

ABSTRACT

BLYSTONE, CHAD ROBERT. Conazole Pesticide Disruption of Testicular Steroidogenesis during Different Stages of Male Development. (Under the direction of L. Earl Gray, Jr. and Gerald A. LeBlanc.)

This work investigated conazole pesticide effects on rat gonadal steroidogenesis and related the observed changes in testosterone production to adverse effects on pubertal and fetal reproductive development. In the initial experiments, prochloraz (PCZ) exposure during male rat pubertal development was investigated to test the hypotheses that PCZ would inhibit testosterone production within the pubertal testis and delay pubertal development.

Furthermore, PCZ antagonism of the androgen receptor (AR) *in vivo* was quantified to assess whether this mechanism contributed to delayed pubertal development. These studies showed that PCZ was a strong inhibitor of steroidogenesis relative to its AR antagonism and unexpectedly the large decrease in testosterone did not necessarily delay pubertal development.

Next, the sensitivity of fetal testosterone production to maternal PCZ exposure was investigated to support the hypothesis that PCZ inhibition of testosterone synthesis in the fetal testis contributes to the reported adverse effects in androgen-dependent tissues of male offspring. PCZ inhibited fetal testosterone production at doses that correspond to those which cause adverse reproductive malformations in the adult. The hormone data suggested that PCZ inhibits the conversion of progesterone to testosterone through the inhibition of CYP17. To test this hypothesis, the effects of PCZ on CYP17 gene expression and CYP17 hydroxylase activity were evaluated. PCZ did not affect gene expression, but did significantly inhibit CYP17 hydroxylase activity. Taken together, these observations support

the hypotheses that PCZ inhibits CYP17 enzymatic activity resulting in reduced testosterone synthesis with adverse consequences to male development.

In the last chapter, two triazole conazoles, myclobutanil and triadimefon, were hypothesized to be steroidogenesis inhibitors similar to PCZ. Both compounds weakly inhibited steroidogenesis *in vitro*, and when myclobutanil was tested to inhibit fetal steroidogenesis *ex vivo*, there were no effects. Myclobutanil, triadimefon, and a third triazole, propiconazole, did increase serum testosterone in the adult rat after developmental exposure. Focusing on triadimefon, the elevated serum testosterone was hypothesized to be from increased testicular testosterone production. Triadimefon exposure to the adult rat increased intra-testicular testosterone levels suggesting the testis contributed to the increased serum testosterone.

In summary, PCZ was unique among the conazoles examined in its ability to reduce testicular testosterone production and alter male reproductive development.

**Conazole Pesticide Disruption of Testicular Steroidogenesis during
Different Stages of Male Development**

by

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*This work is dedicated to my fiancée, family, friends, and a short-eared cat for their support
and encouragement.*

BIOGRAPHY

Chad Robert Blystone was born in St. Petersburg, Florida on October 5th, 1974. He was the oldest of four in a family that moved about and enjoyed the outdoors. In 1996 he graduated with a Bachelor's of Science in Zoology from Auburn University. During his time at Auburn, he enjoyed studying herpetology under Dr. Craig Guyer and foraging into the swamps to find whatever crossed his path. Chad also enjoyed learning about toxicants in the environment while working in Dr. Mary Mendonça's lab. Deciding to pursue herpetology, he left his college job of tending hybridoma cells and went to Miami University in Oxford, OH for a Master's degree. While there he studied the hydration physiology of the red-backed salamander for two years under the direction of Dr. Dennis Claussen. After visiting Boston for a meeting, he thought he would enjoy the city and moved there after completing his degree. Chad found a good job managing Dr. Elazer Edelman's large lab and tending cells (again) at the Biomedical Engineering Center at M.I.T. Several years past in which he learned much, but in the end he understood that a Ph. D. was needed for him to have a successful career in science. Going back to his interest in toxicants and his desire for a more applied degree, he started the Ph. D. program at N.C. State's Department of Environmental and Molecular Toxicology in August, 2001. Several years into the program he met Erica Wyman and they became engaged in December 2005. His time in the graduate program continued and after overcoming the difficulties in his research, the following work was completed for his dissertation.

