiments Tam binds strongly to the recombinant hER from MCF-7 cells with affinities similar to the E_2-17β. However when tested with a rabbit uterus extract, the binding affinity of Tam for this receptor was very low, only 1/6000th the binding affinity of E_2-17β (Andersen et al. 1999). A similar decreased affinity of Tam for the ER in our experimental protein extract may be happening. 4-Hydroxytamoxifen (4-OH-Tam), have been identified as the estrogenic active metabolite of Tam and it has been shown to bind the ER with higher affinity than Tam, DES and E_2-17β itself (Andersen et al. 1999). Its RBA have been reported from 155% to 272% to cloned ER from human, mouse, chicken and green anole ER (Matthews et al. 2000). Again, as can be seen in figure 34, 4OH-Tam has an available reactive OH, which seems to be important for interaction with ER. An interesting modification of our experiment will be to determine the 4-OHTam competitive ability with [^{3}H]E_2-17β for the anoline ER in our cell extract, expecting to observe a higher percent of inductor of lizard Vtg expression (Data not shown).

In summary, we have clearly showed for the first time the presence of an E_2-17β binding activity, an estrogen receptor (ER), in a liver protein extract from the lizard *Anolis pulchellus*. This putative ER was capable of

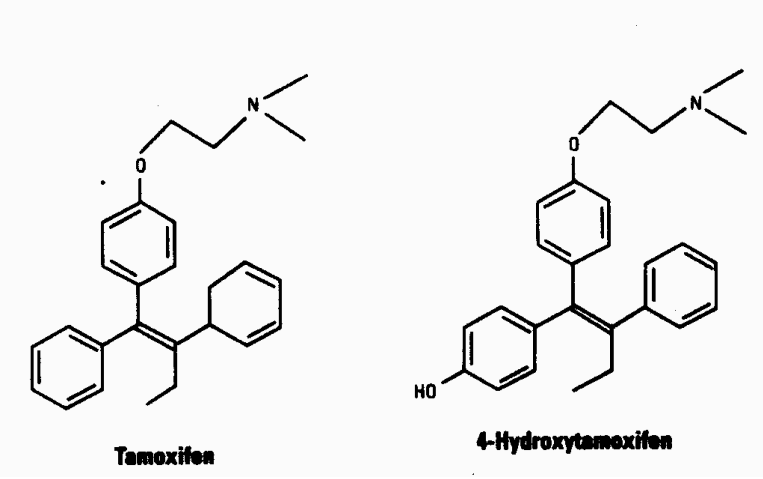

Figure 34 – Structure relationships between Tamoxifen (Tam) and 4-Hydroxytamoxifen (4-OH-Tam). The OH group present in the second can be important for its reported estrogenic activity.

ve interacting with some EDCs/XEs from diverse chemical groups. Almost all XEs that has behave as inductors of Vtg synthesis *in vivo* (BPA, DDT, DDE, DES, Estrone and Estriol) were also able to displace the radio-labeled ligand of the ER complex suggesting a similar mechanism of action than $E_2$17β. One exception was Mtx, who apparently require to be metabolized. The other exception was DEHP, who induce Vtg synthesis only at very large doses and finally, Tam whose interaction with the estrogen binding activity in the extract was expected.

Interaction of EDCs/XEs with Steroid Binding Plasma Proteins.

The steroid hormones in vertebrates can be found free in plasma or bound to some plasma proteins (Wagner, 1978). The [^{3}H]E_2-17β has been extensively used in binding assays for the identification and characterization of sex steroid binding

proteins (SSBP) from plasma (Tollefsen et al. 2004, Dechaud et al. 1999, Hyrb et al. 1990, Ho et al. 1987, Riley et al. 1988, Siiteri et al. 1982). After showing the interaction of E_2-17β and XEs with an intracellular E_2-binding protein from liver we decided to determine if an interaction could also be shown between the XEs and an E_2 binding SSBP from *Anolis*. The rational for this experiment is the hypothesis that if the EDC as well as the ligand (E_2) binds to the SSBP it may be more readily available to the specific target tissue to where the endogenous ligand should be transported. On the other hand, if the XE is not bound to the SSBP then even freely circulating it may be more easily available for bioaccumulation on non-specific tissues from where later can be mobilized to other more sensitive sites. Nevertheless if the EDC binds to the SSBP or not it can still disrupt the endocrine balance of the organism.

Our results reveal the presence of an activity in the plasma of *Anolis* lizard that binds E_2-17β. The displacement of approximately 50% of the total bound labeled E_2-17β by the unlabeled E_2-17β (Figure 21, Table VII) suggests that the E_2-17β plasma binding activity is a SSBP, and E_2 is one specific ligand. The amount of labeled E_2-17β displaced by the cold E_2-17β in our experiments seems to be low when compared with the findings of other reported studies (see below). This is likely due to the fact that only 100X concentrations of the unlabeled E_2-17β or XEs were used in our competition assays, instead of 500X – 1000X used in other studies (Salhenick and Callard, 1980). An alternative explanation for the lower levels of competency showed by E_2-17β in this experimental setting could be that our plasma samples were not incubated with dextran-coated charcoal (DCC) previously to the assays to remove the endogenous estrogens that are already bound to the SSBP. It has to be

pointed out that on early experiments for the study of SSBP the incubation with DCC was part of the general protocol for the removal of the endogenous steroids (Hyrb et al. 1990, Ho et al. 1987, Riley et al. 1988, Siiteri et al. 1982). However, in a more recent study, Laidley and Thomas (1994) reported that the stripping of endogenous steroids by incubation with DCC prior to assay, results in greater ligand binding in fluids associated to estrogen producing tissues, like ovarian interstitial fluid. However, this technique resulted in partial loss of steroid binding activity when plasma samples are used.

Since the main purpose of this experiment was to test if any kind of interaction occur between the EDCs/XEs with an E_2 binding activity in the plasma of *Anolis*, a quantitative comparison of binding affinities and the like, with what is reported in the literature was not intended. However some qualitative similarities and differences could be established between our results and what is reported in the literature based on our static analysis.

Our result of a 50% of labeled ligand displacement by unlabeled E_2-17β is in someway consistent with some observations. Ho et al., (1987), reported the presence of a SSBP in plasma of *Alligator mississippiensis* that binds E_2-17β and Testosterone (T) with medium high affinity. They also observed a 40% to 70% of labeled ligand displacement by 100X concentration of unlabeled T and E_2-17β while E_3 and DES were described as poor competitors. An 80% of [^{3}H]E_2-17β displacement by E_2 17β (while E_1, E_3 and DES were poor competitors with 20% and 40% 10% respectively) from the SSBP of the red eared turtle (Salhenick and Callard, 1980) and a 92% by

E_2-17β (while 37%, 16%, 2% by DES, E_1 and E_3 respectively) in *Nerodia snake* have been reported (Riley et al. 1988).

Only five more recently studies on the interaction between SSBPs binding with a wider range of steroids molecules and EDCs tested were found. Laidley and Thomas (1994) identify and partially characterized a SSBP in the plasma of the spotted sea trout (*Cynoscion nebulosus*). High relative affinities were reported for E_2-17β (167%), an E_3 (118%) among the twenty-two steroid molecules tested. In the same study, very low relative affinities were reported for E_1 (3.3%) and DES (<0.2%). Our results for the [^{3}H]-E_2-17β relative binding by E_3 (78%) was among the highest while for E_1 (43%) and DES (33%) were relative low but not as low like it has been reported. Differing from Crain et al. (1998) and Arnold et al. (1996) who reported that most of the environmental contaminants (i.e. DDT) as well as DES did not interact with the human sex hormone binding globulin (hSHBG) in the *in vitro* YES assay, in our study DDT (81%) and DDE (86%) were among the compounds with the higher level of binding. Finally Dechaud et al. (1999) identified two alkylphenols (4-nonyphenol and 4-tertoctylphenol), and two plasticizers (BPA and O-hidroxybiphenyl) as potent hSHBG ligands. In our study BPA (67%) shows the fourth higher binding. The general function assigned for these proteins is that by binding to plasma proteins the bioavailability for specific tissue cells of these XEs may be modulated as it happens with endogenous ligands. On the other hand, since these proteins are not estrogen specific but steroid specific the binding of these EDCs to the hSHBG may also displace other endogenous sex steroid hormones disrupting androgen-to-estrogen balance.

In summary, **a steroid binding activity that binds E_2-17β was demonstrated for the first time in the blood plasma of anoline lizards.** Our putative SSBP also interacted with E_3, BPA, DDT and DDE, all of them Vtg inductors. Lower interaction with some Vtg inductors such as DES, E_1 was observed. DEHP and Mtx both of them Vtg inductors did not interact. Interestingly a very small but significant interaction was observed with Tam, a non VTG inductor.

