

**ENDOCRINE EFFECTS OF THE PYRETHROID PESTICIDE
ESFENVALERATE METABOLITES; 3-PHENOXYBENZYL
ALCOHOL AND 3-PHENOXYBENZOIC ACID**

A Senior Scholar Thesis

by

MARCO ANTONIO CHAVEZ

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2008

Major: Biomedical Science

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Approved by

Research Advisor:
Associate Dean of Undergraduate Research:

Michelle Pine
Robert C. Webb

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ABSTRACT

Endocrine Effects of the Pyrethroid Pesticide Esfenvalerate Metabolites; 3-Phenoxybenzyl Alcohol and 3-Phenoxybenzoic Acid (April 2008)

Marco Antonio Chavez
Department of Veterinary Integrative Biosciences
Texas A&M University

Research Advisor: Dr. Michelle Pine
Department of Veterinary Medicine

Much research has been conducted on type II pyrethroid pesticides but there is a paucity of research conducted on metabolites from these pesticides. 3-phenoxybenzoic acid and 3-phenoxybenzyl alcohol are two metabolites common to many of the type I and type II pyrethroid pesticides.

In vitro experiments were conducted in our laboratory using MCF-7 estrogen dependent breast cancer cells. These cells were grown in 96 well plates and treated with 1mM, .1mM, .01mM, and .001mM concentrations of pyrethroid metabolites, 3-phenoxybenzoic acid and 3-phenoxybenzyl alcohol. Cell proliferation was measured and compared to cells grown in media only and media with 1nM estradiol. By day 10 the metabolite 3-phenoxybenzyl alcohol at a concentration of .001 mM showed proliferation, even larger than 1nM estradiol at incubation day 10 (2.69 nm versus 1.15 nm respectively). Metabolite 3-phenoxybenzoic acid at concentrations of .01 mM (.63

nm) and .001 (.56 nm) mM both also showed proliferation greater than 1nM (.54 nm) estradiol at day 5.

In vivo experiments were conducted by dosing immature female Sprague Dawley rats with 10mg/kg esfenvalerate, 10mg/kg 3-phenoxybenzyl alcohol, and 10mg/kg, 5mg/kg, 1mg/kg 3-phenoxybenzoic acid. Vaginal opening (VO) was measured to indicate the onset of puberty. Previous data from our laboratory show that parent compound esfenvalerate delays puberty at a statistical significance of $p < 0.05$. Neither metabolite had any effect on the onset of puberty in our experiment.

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CHAPTER I

INTRODUCTION

Many environmental chemicals including pesticides have been reported to possess hormonal activities, causing them to be classified as endocrine disruptors in humans and animals.¹ Pesticides are applied extensively. In 1997 approximately 129 million pounds of insecticides were sold in the United States, and 17 million pounds were used specifically to treat homes and gardens. Of the pesticides used, the four most common are the carbamates, organochlorines, organophosphates, and pyrethroids.

Synthetic pyrethroids are among the most commonly used insecticides worldwide. They are used to control agricultural and household pests. Pyrethroids are synthetic analogs of the naturally occurring chemical, pyrethrin, derived from the toxin produced in the flowers of *Chrysanthemum cinerariaefolium*. Pyrethroids can be divided into two classes (type I and type II) based on the symptoms of toxicity exhibited and the presence or absence of a cyano group at the carboxyl alpha position. Symptoms of toxicity by type I pyrethroids (non-cyano compounds) are characterized by tremors and increased sensitivity to external stimuli, while toxicity caused by exposure to type II pyrethroids (cyano compounds) is characterized by salivation, rapid jerky movements, and seizures.

This thesis follows the style of *Journal of Environmental Monitoring*.

The More toxic type II pyrethroid esfenvalerate [(*S*)- α -cyano-3-phenoxybenzyl (*S*)-2-(4-chlorophenyl)-3-methylbutyrate] has replaced the use of fenvalerate in the United States. Both esfenvalerate and fenvalerate contain the same components; the only difference is that esfenvalerate contains 84% of the insecticidally active isomer whereas fenvalerate contains 22%.² Esfenvalerate is highly toxic to fish and bees while less so for mammals.³