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TABLE OF CONTENTS

| | Page |
|---|-------------|
| LIST OF TABLES..... | vii |
| LIST OF FIGURES..... | ix |
| INTRODUCTION..... | 1 |
| References..... | 12 |
| CHAPTER I: Prochloraz inhibits testosterone production at dosage below those that affect androgen-dependent organ weights or the onset of puberty in the male Sprague Dawley rat. | |
| Abstract..... | 20 |
| Introduction..... | 21 |
| Materials and Methods..... | 22 |
| Results..... | 26 |
| Discussion..... | 29 |
| Acknowledgement..... | 33 |
| References..... | 34 |
| CHAPTER II: Sensitivity of fetal rat testicular steroidogenesis to maternal prochloraz exposure and the underlying mechanism of inhibition. | |
| Abstract..... | 48 |
| Introduction..... | 49 |
| Materials and Methods..... | 50 |
| Results..... | 56 |
| Discussion..... | 58 |
| Acknowledgement..... | 61 |

| | |
|---|-----|
| References..... | 62 |
| CHAPTER III: The <i>in vitro</i> and <i>in vivo</i> effects of triazole pesticides on male rat gonadal steroidogenesis and development. | |
| Abstract..... | 75 |
| Introduction..... | 77 |
| Materials and Methods..... | 79 |
| Results..... | 85 |
| Discussion..... | 91 |
| Acknowledgement..... | 95 |
| References..... | 113 |
| CONCLUSION..... | 118 |
| References..... | 121 |

LIST OF TABLES

| | Page |
|---|------|
| CHAPTER I | |
| Table 1. Mean age and weight at the initiation and completion of PPS during the first pubertal PCZ exposure..... | 38 |
| Table 2. Mean reproductive organ weights at different time points from the first pubertal PCZ exposure and in the second pubertal exposure | 39 |
| Table 3. Mean body and non-reproductive organ weights at different time points from the first pubertal PCZ exposure and second pubertal exposure..... | 40 |
| Table 4. Mean body and organ weights from the Hershberger assay (castrated male + testosterone propionate) after 10-11 day exposure to PCZ..... | 41 |
| Table 5. The LOEL (mg/kg/day) for various endpoints in each experiment..... | 42 |
| CHAPTER II | |
| Table 1. Primer set sequences for quantitative real-time RT-PCR..... | 66 |
| Table 2. Summary of PCZ effects on mean (\pm SEM) dam weight and post-implantation loss..... | 67 |
| Table 3. Summary of PCZ effects on testosterone production in relation to reported morphological changes..... | 68 |
| CHAPTER III | |
| Table 1: Summary of myclobutanil effects on maternal and litter endpoints..... | 96 |
| Table 2: Summary of treatment effects on F ₁ body weight at different ages within the multi-triazole study..... | 97 |

| | |
|---|-----|
| Table 3: Summary of treatment effects on F ₁ male liver weight at different ages within the multi-triazole study..... | 98 |
| Table 4: Summary of treatment effects on F ₁ testes weight at different ages within the multi-triazole study..... | 99 |
| Table 5: Mortality and parturition data after treatment to Triadimefon 1800ppm and litter average of F ₁ male weight and AGD..... | 100 |
| Table 6: Fertility indices of F ₁ males after two different exposures to triadimefon 1800ppm..... | 101 |
| Table 7: Litter averages at PND 90 after two different exposures to triadimefon 1800ppm..... | 102 |
| Table 8: Average weight and hormone measurements from control and animals treated via feed to triadimefon 1800ppm..... | 103 |