The literature review shows that the test of other EDCs like BPA, Mtx, DEHP, DDT and DDD in binding to SSBP proteins was scarcely reported. This present study appears to be the third one reporting a competitive interaction of BPA, DDT and DDD with a plasma steroid binding activity. Both, the similarities and differences between the results reported in this study and the results previously reported, demonstrate the need for more detailed studies to clarify the interaction between the SSBP and EDCs. Given the role putative role of SSBP as specific transporters of steroids, this binding is an important preliminary step in the mechanism of the estrogenic activity of XEs.

Qualitative Ranking of EDCs for Estrogenicity.

Taking together the results on VTG induction and on interaction with the ER and with the SSBP, the EDCs tested in this study were qualitatively ranked in terms of their estrogenic potential. Four plus (+) signs were assigned to the ones that induce Vtg with the lower doses amounts. For examples E_1, E_3 and DES induced Vtg with less than a µg so we assigned (++++) (Table IX). DEHP induced VTG with mg doses amounts so we assigned (+) and BPA with µg amounts received (++). In terms

of interaction with the ER or the SSBP a (+) was assigned for each 25% of relative binding. So an EDC with more than 75% but less than 100% of relative binding received (+++), an EDC with more than 25% but less than 75% of relative binding received (++) and so on. Then a relative potency order is suggested as follows: E_2>E_3>DDT>E_1=DES=DDE>BPA>Mtx>DEHP>Tam. In summary based on their estrogenic potential the EDCs can be distributed in three groups: E_3 and DDT are in group I classified as the more potent, an intermediary group (II) that included several EDCs with similar potential and the last group (III) with basically no estrogenic potential. As expected the natural hormonal compounds are the most potent XEs. Mtx and DEHP, both of them were Vtg inductors but show very low interaction with the ER or SSBP. Mtx is over DEHP because lower doses (µg amounts) are needed for its effects.

Recognition of a consensus ERE by the E_2/ER complex from *Anolis'* liver.

Molecular studies on the mechanism of action of the steroid hormones have shown that once $E_2$17β binds to its receptor, a conformational change occurs and the ER homo-dimerizes (Jensen et al; 1982, O'Malley and Tsai, 1992). These changes lead the ER to recognize and interact with a specific DNA sequence in the promoter region of the target genes know as the estrogen response element (ERE) (Sathya et al 1997, Stancel et al, 1995, Martinez and Whali 1989, Evans 1988, Kumar and Chambom, 1988). We postulate that a similar set of changes in the ER could be promoted by 6 of the 9 EDCs/XEs that we tested in this study, that in addition to

Table IX –. According to the doses needed for Vtg Induction and their relative binding affinities for the ER and the SSBP the EDCs were qualitative ranked for their estrogenic potential.

Compound	Vtg Ind	ERRB[a]	SSBPRB[b]	Total
$E_2$17β	++++	100%/++++	100%/++++	12
BPA	++	78/+++	67/++	7
DEHP	++	15/-	0/-	2
Mtx	+++	22/-	2/-	3
DDT	+++	67/++	80/+++	8
DDE	+++	45/+	84/+++	7
DES	++++	55/++	33/+	7
E_1	++++	73/++	42/+	7
E_3	++++	77/+++	76/+++	10
Tam	-	11/-	40/+	1

a) **ERRB**-Estrogen Receptor Relative Binding
b) **SSBPRB**-Serum Steroid Binding Protein Relative Binding

behave as Vtg synthesis inductors also interacted specifically with the ER present in the liver protein extract. The next step in this study was to demonstrate that once formed the ER-$E_2$17β complex in the *Anolis* extract was able to recognize a consensus sequence derived from the EREs of estrogen regulated genes. The rational for this experiment was the hypothesis that if $E_2$17β is able to induce Vtg in male lizards and interact *in vitro* with the ER in the lizard liver protein extract, then, if this activated complex is functional it should be able to recognize the ERE of the vertebrate Vtg gene. In order to demonstrate the recognition of an ERE by the ER-

$E_2$17β complex EMSA assays were performed. The binding assay conditions described by the Promega Gel Shift Assay kit promoted the interaction of proteins from the HeLa nuclear extract and the oligonucleotide for transcription factors (SP1 and TFII) that came as controls in the kit. However no shift was obtained in the liver protein extract, meaning that an optimization process have to be carry on. This process included 1) changes in the *Anolis* liver protein extraction protocol (i.e. temperatures, incubation times of the tissue before homogenization), 2) changes in protein concentrations e used in EMSAs, 3) changes in size and sequence of oligonucleotide used as a probe in the EMSAs for ER, as well as 4) changes in the reaction buffer components.

For example, in earlier experiments before the protein extractions were started, the dissected livers were frozen in liquid nitrogen and stored at -80^{o}C until the dissection process finished. This period of time could be from 2 to 4 hr depending on how many animals (generally 40) were sacrificed. In later experiments only 15-20 animals were sacrificed. This amount takes only 60-90 minutes for the complete process. Another change introduced in the process was not to freeze the dissected livers but to incubate them in ice chilled NaCl 0.60%. The ER belongs to the intracellular super family of receptors that acts as ligand-activated transcription factors (Rachel and Jacob, 1993; and Mangelsdorf et al. 1996). This introduce the incubation of the protein extract with E_2-17β (for the activation of the ER) as a step previous to the incubation with the ERE. Thus several experiments were done with gradual changes from 1-12 hr in the incubation time of the extracted proteins with $E_2$17β, and also testing different protein concentrations (Figure 23). After these

adjustments the result obtained suggested the presence of a retarded complex for ER but due to an intense background this experiment was not considered conclusive.

At the same time, a comparison with other five (5) EREs found in the literature (several Vtgs and others E_2-17β induced genes) demonstrated that the oligonucleotide that we were using was 13bp shorter. It was suggested to us that this space reduction could be affecting the ER-E_2-ERE complex stability (C. Santiago, personal communication) and so the sensitivity of the assay. Using the sequences of these EREs, a new ERE_CS, larger and with changes in the sequence was designed. More details are included in figure 24.

In a first experiment using the new oligonucleotide the ERE_CS show to be functional using the HeLa cells nuclear extract from the Promega Kit, but not with the liver protein extract from *Anolis*. Then, the process of optimization leads us to verify and compare the binding reaction buffer provided by Promega (P) with some other buffers used in successful studies reported in the literature. The test of these reported binding buffers (Ruiz-Echevarria, RE, 2000 and Kumar and Chambon, KC, 1988) allow us to show the presence of the two general transcription factors, SP1 and TFII for the first time in our protein extract (Figure 26) using the oligonucleotides provided in the Promega kit. These results confirmed that the transcription factors in our liver extract were functional. Apparently the conditions generated by the absence of EDTA (cations presence) in this new binding reaction buffers were important for the interactions in the *Anolis* extract to occur.

Finally, using the RE and KC buffers we were able to demonstrate for the first time in our system the presence of a gel shift that corresponded to the ER-E_2-

ERE_CS complex formation. The absence of a shift using the binding buffer from the Promega Kit indicates these conditions were not appropriate for the *Anolis* ER preparation. Interestingly, these Promega buffers conditions work well with the new ERE_CS and the HeLa cell nuclear extract. These optimization experiments allowed to obtain the appropriate binding reaction conditions for the consensus ERE recognition by the $E_2$17β activated ER in the *Anolis pulchellus* liver extract.

As observed in Table VIII there are three (3) buffer components in the P buffer that are absent in the other two buffers: NaCl, poly dI/dC, and EDTA. It is interesting to determine which of these three component is the limiting one for the interaction between the ER/E_2 with the ERE. In first place, it seems unlikely that NaCl be limiting since there are other salts ($MgCl_2$ and KCl) in the other two buffers. The poly dI/dC (polydeoxiinosinic-deoxicytidylic acid) is a molecule that used to reduce the non-specific binding, providing more DNA surface for non-specific protein/DNA interaction and so, reducing the background contamination in the assays. The specificity of our ER-E_2-ERE_CS interaction was shown with the specific and non-specific competition assays (Figure 28, Sp, NSp lanes) although some level of background is observed. To confirm the specific relationship of the observed ER-E_2-ERE_CS interaction, an experiment with increasing concentrations of the specific unlabeled oligonucleotide as competitor was designed. It was found that as low as 100 fm of unlabeled ERE_CS was able to almost completely compete off the labeled ERE_CS, demonstrating that the gel shift observed is specific for the new consensus ERE_CS. Therefore, this analysis suggests as the culprit factor for the lack of interaction the presence of EDTA in the P buffer. Apparently the presence of divalent cations (Mg^{+2})

is strictly necessary for the interaction to occur. In the P buffer there is 5 mM $MgCl_2$ present but EDTA is also included, apparently leaving free lower levels of $MgCl_2$ than required for the interaction. In the RE buffer no EDTA its present and 3 mM $MgCl_2$ also is included. Although in KC buffer no divalent cations are added, neither EDTA was included in the buffer. It is probably that the divalent cations ($MgCl_2$) in the extraction buffer are still in the liver protein extract at a sufficient level for the interaction to occur.