Exposure to these pyrethroids can be dangerous, especially in children. Data based on animal studies indicates that alterations of sexual development may result from exposure to pesticides.^{4,5,6} These pesticides are able to disrupt the endocrine system by mimicking or blocking natural hormones or by interfering with the mechanisms that regulate hormone availability to responsive cells.^{4,5,6} Studies have shown that pyrethroid metabolites are capable of interacting with the human estrogen receptor, and so might also present a risk to human and animal health. Findings like these lead to the accession of synthetic pyrethroids, listed by the United States Environmental Protection Agency (U.U. EPA), as possible endocrine disrupting chemicals.⁷ Sexual development during the prenatal and early postnatal periods is sensitive to endocrine disruptor chemicals since sexual development is under hormonal control. Certain subpopulations, such as children living in agricultural communities or children whose parents work with pesticides, may have higher exposure to these pesticides.⁸

Few *in vivo* and *in vitro* studies have been conducted to determine if type II pyrethroids have endocrine effects and the results are conflicting. *In vitro* experiments

demonstrated fenvalerate and deltamethrin induced proliferation in the estrogen-dependent breast cancer cell line, MCF-7.⁹ Similarly, fenvalerate stimulated the estrogen responsive pS2 gene in MCF-7 cells.¹⁰ Causing the mRNA levels to rise. Likewise, fenvalerate exposure caused significant cell proliferation in human endometrial cancer, Ishikawa Var-I, cells which are also estrogen-dependent.¹¹ However, separate studies using estrogen-dependent MCF-7 BUS breast cancer cells and the pesticide fenvalerate, did not find any estrogenic effect on the cells.

Although studies have been conducted on several of the pyrethroid pesticides, a paucity of data exists on the effects of their metabolites. As noted, conflicting results in the studies of pyrethroids have led us to examine a more detailed effect of these pesticides by examining their metabolites (Fig. 1). Only one study was found in which the estrogenicity of pyrethroid metabolites was examined. 3-phenoxybenzoic acid, which is a common metabolite to many type II pyrethroids, was evaluated using a yeast assay and results demonstrated the metabolite behaved as an antiestrogen.¹² However, the metabolites 3-phenoxybenzyl alcohol, 3-(hydroxyl-3phenoxy)benzyl alcohol, and 3-phenoxybenzaldehyde were shown to have estrogenic activity of approximately 10^5 less than that of 17 β -estradiol.¹²

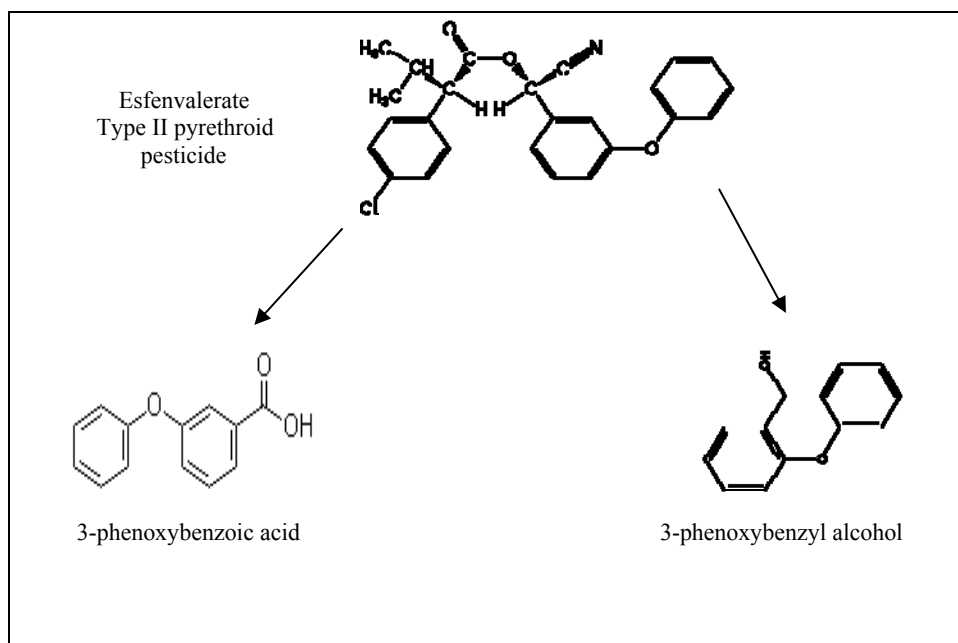


Fig. 1. Esfenvalerate and two of its metabolites. Esfenvalerate is hydrolyzed in the liver to 3-phenoxybenzyl alcohol. 3-Phenoxybenzyl alcohol is further metabolized to 3-phenoxybenzoic acid.