LIST OF FIGURES

| | Page |
|---|------|
| INTRODUCTION | |
| Figure 1. The pathway of steroidogenesis starting with pregnenolone..... | 8 |
| Figure 1. The enzyme activity of CYP17..... | 9 |
| Figure 3. Common chemical structure of an imidazole and triazole fungicide..... | 10 |
| Figure 4. Chemical structures of prochloraz, an imidazole, and the triazoles: triadimefon, myclobutanil, and propiconazole..... | 11 |
| CHAPTER I | |
| Figure 1. Mean serum hormones from mid-puberty and post-puberty of first peripubertal exposure and mid-puberty of second peripubertal exposure..... | 43 |
| Figure 2. Mean <i>ex vivo</i> hormone levels at mid-puberty and post-puberty from the first peripubertal exposure..... | 44 |
| Figure 3. Mean <i>ex vivo</i> hormone levels at mid-puberty from the second peripubertal exposure..... | 45 |
| Figure 4. The relationship of mean serum testosterone levels and <i>ex vivo</i> testosterone levels with hCG stimulation and without hCG stimulation from the second peripubertal exposure..... | 46 |
| CHAPTER II | |
| Figure 1. Mean serum estradiol and progesterone levels at GD18 in the dam after PCZ treatment..... | 69 |
| Figure 2. Litter mean <i>ex vivo</i> levels of fetal progesterone, 17 α -hydroxyprogesterone, androstenedione, and testosterone at GD 18 after a 3 hr incubation..... | 70 |

| | |
|---|----|
| Figure 3. Litter mean mRNA levels of StAR, CYP 11A, and CYP17 in the fetal testes..... | 71 |
| Figure 4. The mean hydroxylase activity of CYP17 at various progesterone and PCZ concentrations. Lineweaver-Burke plot of the same plot..... | 72 |
| Figure 5. The mean PCZ concentration in maternal serum and amniotic fluid. The PCZ concentration within the amniotic fluid and corresponding <i>ex vivo</i> testosterone production from the fetal testes of the same litter..... | 73 |

CHAPTER III

| | |
|---|-----|
| Figure 1: <i>In Vitro</i> testosterone production by the adult and neonatal testis after myclobutanil and triadimefon exposure..... | 104 |
| Figure 2: <i>In Vitro</i> androstenedione production by the adult and neonatal testis after myclobutanil and triadimefon exposure..... | 105 |
| Figure 3: <i>In Vitro</i> 17 α -hydroxyprogesterone production by the adult and neonatal testis after myclobutanil and triadimefon exposure..... | 106 |
| Figure 4: <i>In Vitro</i> progesterone production by the adult and neonatal testis after myclobutanil and triadimefon exposure..... | 107 |
| Figure 5: Average serum testosterone levels at PND 50 from the multi-triazole experiment..... | 108 |
| Figure 6: Average serum testosterone levels at PND 92 and PND 99 from the multi-triazole experiment..... | 109 |
| Figure 7: Average calculated maternal and F ₁ male dose and the effects on maternal and F ₁ male body weight..... | 110 |

Figure 8: Serum testosterone levels at PND 53 and PND 90, testicular testosterone at PND 90, serum estradiol at PND 90, and serum LH at PND 90 after GD 6 – PND 90 or PND 23 – 53 triadimefon 1800ppm exposure.....111

Figure 9: Serum testosterone levels at PND 74 and PND 90, testicular testosterone at PND 90, serum estradiol at PND 90, and serum LH at PND 90 after PND 60 – 90 exposure to triadimefon 1800ppm.....112

INTRODUCTION

Endocrine glands secrete hormones that act on target tissues in order to stimulate growth, differentiation and maturation, maintain homeostasis, or promote other physiological processes (Kacsoh 2000). Exposure to exogenous chemicals that disrupt the function of the endocrine system might be affecting wildlife and human populations (Colborn *et al.* 1993; Guillette and Gunderson 2001). An unsettling outcome of this disruption is that these chemicals might alter hormone levels or receptor function leading to dramatic changes during organizational periods of sexual development (Colborn *et al.* 1993; Sharpe 2006). The Environmental Protection Agency (EPA) was charged with screening chemicals for endocrine disruption under the Food Quality and Protection Act of 1996. In 1998, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) presented recommendations for screening and testing of chemicals, and the EPA's Endocrine Disruptor Screening Program (EDSP) is currently validating tests for use (F.R. 1998). Using some of these proposed assays, this research investigates conazole pesticide disruption of testosterone production during different stages of male development in order to quantify the sensitivity of testosterone production to exposure and elucidate the sensitivity of development to reduced testosterone and AR antagonism.