It seems likely that the liver cell protein extract was always functional as shown by its activity in the competitive binding assays. As the EMSA were always performed with a cytoplasmic protein extract and not with a purified nuclear extract or pure transcription factors it is very probable that the increase in the oligonucleotide size also improved the sensitivity of the assay. It is likely that while the ER-E_2-17β complex started to be formed other soluble protein factors present in the extract begin to associate thus providing more stability or, may be, the complex just needed more lineal space to accommodate the proteins. To overcome this transient interaction problems during complex formation, UV treatment are used to generate protein-protein and protein-DNA cross-links, but in this case UV treatment did not improve our results.

A question was raised about the similarity of this ER-E_2-ERE_CS formed in our extract from E_2-17β induced male with a complex formed in recently captured vitellogenic females. The EMSA was performed side by side with protein extracts from both E_2-17βtreated males and Vtg females. The presence of a retarded complex is quite apparent in both preparations, however the bands are not nicely compacted

thus preventing or making more difficult the comparison of the complexes (Figure 28). Therefore we cannot asseverate from this assay that the complex formed in both male and female extracts is exactly the same or that the same ER is being activated by E_2-17β in both sexes. However both complexes are specific as can be observed in the competition lane. A differential expression of three estrogen receptors iso-types (α, β, γ) was recently reported in fish (Sabo-Attwood et al. 2004). The authors reported that in females ER α and β expression predominated in liver, while β and γ predominate in other tissues. They also found that ER α is highly up-regulated, γ is slightly up regulated while β remained unchanged in the liver when plasma $E_2$17β and Vtg levels were elevated. Similarly, when males where injected with $E_2$17β, ER α was highly inducible, γ was moderately up regulated and ERβ levels were not affected. It can then be stated that the E_2-ER-ERE_CS complexes that we observed in both, E_2 17β induced males and Vtg females could contain the ER α.

A concluding experiment that should follow this present study to complete the analysis of the mechanism of actions of the EDCs/XEs is to testing them as activating compounds for *Anolis* ER. This experiment requires the pretreatment of the liver protein extract with DCC or other available method, to remove endogenous E_2 from females or from males that were treated with exogenous E_2-17β previous to the protein extraction. The presence of high E_2-17β levels will interfere with the EDCs/XEs interaction and thus confound the results. It is reasonable to extrapolate that the EDC/XE that induced Vtg synthesis and that as well as E_2-17β interact with the ER should be capable of activating it and lead to the formation of an EDC/ER complex that is able to recognize the consensus ERE.

In summary **this is the first time that in a liver protein extract from the tropical lizard *Anolis pulchellus* an E_2-17β binding activity (ER) is activated by E_2-17β and shown to recognize a consensus ERE inducing a electrophomobility shift.**

The fact that E_2-17β was able to promote this line of events is a premise to support the possibility that at least the EDCs/XEs that as E_2, 1) show to be Vtg inductors (DDT, DDE, BPA, Estriol, Estrone, DES) and 2) competed with E_2-17β for the ER in the competitive binding assay could be carry on their estrogenic effects following a similar cascade of activities.

$E_2$17β-EDCs/XEs in *Anolis pulchellus* in the context of the steroid mechanism of action

To summarize the information obtained in this study in the context of the accepted mechanism of action of estrogen the following illustration (Figure 35) was developed.

E_2-17β was shown, (a) to be estrogenic in males, as well as (b) reverse captivity effects in females, by the induction of Vtg mRNA and Vtg protein synthesis *de novo*. We propose that $E_2$17β (green arrow) accomplished this task in *Anolis* by (1) interacting with a SSBP (burgundy) in the lizard blood plasma that make it specifically available for (2) the interaction with a cytoplasmic ER (red arrow) in the liver cell. After the (3a) complex E_2-ER is organized (green/red as a dimmer (3b)),

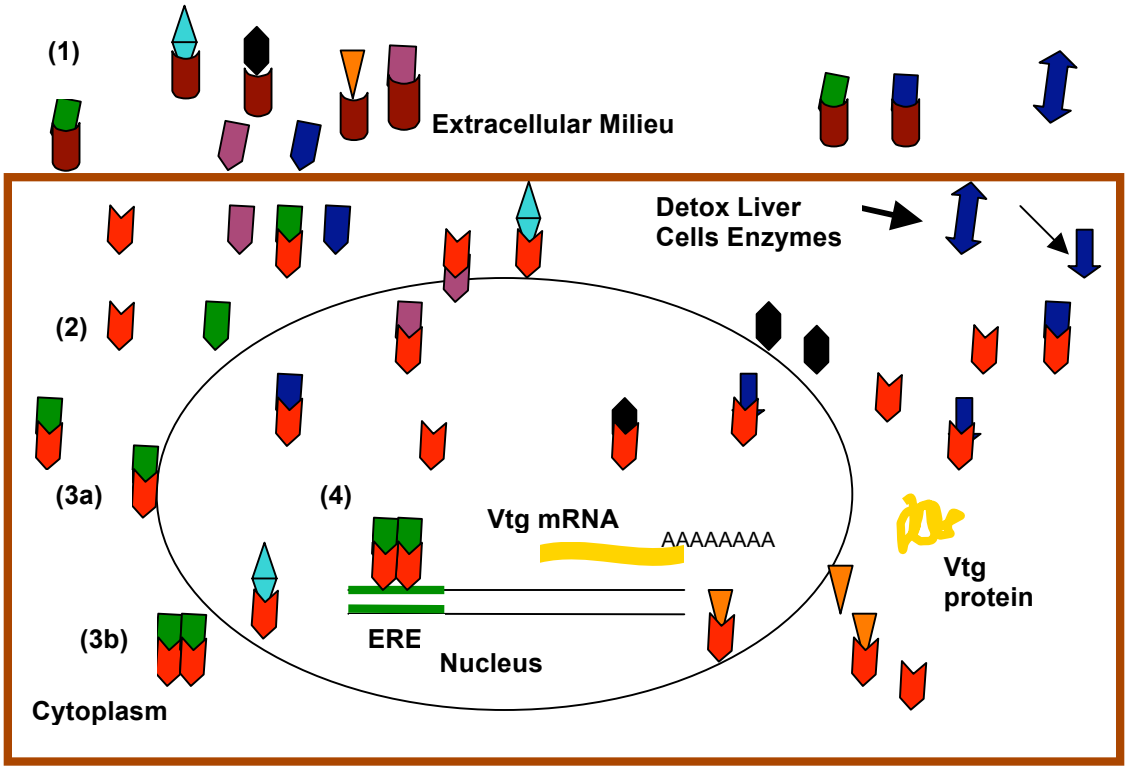

Figure 35 – E_2 and EDC in *Anolis pulchellus*; insights on the mechanism of action. The steps for the accepted mechanism of action of E_2 for Vtg gene regulation demonstrated in this study are shown (numbered, except for (3b)). The variety of structures, metabolic activation and bioavailability of the EDCs/XEs molecules are represented in the diagram to show the proven steps for XEs. See text for details. (E_2-green arrow, SSBP-burgundy, ER-red arrow, DDT,DDE-aqua blue double triangles, BPA-black hexagons, Estriol and Estrone-blue and purple arrows, DES-orange triangles, Mtx-double headed blue arrow, HPTE-one headed blue arrow,

it moves to the nucleus where is capable of (4) recognizing the ERE of the Vtg gene with the consequent induction of Vtg mRNA synthesis. We also propose that XEs that induced Vtg mRNA and protein synthesis and displaced radio labeled ligand from the anoline ER, follows the same mechanism of action of E_2-17β. These XEs may share many structural similarities with E_2-17β, like shown for Estriol, and Estrone (blue and purple arrows); or may not share so many structural features with E_2-17β like DES (orange triangles), DDT and DDE (aqua double triangles) and BPA (black hexagons), nevertheless they can interact with the SSBP and with the ER. Our extrapolation that EDC/XE/ER would recognize EREs an so induce gene expression can be verified

testing the capabilities of the XEs to activate the ER for the recognition of the Vtg ERE the EMSAs. The metabolic conversion of Mtx (blue double arrow) to a more estrogenic metabolite is also illustrated in the diagram.