While *in vitro* studies have shown the parent compounds and metabolites to have estrogenic and antiestrogenic activity, recent *in vivo* experiments conducted in our laboratory revealed that immature female Sprague Dawley rats dosed with 1.0 mg/kg esfenvalerate had delayed vaginal opening (VO), decreased serum estradiol and a suppression in the afternoon rise of luteinizing hormone, thus indicating that this pesticide is not acting as an environmental estrogen. 3-Phenoxybenzoic acid and 3-phenoxybenzyl alcohol are metabolites common to many type II pyrethroid pesticides including esfenvalerate. Because of the findings in our laboratory and because few studies evaluating pyrethroid metabolites have been done, we chose to perform both *in vivo* and *in vitro* analysis of 3-phenoxybenzoic acid and 3-phenoxybenzyl alcohol.

CHAPTER II

METHODS AND MATERIALS

In vitro Cell Proliferation Assay

MCF-7 cells purchased from American Type Tissue Culture Bank were maintained in minimum essential medium with phenol red (MEM) supplemented with 5% fetal bovine serum (FBS), 1 % penicillin/streptomycin antibiotic and 1x sodium pyruvate all purchased from Gibco chemical company in Grand Island, N.Y. This cell colony was the source for cells used in our experiments. Cells were maintained in an environment with 5% carbon dioxide and a temperature of 38 °C, fed MEM, and passaged 1-2 times a week. Using an improved Neubauer hemacytometer, cells were counted and seeded onto 96 well plates at 5,000 cells per well and allowed to attach over night. The next day test medium which consisted of Dulbecco's modified eagle medium (DMEM) without phenol red (Gibco), 2.5 % charcoal stripped dextran FBS (Aiken Biologicals in Texarkana, Arkansas) was added. Cells were challenged with 0, 1.0 mM, 0.1 mM, 0.01 mM, and 0.001 mM concentrations of 3-phenoxybenzoic acid and 3-phenoxybenzyl alcohol dissolved in dimethyl sulfoxide (DMSO Sigma chemical company in St. Louis, MO). Cells treated with 1nM Estradiol (Sigma, St, Louis, MO) were used as a positive control. Medium was changed on day 3 for 3-phenoxybenzoic acid and day 5 for 3-phenoxybenzyl alcohol. Proliferation was assessed using the CellTiter 96® Aqueous One Solution (Promega, Madison, WI) according to manufacture protocol. Briefly cells were incubated for 3 hours and absorbance at 490 nm was read on days 1, 3, and 5 for 3-phenoxybenzoic acid and days 2, 5, and 10 for 3-phenoxybenzyl alcohol.

***In vivo* Animal Studies**

Female Sprague Dawley rats raised in our colony at the Texas A&M Department of Comparative Medicine were used. Twenty-two day old female rats were randomly assigned to treatment groups. Animals were weighed and dosed orally once a day either with 0, 1, 5, or 10 mg/kg concentrations of 3-phenoxybenzoic acid or 10 mg/kg 3-phenoxybenzy benzyl acid both dissolved in corn oil. Control animals received an equal volume of corn oil. Sprague Dawley rats in our colony reach puberty at an average of 34-35 days of age; therefore, animals were inspected daily beginning on post natal day (PND) 27 for vaginal opening (VO) which indicates the onset of puberty. After VO occurred, vaginal cytology was examined to determine that the rats were cycling. All procedures were approved by the University Animal Care and Use Committee, and are in accordance with the National Institutes of Health Guidelines for Care and Use of Laboratory Animals.

Statistical Analysis

Cellular proliferation was analyzed by Kruskal-Wallis nonparametric ANOVA followed by Dunn's multiple comparisons testing. Vaginal opening data was analyzed by Paired Student's t-test or ANOVA followed by Student-Newman-Keuls multiple comparisons testing. Values with $P < 0.05$ were considered significant.