Androgens and Male Development

Androgen hormones (e.g. testosterone) play an important role in sexual differentiation during gestation and sexual maturation during puberty. Sexual differentiation is initiated by the Sex Determining Region of the Y chromosome (SRY) gene which leads to the development of the testis (Hiort and Holterhus 2000; Koopman *et al.* 2001; Brennan and Capel 2004). Within the fetal testis, Leydig cells produce testosterone, which furthers male differentiation. Testosterone is necessary for the development of the phallus and sustains the Wolffian ducts, which develop

into the epididymides, vas deferens, seminal vesicle, and ejaculatory duct (Barsoum and Yao 2006). Sexual maturation during pubertal developmental also requires testosterone to stimulate growth of reproductive organs, sperm production, appearance of secondary sex characteristics, and influence behavioral development (Stoker *et al.* 2000; Dohle *et al.* 2003; Schulz and Sisk 2006).

The production of testosterone starts with transport of cholesterol by StAR protein into the mitochondria. Within the mitochondria, cholesterol is cleaved by CYP11A to form the 21-carbon steroid, pregnenolone. This steroid is further converted to testosterone by dehydrogenases and cytochrome P450's (CYPs) that reside outside the mitochondria (Payne and Hales 2004). The pathway of steroid metabolism is species specific; rat CYP17 lyase prefers 17 α -hydroxyprogesterone as a substrate, while human CYP17 prefers 17 α -hydroxypregnenolone (Flück *et al.* 2003). Both pathways ultimately lead to testosterone production (figure 1). Further conversion of testosterone to dihydrotestosterone (DHT) by 5 α -reductase occurs in peripheral tissues, which leads to greatly enhanced binding to the androgen receptor (Payne and Hales 2004). Although there are a multitude of enzymes involved in steroidogenesis, this research will ultimately focus on the activity of CYP17. CYP17 hydroxylase converts progesterone (or pregnenolone) to a 17 α -hydroxy form, and then CYP17 lyase activity cleaves these 21 carbon progestins into the 19 carbon androstenedione (or dehydroepiandrosterone) (figures 1,2) (Miller 2002; Payne and Hales 2004).

Anti-Androgen Chemicals

There are several mechanisms by which environmental chemicals can act on androgen function (Guillette and Gunderson 2001) and notable ones include antagonism of the androgen receptor

and inhibition of testosterone production. The dicarboximide fungicides vinclozolin and procymidone antagonize the androgen receptor and exposure results in disrupted differentiation and maturation (Kelce *et al.* 1997; Gray *et al.* 1999; Monosson *et al.* 1999; Ostby *et al.* 1999; Mylchreest *et al.* 2000; Mylchreest *et al.* 2002; Kang *et al.* 2004). Maternal exposure to certain phthalates decreases testosterone production in the fetal rat testis and also results in male reproductive abnormalities, which include reduce reproductive organ weights, reduced anogenital distance (AGD), retention of nipples/areolae, cryptorchidism (non-descent of the testes), and malformations of the reproductive tract such as hypospadias (the urinary tract opening is not located at the tip of the penis.) (Gray *et al.* 2000; Parks *et al.* 2000; Mylchreest *et al.* 2002; Barlow *et al.* 2003; Lehmann *et al.* 2004; Foster 2006). The occurrence of these effects within the male rat might be predictive of effects in children. Testicular Dysgenesis Syndrome (TDS) composed of cryptorchidism, hypospadias, impaired spermatogenesis, and testis cancer is hypothesized to be the result of altered prenatal testicular development, which might result from maternal exposure to anti-androgens (Skakkebaek *et al.* 2001; Toppari *et al.* 2001; Bay *et al.* 2006; Skakkebaek *et al.* 2006).