Concluding remarks

The general problem considered in this study was the need to develop methods that help to better determine the estrogenic potential of different natural as well as man made chemical compounds. The induction of the Vtg gene expression in the tropical lizard *Anolis pulchellus* was shown to be very useful for the assessment of the estrogenic potential of EDCs. Especially compounds that can be estrogenic a low doses like DDT, DDE, and hormonal derived chemicals can be tested *in vivo*. This *in vivo* model is especially suited to test chemicals like Mtx that needs to be metabolically activated and thus give false negative results *in vitro*. The anoline liver protein extract seems to have high enough levels of ER to be used successfully to assess the interaction of the EDCs with the ER *in vitro* and possibly this type of assay could be adapted to assess the interaction of the EDCs with other steroid hormone receptors for example the androgen receptor. Finally conditions to test the ER of the anoline lizard as a ligand-dependent transcription factor were preliminary worked out with the optimization of the EMSA. The enrichment of the ER concentration or even purification of the ER should be an easy step to continue further research in this area.

Therefore the use of this tropical lizard as *in vivo* model to test estrogenicity in a laboratory experimental setting proves to be really successful. Lizards are abundant in all regions of the island, during all the year and are very easy to collect and

maintain in economic facilities. They survive captivity, and maintained in small groups and well nourished, they last long time. A situation to be considered is that individual variations in wild populations like age, hormonal and stress levels and metabolic states can influence the experiment results. So as in all scientific studies, experiments have to be repeated several times and animal and experimental conditions have to be maintained as steady as possible. A limitation could be their small size. This is not a good model to test substances that have to be administered in large volumes to see their effects.

One constraint that we confronted in the consideration of lizards as sentinel species is that their habitat is completely terrestrial and the environmental contamination that is related to EDCs/XEs is mainly associated to rivers, lakes, ponds and therefore is more significant to the aquatic organisms whose life cycle is associated to them. The probability that one lizard at random be directly sprayed with some XE and “live to tell”, in other words survive until a researcher capture it, and analyze its blood for Vtg is quite remote. As a matter of fact during the time span of this study approximately only in 1-2% of the experiments a Vtg positive male lizard (from about 1000 animals) was detected by analyzing it just as it comes from the field. This suggests that the levels of contamination in the soil or areas from where we collected the animals do not exist or are extremely low, below to our animal sensitivity. This idea should be tested by collecting animals from specific areas suspected of being contaminated and analyze lizards for Vtg. For example areas near plastic or pharmaceutical industries are good candidates for this kind of assessment. However after the observations and preliminary surveys related to this

research we do consider that anoline lizards not behave as good sentinel species at the open field or free environment in Puerto Rico.

The following conclusions and/or contributions are drawn from the present study:

1. A transdermal mode of administration for $E_2$17β and suspected EDCs was developed and tested. The maximum amount (about 60%) of the total dose exogenously administered was available in the interior of the animals in about 5 minutes.
2. The expression of the Vtg gene *de novo* in males can be used as a confident endpoint of Axes activity in this totally terrestrial anoline lizard at both mRNA and protein synthesis levels.
3. Pesticides and similar compounds as well as hormone-like compounds that are strongly estrogenic are more likely to be effectively and accurately tested in this model system. This system is not appropriate for plasticizers and substances that require to be applied at high volume or doses.
4. The system is specially fitted to test EDCs that require to be metabolized to show its activity like was the case of Mtx/HPTE.
5. Variability in responses to a same experimental agent in animals from wild populations was observed specially with compounds weakly estrogenic. Factors responsibly for this variability has to be taken into account and minimized as much as possible.

6. The presence of an $E_2$17β binding activity (ER) was demonstrated in a liver cytosolic protein extract from *Anolis pulchellus* for the first time. The interaction of 5 of 9 EDCs/XEs with the putative ER was also shown by this binding assay.

7. The presence of an $E_2$17β binding activity (SSBP) was demonstrated in the blood plasma of the *Anolis pulchellus* for the first time. Interaction with some of the EDCs/XEs with the SSBP was also demonstrated.

8. The formation of an $E_2$17β-ER complex using the liver cytosolic protein extract, capable of the recognition of a consensus ERE for estrogen-regulated genes was demonstrated. A consensus ERE appropriate for the anoline ER, was designed and the definition of buffering conditions adequate for this interaction were achieved. These are important technical tools for future studies on XE/ER interactions.

9. The Vtg expression in the tropical lizard *Anolis pulchellus* has proven to be excellent for the assessment of estrogenic potential of suspected EDCs in a laboratory/captivity scenario. However the anole lizard may not perform well as a sentinel species for estrogenic environmental contamination due to its strictly territorial/aerial distribution and its limited interaction with water bodies. An exception could be areas dedicated to agriculture where spraying of pesticides or other EDCs is routinely done.

Literature cited

Almstrup K, Fernandez MF, Petersen JH, Olea N, Skakkebaek NE, Leffers H. (2002) Dual effects of phytoestrogens result in u-shaped dose-response curves. Environ Health Perspect.110:743-8.

Andersen HR, Andersson AM, Arnold SF, Autrup H, Barfoed M, Beresford NA, Bjerregaard P, Christiansen LB, Gissel B, Hummel R, Jorgensen EB, Korsgaard B, Le Guevel R, Leffers H, McLachlan J, Moller A, Nielsen JB, Olea N, Oles Karasko A, Pakdel F, Pedersen KL, Perez P, Skakkeboek NE, Sonnenschein C, Soto AM, (1999) Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. Environ Health Perspect.107 Suppl 1:89-108.

Arnold SF, Collins BM, Robinson MK, Guillette LJ Jr, McLachlan JA. (1996) Differential interaction of natural and synthetic estrogens with extracellular binding proteins in a yeast estrogen screen. Steroids. 61:642-6.

Ashby J, Houthoff SJ, Stevens J, Bars R, Jekat FW, Campbell P, Van Miller J, Carpaninni FM, Randall GLP. (1997). The challenge posed by endocrine disrupting chemicals. Environm Health Perspec 105:164-169.

Autian J. (1973)Toxicity and health threats of phthalate esters: review of the literature. Environ Health Perspect. 4:3-26.

Baerga-Santini C, Hernandez de Morales M. (1991) Vitellogenin diversity in tropical lizards (Anolis pulchellus): identification and partial characterization. Comp Biochem Physiol B.;100(2):347-59

Beato, M.,. Chávez S, Truss M. (1996). Transcriptional regulation by steroid hormones. Steroids, 61: 240-251.

Bergeron JM, Crews, D McLachlan JA. (1994.) PCBs as environmental estrogens: Turtle sex determination as a biomarker of environmental contamination. Environ Health Perspect. 102:780-781.

Bentrem DJ, Dardes RC, Liu H, Macgregor-Schafer J, Zapf JW, Jordan C. (2001). Molecular mechanism of action at estrogen receptor α of a new clinically relevant antiestrogen related to tamoxifen. Endocrinology, 142: 838-846)

Bevan CL, Porter DM, Howard MJ, and Henderson LP. (2002) Environmental estrogens alter early development in *Xenopus laevis*. Environ Health Perspect. 111:488-496.

Bitman J, Cecil HC, Harris SJ, Fries GF. (1968). Estrogenic activity of o,p'-DDT in the mammalian uterus and avian oviduct. Science, 162:371-2.

Bongiovanni AM. (1983). An epidemic of premature thelarche in Puerto Rico. J Pediatr. 103:245-246.

Bowers JL, Tyulmenkov VV, Jernigan SC, Klinge CM. (2000) Resveratrol acts as a Mixed agonist/antagonist for estrogen receptors alpha and beta. Endocrinology, 141:3657-67.

Bourdony CJ. (1998). Premature Thelarche and Early Sexual Development Registry. Annual Report. San Juan, Puerto Rico: Department of Health

Brody, JG and Rudel RA. (2003). Environmental Pollutants and Breast Cancer. Environ Health Perspec. 111:1007-1019.

Brotons, J. Olea-Serrano M., Villalobos M., Pedraza V., and Olea N.. (1995). Xenoestrogens released from lacquer coatings in food cans. Environmental Health Perspectives 103: 608-612.