CHAPTER III

RESULTS

***In vitro* Results for 3-phenoxybenzoic Acid**

Results for 3-phenoxybenzoic acid were statistically significant only for .01 mM and .001 mM concentrations when compared to control cells. Figure 2 shows the proliferation of cells challenged metabolite 3-phenoxybenzoic acid at concentrations of .01mM and .001mM from incubation day 1 to day 5. Graph shows 1nM concentration of E₂ had greater proliferation than control and very similar proliferation to .01mM concentration of metabolite indicating 1nM E₂ induces MCF-7 cell proliferation. Cells treated with .001mM (0.55 ± 0.01) and .01mM (0.623 ± 0.09) metabolite showed an increase in proliferation on day 5 as compared to controls (0.49 ± 0.01) $P < 0.05$ and $P < 0.01$ respectively. Cells treated with higher concentrations of 3-phenoxybenzoic acid (1 mM and .1 mM) did not induce any cell proliferation (not shown). Media was changed after day 3. No statistical significance was seen on cells treated with 1nM estradiol. Cells were seeded at 5,000 cells per well in 96 well plates.

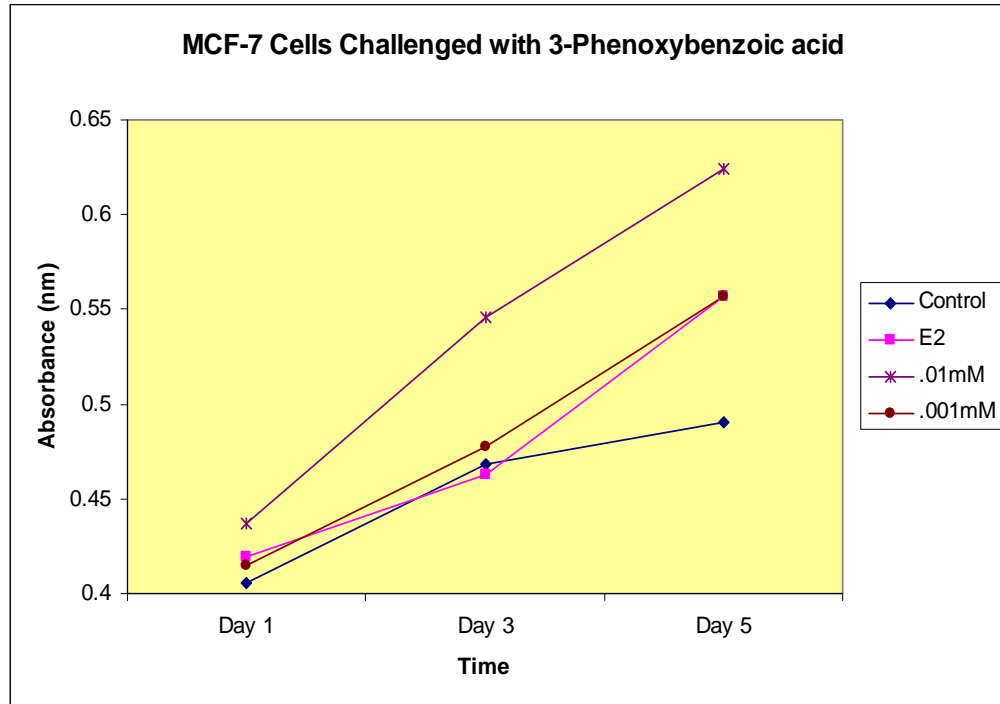


Fig 2. Effect of 3-Phenoxybenzoic Acid on MCF-7 cell proliferation. Estrogen dependent MCF-7 breast cancer cells seeded at 5,000 cells per well on 96 well plates were incubated in two concentrations of 3-phenoxybenzoic acid. Control cells were treated with media only or media plus 1nM E₂. Cell proliferation was assessed by measuring absorbance (490 nm) using the Cell Titer® Aqueous assay. Cells treated with E₂ showed increased proliferation over controls on day 5. (P<0.05). Cells treated with .001mM (0.55 ± 0.01) and .01mM (0.623 ± .09) metabolite showed an increase in proliferation on day 5 as compared to controls (0.49 ± 0.01) P<0.05 and P<0.01 respectively. However, there was no statistical difference between metabolite treated and E₂ treated cells on day 5. All values are the mean ± SEM of 12-16 wells.

***In vitro* Results for 3-phenoxybenzyl Alcohol**

Figure 3 shows cell proliferation of MCF-7 cells challenged with 3-phenoxybenzyl alcohol at concentrations of .001mM and .01mM which show increased proliferation after day 5. 3-Phenoxybenzyl alcohol concentration of .01mM ($1.91 \pm .96$) has a $P < .05$ value when compared to control on day 10. No significance was seen when comparing E_2 with .01mM concentration of 3-phenoxybenzyl alcohol. However, when comparing metabolite concentration of .001mM ($2.79 \pm .6$) with a $P < .05$ value versus control and 1nM estradiol indicated a much higher proliferation than 3-phenoxybenzoic acid metabolite concentrations as seen by comparing figure 2 and figure 3. This is different from the cells treated with 3-phenoxybenzoic acid (previous page) where the cells proliferated until day five then plateaued by day seven (not shown).

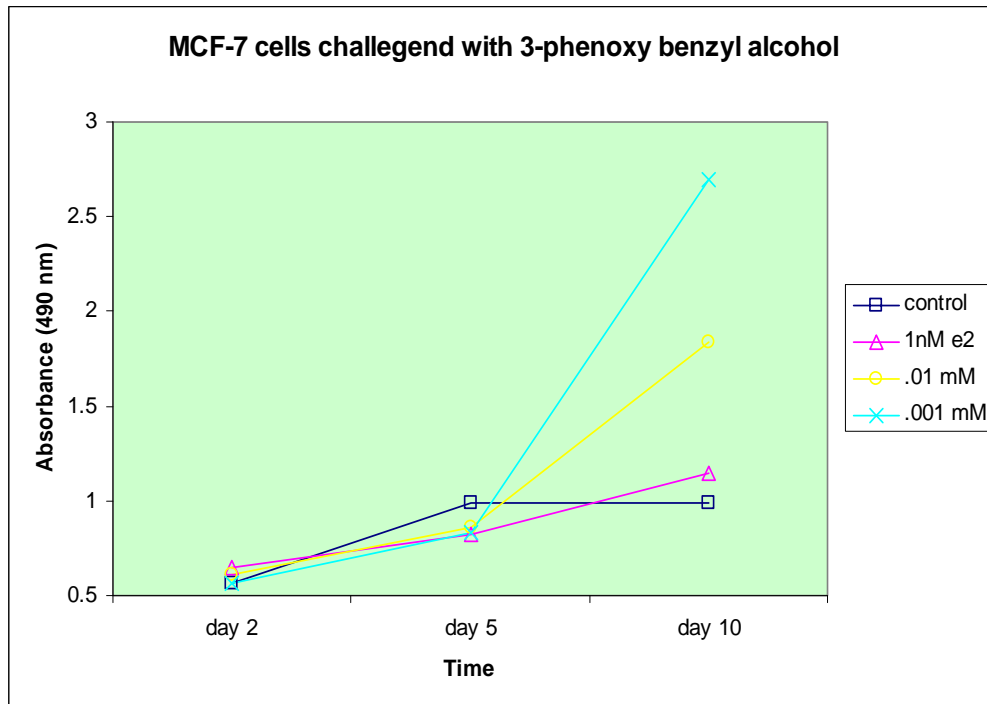


Fig. 3 Effect of 3-phenoxy benzyl alcohol on MCF-7 cell proliferation. Estrogen dependent breast cancer cells seeded at 5,000 cells per well on 96 well plates were incubated in two concentrations of 3-phenoxybenzylalcohol. Control cells were treated with media only or media plus 1nM E₂. Cell proliferation was assessed by measuring absorbance (490 nm) using the Cell Titer® Aqueous assay. Cells treated with E₂ showed increased proliferation over controls on day 5 ($P < 0.05$). Cells treated with .001mM ($2.79 \pm .6$) and .01mM ($1.91 \pm .96$) concentrations had an increase proliferation on day 10 compared to control.

In vivo

Sprague Dawley Rats dosed with 5mg/kg esfenvalerate showed a delay the onset of puberty (Fig. 4). Rats while dosed with 3-phenoxybenzoic acid at concentrations of 10 mg/kg, 5 mg/kg, and 1 mg/kg had not change in VO.