Bulger WH, Muccitelli RM, Kupfer D. (1978). Studies on the *in vivo* and *in vivo* estrogenic activities of methoxychlor and its metabolites. Role of hepatic mono-oxigenase in methoxychlor activation. Biochem Pharmacol 27:2417-2423

Burlington H and Lindenman V. (1950) Effect of DDT on testes and secondary sex characteristics of white leghorn cockerels. Proceedings of the Society for Experimental Biology and Medicine. 74:48-51.

Calabrese EJ, Baldwin LA. (2001) The frequency of U-shaped dose responses in the toxicological literature. Toxicol Sci. 62:330-8.

Callard IP, Riley D, Perez L. (1990) Vertebrate vitellogenesis: molecular model for multihormonal control of gene regulation. Prog Clin Biol Res.;342:343-8.

Cavailles V, Dauvois S, Horset F L, Lopez G, Hoare S, Kushner PJ, Parker MG. (1995).
Nuclear factor RIP140 modulates transcriptional activation by ER. EMBO J, 14:3741-3751.

Colborn T, Clement C. (1992.) Chemically induced alterations in sexual and functional development: The Wildlife/Human Connection. Colborn T, Clement C, eds. Princeton Scientific Publishing, New Jersey, pp 1-8.

Colborn, T., von Saal F., and Soto A. (1993). Developmental effects of endocrine-Disrupting chemicals in wildlife and humans. Environ Health Perspect, 101:378-
384.

Coldham NG, Sivapathasundarama S, McDonell DP, Conor C, Sauer MJ. (1997) Evaluation of a recombinant yeast cell estrogen screening assay. Environ Health Perspec 105:734-742.

Colon I, Caro D, Bourdony CJ, Rosario O. (2000). Identification of phthalate esters in the serum of young Puerto Rican girls with premature breast development. Environ Health Perspect. 2004 Jul;112(10):A541-3.

Crain DA, Guillette LJ, Pickfrod DB, Percival HF, Woodward AR. (1998) Sex steroid And thyroid hormone concentrations in juvenile alligators (*Alligator mississippiensis*) from contaminated and reference lakes in Florida, ESA. Environmental Toxicology and Chemistry, 17:446-452

Crain DA, Noriega N, Vonier PM, Arnold SF, Mclachlan JA, Guillette LJ. (1997) Cellular bioavailability of natural hormones and environmental contaminants as a function of serum and cytosolic binding factors. Toxicol Ind Health, 14:261-273.

Crews D, Cantu AR, Rhen T, Vohra R.(1996). The relative effectiveness of estrone, estradiol-17 beta, and estriol in sex reversal in the red-eared slider (Trachemys scripta), a turtle with temperature-dependent sex determination. Gen Comp Endocrinol. 102:317-26.

Crews D, Willingham E, Skipper JK (2000) Endocrine disruptors: present issues, future directions. Quartely Rev Biol.75:243-60

Curtis, S.W., Washburn T., Sewall C., DiAugustine R., Lindzey J., Couse J.F., and Korach K.S. (1996). Physiological coupling of growth factor and steroid receptor signaling pathways: Estrogen receptor knockout mice lack estrogen-like response to epidermal growth factor. Proc. Natl. Acad. Sci. USA. 93: 12626-12630.

Das, SK, Taylor JA, Korach KS, Paria BC, Dey SK, and Lubhan D.B.. (1997). Estrogenic responses in estrogen receptor-α deficient mice reveal a distinct estrogen signaling pathway. Proc. Natl. Acad. Sci. USA. 94:12786-12791.

Davis WP, and Bartone, SA. (1992) Effects of kraft mill effluents on the sexuality of fishes: an environmental easily warning? In Chemically induced alterations in s sexual and functional development: The wildlife/human connection. Colborn T, Clemens C. eds. Princeton Scientific Publishing, Inc. Princeton, NJ. 113-127.

Dechaud H, Ravard C, Claustrat F, de la Perriere AB, Pugeat M. (1999). Xenoestrogen interaction with human sex hormone-binding globulin. Steroids, 64:328-324.

DeRosa, C., Richter P., Pohl H., and Jones D.E. (1998). Environmental exposures that affect the endocrine system: Public health implications. Journal of Toxicology and Environmental Health*, Part B. 1:3-26.*

Daughton CG, Ternes TA. (1999). Pharmaceuticals and personal care products in the environment: agents of subtle change Environ Health Perspect. 107 Suppl 6:907-38.

Eroschenko VP, Rourke AW, Sims WF. (1996) Estradiol or methoxychlor stimulates estrogen receptor expression in uteri. Reprod Toxicol 10:265-271.

Fannon SA, Vidaver RM, Marts SA. (2001). An abridged history of sex steroid hormone receptor action. J Appl Physiol. 91:1854-9.

Feldman, D. and Krishnan A. (1995). Estrogens in unexpected places: possible implications for researchers and consumers. Environ. Health Perspect. 103(Suppl 7):129-133.

Freni-Titulaer LW, Cordero JF, Haddock L, Lebron G, Martinez R, Mills JL (1986). Premature thelarche in Puerto Rico. A search for environmental factors. Am J Dis Child. Dec;140:1263-1267.

Fox, G. (1992). Epidemiological and pathological evidence of contaminant induced alterations in sexual development in free-living wildlife. In: Chemically-induced alterations in sexual and functional development: the wildlife/human connection.

Fry DM, and Toone CD. (1981). DDT-induced feminization of gull embryos. Science 231: 919-924.

Ghosh D, Taylor C, Green J, Lubahn D. (1999) Methxychlor Stimulates Estrogen-Responsive Messenger Ribonucleic Acids in Mouse Uterus through a Non-Estrogen Receptor (Non-ER)α and Non-ERβ Mechanism. Endocrinology 140:3526-3533.

Greco TL, Duello TM, Gorski J. (1993). Estrogen receptors, estradiol, and diethylstilbestrol in early development: the mouse as a model for the study of estrogen receptors and estrogen sensitivity in embryonic development of male and female reproductive tracts. Endocr Rev. Feb;14(1):59-71.

Guillette LJ Jr, Vonier PM, McLachlan JA. (2002) Affinity of the alligator estrogen receptor or serum pesticide contaminants. Toxicology. 181-182:151-4.

Guillette LJ, Arnold SF., McLachlan JA. (1996). Ecoestrogens and embryos - Is there a scientific basis for concern? Animal Reproduction Science 42:13-24.

Guillette, L.J., Gross T.S., Gross D.A., Rooney A.A. and Percival H.F.(1995). Gonadal steroidogenesis *in Vitro* from juvenile alligators obtained from contaminated or control lakes. Environ. Health Perspect. 103(Suppl 4):31-36.

Guillette, LJ, Gross TS, Masson GR, Matter JM, Percival HF, and Woodward AR. (1994). Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ Health Perspect. 102:680-688.

Guillette LJ Jr, Brock JW, Rooney AA, Woodward AR. (1999). Serum concentrations of various environmental contaminants and their relationship to sex steroid concentrations and phallus size in juvenile American alligators. Arch Environ Contam Toxicol. 36:447-55.

Guillette LJ Jr, Woodward AR, Crain DA, Pickford DB, Rooney AA, Percival HF (1999) Plasma steroid concentrations and male phallus size in juvenile alligators from seven Florida lakes. Gen Comp Endocrinol. 116:356-72.

Guo YL, Lambert GH, Hsu CC. (1995) Growth abnormalities in the population exposed *in uteri* and early postnatally to polychlorinated biphenyls and debnsofurans. Environ Healt Perspect. 103:(Suppl 6): 117-122.

Harris, C. A., Henttu P., Parker M. G. and Sumpter J. P. (1997). The estrogenic activity of phthalates ester *in vitro.* Environ. Health Perspect, 105:802-811.

Haddock L, Lebron G, Martinez R. (1985) Premature sexual development in Puerto Rico: background and current status. In: Estrogens in the Environment II. Influences on Development (McLachlan JA, ed) Elsevier Science Publishing Co. New York, p. 349-357.

Hannon WH, Hill RH Jr, Bernert JT Jr, Haddock L, Lebron G, Cordero JF (1987). Premature thelarche in Puerto Rico: a search for environmental estrogenic contamination. Arch Environ Contam Toxicol. 16:255-62.

Heppell SA, Denslow ND, Folmar LC, Sullivan CV. (1995). Universal assay of vitellogenin as a biomarker for environmental estrogens. Environ Health Perpec 103(Suppl 7):9-15.

Ho S-M, Lance V, Megaloudis M. (1987). Plasma sex-steroid binding protein in a seasonally breeding reptile, *Alligator mississippiensis.* Gen Comp Endocrinol, 65:121-132.