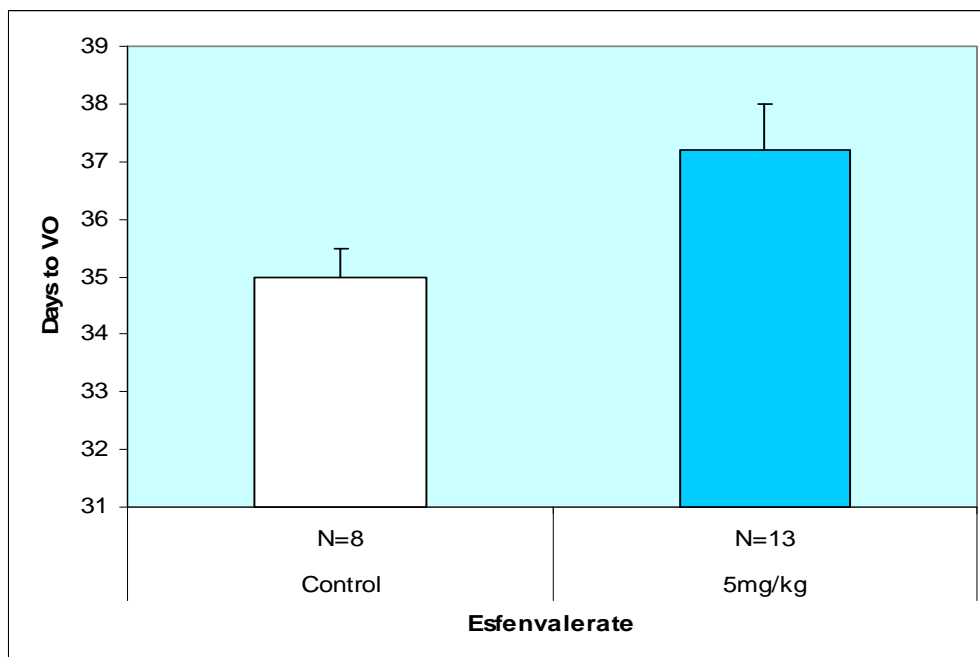


Fig. 4. The effect of esfenvalerate on the onset of puberty in female Sprague Dawley Rats. All animals were dosed beginning on post natal day until the onset of puberty as denoted by vaginal opening (VO). Esfenvalerate at 5mg/kg delayed the onset of puberty by approximately two days ($P<0.05$).

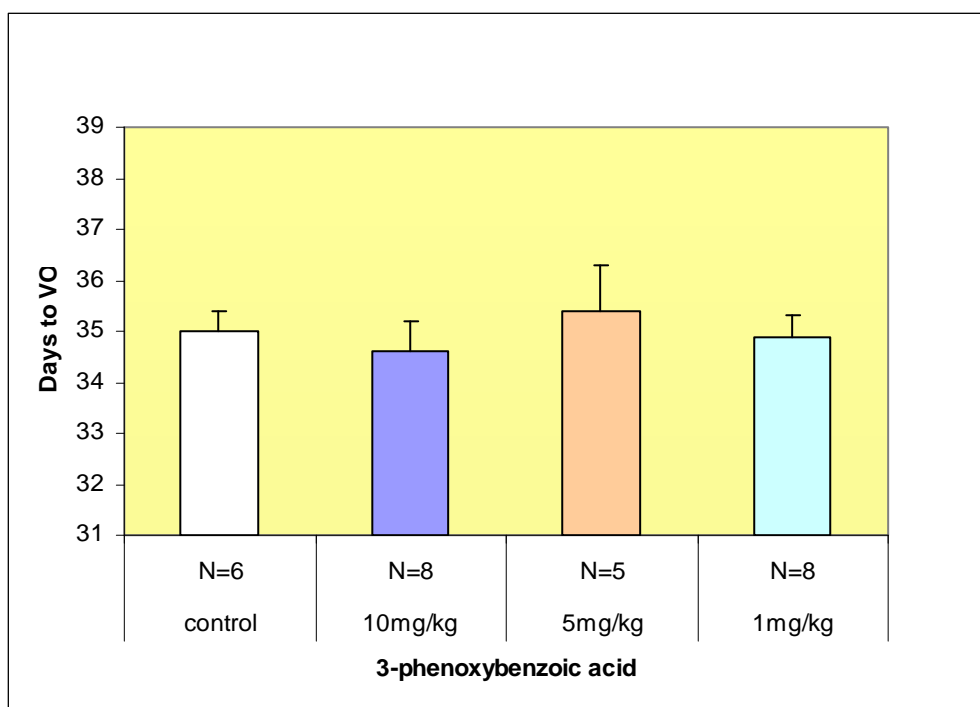


Fig. 5. The effect of 3-phenoxybenzoic acid on the onset of puberty in female Sprague Dawley Rats. All animals were dosed beginning on post natal day 22 until the onset of puberty as denoted by vaginal opening (VO). Metabolite had no effect on the onset of puberty as seen by graph.