Hong H, Kohli K, Trivedi A, Johnson DL, Stallcup MR. (1996). GRIP1, a novel mouse protein that serves as a transcriptional co-activator in yeast for the hormone binding domain of steroids receptors. Proc Natl Acad Sci USA, 93:4948-4952.

Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenbergh JG, vom Saal FS (1999). Exposure to bisphenol A advances puberty. Nature. 1999 Oct 21;401(6755):763-4

Hughes CL. (1988). Phytochemical mimicry of reproductive hormones and modulation of herbivore fertility by phytoestrogens. Environ Health Perspect. 78:171-175.

Hyrb DJ, Khan MS, Romas NA, Rosner W. (1990). The control of the interaction of sex hormone-binding globulin with its receptor by steroid hormones. J Biol Chem., 265:6048-6054.

Ignar-Trowbridge, D.M., Nelson K.G., Bidwell M.C., Curtis S.W., Washburn T.F., McLachlan J.A. and Korach K.S. (1992). Coupling of dual signaling pathways: epidermal growth factor actions involves the estrogen receptor. Proc. Natl. Acad. Sci. USA. 89:658-4662.

Ignar-Trowbridge, D.M., Pimentel M., Teng C.T., Korach K.S. and McLachlan J.A. (1995).Cross talk between peptide growth factor and estrogen receptor signaling systems. Environ. Health Perspect. 103(Suppl 7):35-38.

Irwin LK, Gray S, Oberdorster E. (2001) Vitellogenin induction in painted turtle, Chrysemys picta, as a biomarker of exposure to environmental levels of estradiol. Aquat Toxicol 55(1-2):49-60

Jaeger RJ, and Rubin RJ. (1972). Migration of a phthalate ester plasticizer from polyvinyl chloride blood bags into stored human blood and its localization in human tissues. New England J Med. 287:1114-1148.

Jensen, T. K., Toppari J., Keiding N. and Skakkebaek N. E. (1995). Do environmental estrogens contribute to the decline in male reproductive health?. Clin. Chem. 41:1896-1901.

Jobling, S, Reynolds T, White R, Parker MG, and Sumpter JP. (1995) A variety of environmentally persistent chemicals, including some phthalates plasticizers, are weakly estrogenic. Environ Health Perspect. 10:582-587.

Jordan VC and Morrow M. (1999). Tamoxifen, raloxifen and the prevention of breast cancer. Endocrine review 20:253-278

Jordan VC, Gosden B. (1982). Importance of the alkylaminoethoxy side-chain for the estrogenic and antiestrogenic actions of tamoxifen and toxifene in the immature rat uterus. Mol Cell Endocrinology 140:5828-5840

Jordan VC, Phelps E, Lindgren JU. (1987) Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 10:31-35

Kalling M, Kugler W, Ross K, Zoidl C, and Ryffel G. (1991). Liver-specific gene expression: A-activator-binding site, a promoter module present in vitellogenin and acute phase genes. Molecular and Cellular Biology 11:93-111.

Kapoor IP, Metcalf RL, Sangha GK. (1970) Comparative metabolism of methoxychlor, methoxychlor and DDT in mouse, insects, and in a model ecosystem. J. Agric Food Chem 18:1145-1152.

Katzenellenbogen, J.A. (1995). The Structural Pervasiveness of Estrogenic Activity. Environ. Health Perspect. 103(Supp 7):93-101.

Kelce WR, Stone CR, Laws SC, Gray LE, Kemppainen JA, Wilson EM. (1995) Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist Nature. 375):581-585.

Kleinsmith LJ, Kerrigan MS, Kelly J.(2002). Understanding Estrogen, Receptors, Tamoxifen, Raloxifen. National Cancer Institute News center. Pp1-22.

Klinge CM. (2000). Estrogen receptor interaction with co-activators and co-repressors. Steroids. May;65(5):227-51.

Klock G, Strahle U, Schmid W, Schutz G. (1987) Similarities and differences of steroid response elements. Horm Metab Res Suppl.;17:11-3.

Klotz DM, Beckman BS, Hill SM, McLachlan JA, Walters MR, and ArnoldSF. (1996). Identification of environmental chemicals with estrogenic activity using a combination of *in vitro* assays. Environ Health Persp 104:1084-1089.

Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT (2002).Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S.streams, 1999-2000: a national reconnaissance. Environ Sci Technol. 15;36(6):1202-11

Korach, K.S. and McLachlan J.A. (1995). Techniques for Detection of Estrogenicity. Environ. Health Perspect, 103(Suppl)7: 5-8.

Krishnan, AV; Stathis P, Permuth SF, Tokes L and Felman D. (1993) Bisphenol A: and estrogenic substance is release from polycarbonate flasks during autoclaving. Endocrinology 132:2279-2286.

Kuhnau J. (1976). The flavonoids. A class of semi-essential food components: their Role in human nutrition World Rev Nutr Diet. 1976;24:117-91.

Kuipper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925-5930.

Kumar V and Chambon P. (1988). The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell 55:145-156.

Laidley CW, Thomas P. (1994) Partial characterization of a sex-steroid binding protein in the spotted sea-trout (Cynoscion nebulosus). Biol Reprod. Nov;51(5):982-92.

Landel, C.C.; Kushner P.J., and Greene G.L. (1994). The interaction of human estrogen receptor with DNA is modulated by receptor-associated proteins. Molecular Endocrinology 8: 1407-1419.

Landel CC, Kushner PJ, Greene GL. (1995) Estrogen receptor accessory proteins: effects on receptor-DNA interactions. Environ Health Perspect.103 Suppl 7:23-8.

Larkin P, Knoebl I, Denslow ND (2003). Differential gene expression analysis in fish exposed to endocrine disrupting compounds Comp Biochem Physiol Biochem Mol Biol. Oct;136(2):149-61.

Lazier CB, Lonergan K, Mommsen TP. (1985). Hepatic estrogen receptors and plasma estrogen-binding activity in the Atlantic salmon. Gen Comp Endocrinol. 57:234-45.

Leblanc. G. A. and Blain L.J.. (1997). Chronic toxicity of environmental contaminants: sentinels and biomarkers. Environ. Health Perspect, 105(Suppl. 1): 65-80.

Le Douarin B, Nielsen AL, Garnier JM, Ichinose H, Jeanmougin F, Losson R, Chambon P. (1996) A possible involvement of TIF1 alpha and TIF1 beta in the epigenetic control of transcription by nuclear receptors EMBO J. Dec 2;15(23):6701-15.

Le Guellec K, Lawless K, Valotaire Y, Kress M, Tenniswood M. 1988 Vitellogenin gene expression in male rainbow trout (Salmo gairdneri). Gen Comp Endocrinol. 71:359-71.

Li J, Gomes PJ, Chen JD, (1997). Rac3, a steroid/nuclear receptor-associated co-activator that is related to SRC-1 and TIF2. Proc Natl Acad Sci USA 94:8479-8484.

Lind PM, Milnes MR, Lundberg R, Bermudez D, Orberg JA, Guillette LJ Jr. (2004) Abnormal bone composition in female juvenile American alligators from a pesticide-polluted lake (Lake Apopka, Florida).Environ Health Perspect. 112:359-62.

Lieberher M, Grosse B, Kachkache M, Balsan S. (1993). Cell signaling and estrogen In female rate osteoblasts: a possible involvement of unconventional non-nuclear receptors. J Bone Mier Res 8:1365-1376.

Martin B, Xavier F. (1981) High-affinity binding of progesterone, estradiol-17 beta and testosterone by plasma proteins of the reptile Lacerta vivipara J Gen Comp Endocrinol. 43:299-307.

Martinez E. and Wahli W. (1989) Cooperative binding of estrogen receptor to imperfect estrogen-responsive DNA elements correlates with their synergistic hormone-dependent enhancer activity. The EMBO Journal 8:3781-3791

McLachlan JA, Newbold RR, Bullock BC (1980) Long-term effects on the female mouse genital tract associated with prenatal exposure to diethylstilbestrol. Cancer Res. 40(11):3988-99.

McLachlan, JA. (1993). Functional toxicology: a new approach to detect biologically active xenobiotics. Environ Health Perspect. 101:386-387.

McLachlan JA, Arnold SF. (1996) Endocrine Disruptors in the Environment. American Scientist, 65:38-78.

McLachlan JA, Korach KS. (1995) Symposium on estrogens in the environment, III Environ Health Perspect;103 Suppl 7:3-4.

Mangelsdorf, D.J., Thummel C., Beato M., Herrlich P., Schutz G., Umesono K., Blumberg B., Kastner P., Mark M., Chambon P. and Evans R.M. (1996). The nuclear receptor superfamily: The Second Decade. Cell, 83:834-839.

Mangelsdorf, and Evans R.M.. (1995). The RXR heterodimers and orphan receptors Cell, 83:841-850.

McKinney, J. and WalleC. r. (1994). Polychlorinated biphenyls as hormonally active structural analogues. Environmental Health Perspectives, 102: 290-297.

Matthews J, Celius T, Halgreen R, Zacharewski T. (2000). Differential estrogen receptor binding of estrogenic substances: a species comparison. Journal of Steroid Biochemistry & Molecular Biology. 74:223-234.

Mathews J and Zacharewski T. (2000) Differential binding affinities of PCBs, HO-PCBs and aroclors with recombinant human, rainbow trout (*Onchorhynkiss mykiss*), and green anolis (*Anolis carolinensis*) estrogen receptors, using a semi-high throughput competitive binding assay. Toxicological Sciences 53:326-339.

Metcalf JL, Law SC, Cummings AM. (1996) Methoxyclhor mimics the action of 17β estradiol on induction of uterine epidermal growth factor receptors in immature female rats. Reprod Toxicol 10:393-399.

Moore A, Bottoms G, Coppoc F, Pohland R, Roesel O. (1982) J. Animal Science 55:124-134.

Moore R, Rudy T, Lin TM, Ko K, Peterson R. (2001) Abnormalities of sexual development in male rats with in uterus and lactational exposure to the antiandrogenic plasticizer Di(2-ethylhexyl) Phthalate. Environ Health Perspect 109:229-237.

Morales MH, Osuna R, Sanchez E. (1991). Vitellogenesis in Anolis pulchellus: induction of VTG-like protein in liver explants from male and immature lizards. J Exp Zool. 260:50-58

Morales MH, Baerga-Santini C, Cordero-Lopez N.(1996) Synthesis of vitellogenin polypeptides and deposit of yolk proteins in Anolis pulchellus. Comp Biochem Physiol. 114B:225-231,

Morales MH, Sanchez EJ.(1996) Changes in vitellogenin expression during captivity-induced stress in tropical anole. General and Comparative Endocrinology, 1003:209-219.

Mosselman S, Polman J, Dijkema R. (1996) ER beta: identification and characterization of a novel human estrogen receptor. FEBS lett. 392-49-53.

Newbold R.(1995). Cellular and molecular effects of developmental exposure to DES: implications for other environmental estrogens. Environ Health Perspect. 103(Suppl 7):83-87.

Norfleet AM, Thomas ML, Gametchu B, Watson CS. (1999) Estrogen receptor-alpha detected on the plasma membrane of aldehyde-fixed GH3/B6/F10 rat pituitary tumor cells by enzyme-linked immunocytochemistry Endocrinology. 140:3805-14.

Orlando, EF, Kolok AS, Binzcik GA, Gates JL, Horton MK, Lambright CS, Gray LE, Soto AM, Guillette LJ. (2004). Endocrine-Disrupting Effects of Cattle Feedlot Effluent on an Aquatic Sentinel Species, the Fathead Minnow. *Environmental Health Perspectives.*112:353-358.

Olea, N., Pulgar R., Perez,P. Olea-Serrano F., Rivas A., Novillo-Fertrell A., Pedraza V., Soto A, and Sonnenschein C.. (1996) Estrogenicity of resin-based composites and sealants used in dentistry. Environmental Health Perspectives, 104:298-305.

O'Malley BW, and Tsai MJ (1992). Molecular pathways of steroid receptor action. Biol Reprod. 46:163-7.

Onate SA, Tsai SY, Tsai MJ, O'Malley B. (1995) Sequence and characterization of a co-activator for the steroid hormone receptor super family. Science 270:1354-1357.

Paech K, Webb P, Kuipper G, Nillson S, Gustafsson J, Kushner PJ, Scalan TS. (1997). Differential ligand activation of estrogen receptors ERα and ERβ at AP1sites. Science 277:1508-1510.

Palmer, BD and Palmer SK. (1995) Vitellogenin induction by xenobiotic estrogens in the red-eared turtle and African clawed frog. Environ Health Perspect. 103:19-25

Pellisero C, Flouriot G, Foucher JL, Bennetau B, Dunogues J, Le Gac F, Sumpter JP. (1993). Vitellogenic synthesis in cultured hepatocytes; and *in vitro* test fir the estrogenic potency of chemicals' Steroid Biochem Molec Biol 44:263-272.

Perez Comas A. (1982) Precocious sexual development: clinical study in the western region of Puerto Rico Bol Asoc Med P R. Sep;74(9):245-51

Pink JJ, and Jordan VC. (1996). Models of estrogen regulation by estrogens and antiestrogens in breast cancer cell lines. Cancer Res 56:2321-2330.

Porter W, Saville B, Hoivik, and Safe DS. (1997). Functional synergy between the transcriptional factor sp1 and the estrogen receptor. Molecular Endocrinology, 11:1569-1580.

Purdom CD, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Sumpter JP. (1994). Estrogenic effects of effluents from sewage treatments works. Chem Ecol 8:275-285.

Ralof J.(2002) Hormones: Here is the Beef: Environmental concerns reemerge over steroids given to livestock. Sciencenews Online, Jan 5 Vol 161:10

Ralof J. (2002) Hormones: Here's the Beef :Environmental concerns reemerge over steroids given to livestock. Sciencenews Online, Jan 5 Vol 161:p10

Rivera-González R, Petersen D, Tkalcevic G, Thompson D, Brown T. (1998) Estrogen induced genes in uterus of ovariectomized rats and their Regulation for Droloxifene and Tamoxifen. J. Steroid Biochem. 64:13024.

Reichel, R.R. and Jacob S.T.. (1993). Control of gene expression by lipophilic hormones. FASEB J. 7: 427-436.

Ren L, Lattier D, and Lech JJ. (1996). Estrogenic activity in rainbow trout determined with a new cDNA probe for vitellogenesis, pSG5Vg51.1. Bull Environm Contam Toxicol 56:287-294.

Reuber, MD.(1980) Carcinogenicity and toxicity of methoxychlor. Environ. Health Perspec. 36:205-219.

Riley D, Kleis-San Francisco SM, Callard IP (1988). A plasma steroid hormone binding protein in the viviparous water snake, Nerodia Gen Comp Endocrinol. 1988 71:419-28.

Riley D, and Callard IP (1988). An estrogen receptor in the liver of the viviparous water snake, Nerodia; characterization and seasonal changes in binding capacity. Endocrinology. 1988 123:753-61.

Romine LE, Wood JR, Lamia LA, Prendergast P, Edwards DP, and Nardulli, AM. (1998). The high mobility group protein 1 enhances binding of the estrogen receptor binding domain to the estrogen response element. Molecular Endocrinology, 12:664-674.

Ruiz-Echevarria MJ, Munshi R, Tomback J, Kinzy TG, Peltz SW. (2000) Characterization of a general stabilizer element that blocks deadenylation-dependent mRNA decay. J Biol Chem. 276:30995-1003.

Sabo-Attwood T, Kroll KJ, Denslow ND. (2004) Differential expression of largemouth bass (Micropterus salmoides) estrogen receptor isotypes alpha, beta, and gamma by estradiol Mol Cell Endocrinol. 218:107-18.

Safe S. (1994). Environmental and Dietary Estrogens and Human Health: Is there a problem. Environ Health Perspec 103:346-351.

Safe S. (1995). Response to: Environmental Estrogens. Environ Health Perspec 103:784-785.

Salhanick ACR, and Callard IP. (1980). A sex-steroid-binding protein in the plasma of the fresh water turtle, *Chrysemys picta*. Gen Comp Endocrinol, 42:163-166

Sathya G, Li W, Klinge CM, Anolik JH, Hilf R, Bambara RA. (1997) Effects of multiple estrogen responsive elements, their spacing and location on estrogen response of reporter genes. Molecular Endocrinology 11: 1994-2003.

Schmidt CW. (2003) The lowdown on low-dose endocrine disruptors. Environm Health Perspect, 109: 420-421

Shapiro D. (1982) Steroid hormone regulation of vitellogenin gene expression. CRC Crit Rev Biochem. 12:187-203.

Shapiro DJ, Barton MC, McKearin DM, Chang TC, Lew D, Blume J, Nielsen DA, Gould L. (1989) Estrogen regulation of gene transcription and mRNA stability. Recent Prog Horm Res. 1989;45:29-58; discussion 58-64.

Sharpe, RM and DS Irvine. 2004. How Strong is the Evidence of a Link Between Environmental Chemical and Adverse Effects on Human Reproductive Health? *British Medical Journal.* 328(21 Feb):447-451.