In figure 6 animals were dosed with 10mg/kg of 3-phenoxybenzyl alcohol. No difference was seen between control and treated.

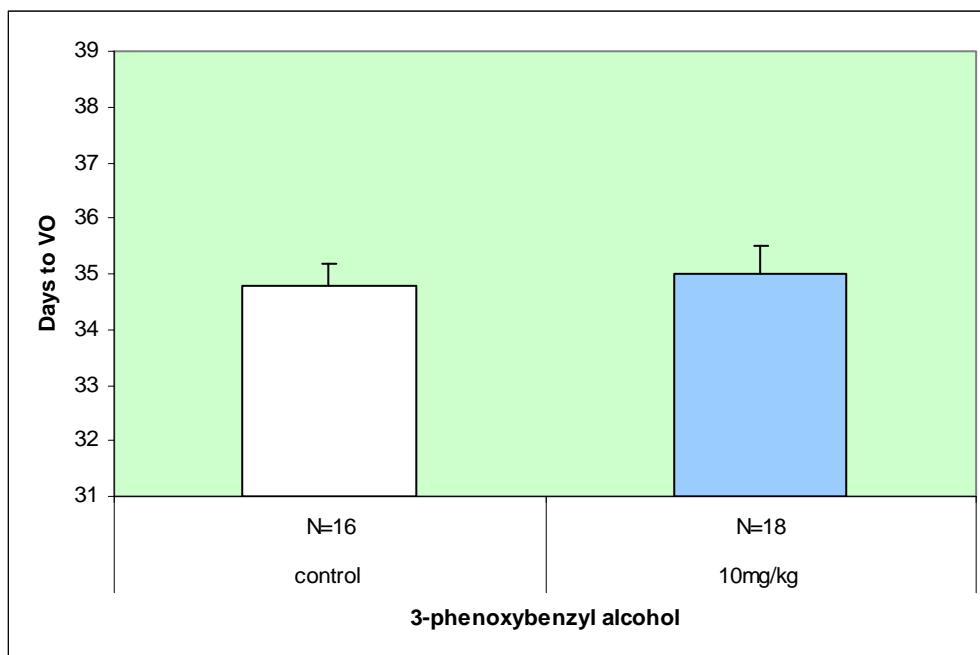


Fig. 6. The effect of 3-phenoxybenzyl alcohol on the onset of puberty in female Sprague Dawley Rats. All rats were dosed beginning on post natal day 22 until the onset of puberty denoted by vaginal opening. Animals treated with 10mg/kg concentration of 3-phenoxybenzyl alcohol had no difference in the onset of puberty as compared to controls.

CHAPTER IV

CONCLUSION

Cells treated with both 3-phenoxybenzoic acid and 3-phenoxybenzyl alcohol at concentrations of .01 mM and .001 mM showed an increase in proliferation over controls by day 5 and day 10. The metabolite treated cells showed a trend of increased proliferation over estradiol treated cells.

Previous studies conducted in our laboratory demonstrated that immature female Sprague Dawley rats dosed with 5.0 mg/kg esfenvalerate had delayed vaginal opening (VO) thus indicating that this pesticide is not acting as an environmental estrogen. On the contrary, immature female rats treated with the metabolites did not show any difference in the onset of puberty. These data would suggest that although the metabolites are able to increase cell proliferation in an estrogen dependent cell line, they are doing so through an estrogen receptor (ER) independent mechanism. If the metabolites were acting through the ER to stimulate cell growth, then the expected results from the *in vivo* experiments would be an advancement in the onset of puberty after exposure to the metabolites. This did not happen. In fact animals treated with a type II pyrethroid pesticide showed a delay in the onset of puberty. Further experiments into the mechanism of this delay are ongoing.

We do not know what the chronic effects of endocrine disruptors are, but what we do know is these synthetic compounds are everywhere in our environments and may be causing endocrine problems.

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CONTACT INFORMATION

Name: Marco Antonio Chavez

Professional Address: c/o Dr. Michelle Pine
Department of Veterinary Integrative
Biosciences
Texas A&M University
College Station, TX 77843-3133

E-mail Address: marco_antonio1986@tamu.edu

Education: B.S. Biomedical Science
Texas A&M University, May 2008
Undergraduate Research Scholar