Sharpe, RM, Fisher JS, Millar MM, Jobling S, and Sumpter JP. (1995). Gestational and lactational exposure of rats to xenoestrogen results in reduced testicular size and sperm production. Environ Health Perspect. 103: 1136 - 1145.

Shannon Atkinson, Marlin J. Atkinson, and Ann M. Tarrant. (2003). Estrogens from Sewage in Coastal Marine Environments. Envioron Health Perspec. 111(April): 531-535.

Shelby, M.D., Newbold R.R., Tully D.B., Chae K. and Davis.V.L. (1996). Assessing environmental chemicals for estrogenicity using a combination of *in vitro* and *in vivo* Assays. Environ. Health Perspect, 104:1296-1300.

Schiffer B, Daxenberger A, Meyer K, Meyer HH. (2001) The fate of trenbolone acetate and melengestrol acetate after application as growth promoters in cattle: environmental studies Environ Health Perspect. 109:1145-51.

Siiteri, PK, Murai JT, Hammond GL, Nisker JA, Raymoure WJ, and Kuhn RW. (1982). The Serum Transport of Steroid Hormones. Recent Prog Horm Res. 38:457-510.

Sone K, Hinago M, Kitayama A, Morokuma J, Ueno N, Watanabe H, IguchiT. (2004) Effects of 17beta-estradiol, nonylphenol, and biphenyl-A on developing Xenopus laevis embryos. Gen Comp Endocrinol. 15;138:228-36.

Soto, AM. et al. 2004. Androgenic and Estrogenic Activity in Water Bodies Receiving Cattle Feedlot Effluent in Eastern Nebraska, USA. Environ Health Perspec. 112(March): 346-352

Soto, A, Chung K., and Sonnenschein C.. (1994). The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environ. Health Perspect. 102: 380-383.

Soto AM, Sonnenschein C., Chung KL, Fernández MF, Olea N, and Olea Serrano F. (1995) The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Persp 103(suppl 7):113-122.

Soto-Caraballo, ML. (1990). Inducción con estradiol 17β de vitelogénesis en el macho del lagartijo tropical *Anolis pulchellus* y su efecto en la albumina de s suero. Master Thesis, Univ of Puerto Rico, Rio Piedras Campus.

Solomon GM, and Schettler T. (2000). Environment and health: Endocrine disruption and potential human health implications. CMAJ 163:1471-1476.

Special Report on Environmental Endocrine Disruption: An effects assessment and analysis. Risk Assessment Forum, US Environmental Protection Agency. (1997) Thomas Crisp (Chair). EPA/630/R-96/012 p. 1-91

Stancel, G.M., Boettger-Tong H.L., Chiappetta C., Hyder S.M., Kirkland J.L., Murthy L., and Loose-MItchell D.S. (1995). Toxicity of endogenous and environmental estrogens: What is the role of elemental interactions? Environ. Health Perspect. 103(Suppl 7):29-33.

Suetsugi M, Su L, Karlsberg K, Yuan Y-C, and Chen S. 2003. Flavone and Isoflavone Phytoestrogens Are Agonists of Estrogen-Related Receptors. Molecular Cancer Research1:981-991(2003),

Sumpter JP and Jobling S. (1993).Male sexual development in "a sea of oestrogen". Lancet. 342(8863):124-5.

Sumpter JP, and Jobling S. (1995). Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ Health Perspect. 103 Suppl 7:173-8.

Tansey WP and W Herr. (1997). TAFs: Guilt by Association. Cell 88:729-732.

Thomas, KB and Colborn T. (1992) Organochlorine endocrine disruptors in human tissue: The Wildlife/Human Connection. Colborn T, Clement C, eds. Princeton Scientific Publishing, New Jersey, pp 365-394.

Tollefsen KE, Ovrevik J, Stenersen J. (2004) Binding of xenoestrogens to the sex steroid-binding protein in plasma from Arctic char (Salvelinus alpinus L.). Comp Biochem Physiol Toxicol Pharmacol. 139:127-33.

Tong, W., .Perkins R, Xing L., Welsh W., and Sheehan D.. (1997). QSAR models for binding of estrogenic compounds to estrogen receptor α and ß subtypes. Endocrinology, 138:4022-4025.

Tullner WW, and Edgcomb JH (1962). Cystic tubular nephropathy and decrease in testicular weight in rats following oral methoxychlor treatment. J Pharmacol Exp Ther.138:126-130

Tyler CR, Jobling S, and Sumpter JP. (1998) Crit Rev Toxicol. Endocrine disruption in wildlife: a critical review of the evidence.28:319-61.

US Department of Human Health and Human Services, 1994. Toxicology Profile for DDT, DDE, DDD; The Hazardous Substances Data Bank, US National Library of Medicine; NIH, 1998),

Van Winter JT, Noller KL, Zimmerman D, Melton LJ 3rd. (1990). Natural history of premature thelarche in Olmsted County, Minnesota, 1940 to 1984. J Pediatr. 116:278-80

Villalobos M, Olea N, Brotons JA, Olea-Serrano MF, Ruiz de Almodovar JM, and Pedraza V P. (1995). The E-SCREEN Assay: a comparison of different MCF7 cell stocks.Environ Health Persp 103:844-850.

Vonier, P., D. Crain, J. Mclahan, L. Guillette and S. Arnold. (1996). Interaction of environmental chemicals with the estrogen and progesterone receptors from the oviduct of the american alligator. Environmental Health Perspectives, 104:1318-1322.

vom Baur E, Zechel C, Heery D, Heine MJ, Garnier JM, Vivat V, Le Douarin B, Gronemeyer H, Chambon P, Losson R. (1996) Differential ligand-dependent Teractions between the AF-2 activating domain of nuclear receptors and the putative transcriptional intermediary factors mSUG1 and TIF1 EMBO J. 15:110-24.

Vom Saal Fs, Timms BG, Montano MM, Palanza P, Thayer KA, Nagle SC, Dhar MD, Ganiam VK, Parmigiani S, Welshons WV. (1995) Prostate enlargement in mice to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. PNAS 94: 2056-2061.

Wagner, RK. (1978). Extracellular and intracellular steroid binding proteins: properties, discrimination, assay and clinical application. Acta Endocrinol. 88(suppl 218) 1 73.

Waller, C.L., Oprea T.I., Chae K., Hae-Kyoung Paark, , Korach K.S., Laws S.C., Wiese T.E. ,Kelce W.R, and Gray, Jr L.E.. (1996) Ligand-based identification of environmental estrogens. Chem. Res. Toxicol. 9:1240-1248.

Walters LM, Rourke AW, Eroschenko VP. (1993). Purified methoxychlor stimulates the reproductive tract in immature female mice. Reprod Toxicol 7:599-606.

Watson, C.S., Pappas T.C. and Gametchu B. (1995). The other estrogen receptor in the plasma membrane: Implications for the actions of environmental estrogens. Environ. Health Perspect. 103(Suppl 7):41-50.

Welch RM, Levin W, and Conney AH (1969) Estrogenic action of DDT and its analogs. Toxicol Appl Pharmacol.14: 358-67.

Welshons, WV, Thayer KA, Judy BM, Taylor JA, Curran EM, and vom Saal FS. (2003). Large Effects from Small Exposures. I. Mechanisms for Endocrine-Disrupting Chemicals with Estrogenic Activity. Environmental Health Perspectives. 111(June):994-1006.

Willingham E, and Crews D. (1999) Sex reversal effects of environmentally relevant xenobiotic concentrations on the red-eared slider turtle, a species with temperature-dependent sex determination. Gen Comp Endocrinol. 113:429-35.

Wine, RN, Li LH, Hommel Barnes L, Gulati DK, and Chapin RE. (1997) The reproductive toxicity of di-n-butylphthalate in a continuous breeding protocol in sprague-dawley rats. Environ Health Perspect. 105:102-107.

Wolf M. (1995). Environmental Estrogens. Environ Health Perspec 103:784

World Health Organization (1979). DDT and its derivatives. Environmental Health Criteria. Geneva, Switzerland, Vol. 9.

Yang, N.N., Bryant H.U., Hardikar S., Sato,M. R. Galvin J.S., Glasebrook A.L. and Termine.J.D. (1996) Estrogen and raloxifene stimulate transforming growth factor-ß3 gene expression in rat bone: A potential mechanism for estrogen or raloxifene mediate bone remodeling. Endocrinology, 137: 2075-2084.

Zacharewski, T. (1997). *In vitro* bioassays for assessing estrogen substances. Environmental Science & Technology, 31:613-623.