Conazole Fungicides

The research presented here focuses on the anti-androgen activity of the conazole fungicides. These chemicals contain a 1,2,4 triazole or 1,3 diazole ring (figure 3) and are divided into triazole and imidazole subclasses. Used in agricultural and pharmaceutical applications, conazoles inhibit fungal CYP51 activity. This leads to a decrease in ergosterols and a build up of 14 α -methylated sterols, which together interfere with the structure and function of the fungal cell membrane (White *et al.* 1998; Sheehan *et al.* 1999; Maertens 2004). Conazoles also inhibit and induce the activity of other CYPs (Ronis *et al.* 1994; Zhang *et al.* 2002; Coulson *et al.* 2003).

The non-discriminate inhibition of other CYP's has raised concern that unintentional exposure to agricultural conazoles disrupts reproductive function (Zarn *et al.* 2003). Supporting this concern are reports that several agricultural conazoles inhibit aromatase activity *in vitro* (Vinggaard *et al.* 2000; Andersen *et al.* 2002a; Trosken *et al.* 2004).

To date, few agricultural conazoles or other pesticides have been shown to inhibit testosterone production or affect male development. Flusilazole and hexaconazole (triazoles) increase the incidence of Leydig cell tumors, purportedly through this inhibiting testosterone production (FAO/WHO 1990, 1995; Cook *et al.* 1999a). There are a few studies that have examined the effect of the imidazole prochloraz (PCZ) (figure 4A) on sexual differentiation. Maternal exposure to PCZ results in reproductive malformations in male offspring and this chemical reportedly acts through two anti-androgenic mechanisms: androgen receptor antagonist and inhibitor of steroidogenesis (Vinggaard *et al.* 2002a; Wilson *et al.* 2004; Vinggaard *et al.* 2005a; Laier *et al.* 2006). The contribution of these two mechanisms to the malformations in male offspring is not known. Additionally the effects of PCZ and many other conazole pesticides on pubertal development are also not known. The research presented here will address these unknowns and other questions regarding conazole disruption of androgen function and male development.

Research Outline

In chapter one, PCZ exposure was hypothesized to delay rat pubertal development and inhibit gonadal steroidogenesis. These hypotheses were tested using a modified version of the rat pubertal protocol, which was recommended as an alternative test for the EDSP. A mid-puberty time point and serum hormone and *ex vivo* testosterone production measurements were added to

the protocol for a better assessment of prochloraz's effects on puberty. We hope to demonstrate with this modified protocol that the hormonal effects of a steroid synthesis inhibitor can be detected, and that the sensitivity of the protocol was enhanced by including hormone endpoints. In addition to determining if prochloraz exposure could delay pubertal development, the relative contribution of each anti-androgen mechanism to pubertal delay was assessed. First, steroidogenesis was quantified by measuring serum hormones and *ex vivo* testis testosterone production to determine the prochloraz dosage that would reduce testosterone production. Second, PCZ was hypothesized to antagonize the AR *in vivo* and this was tested using the Hershberger assay, another EDSP protocol. Together, the experiments were designed to elucidate PCZ effects on puberty and to examine the mechanism behind the effects by comparing the results of the Hershberger assay to the steroidogenesis results within the intact male.

In chapter two, the sensitivity of fetal gonadal steroidogenesis to maternal PCZ exposure was investigated to test the hypothesis that reduced fetal testosterone production contributes to the reported PCZ induced malformations of androgen-dependant tissues in male offspring.

Testosterone production was assessed by incubating fetal testes *ex vivo* after maternal prochloraz exposure, similar to the pubertal *ex vivo* experiments. In addition, the underlying mechanism of reduced testosterone production, as reported previously, was investigated. Hormone data suggested that CYP17 is inhibited by PCZ, but the mechanism of this inhibition has not been identified. We hypothesized that PCZ inhibition of CYP17 was not at the gene expression level, but due to altered enzymatic activity as found with other imidazoles (Ayub and Levell 1987a). To test this hypothesis, CYP17 gene expression within the fetal testis was measured by qRT-PCR after maternal PCZ exposure. Furthermore, CYP17 enzymatic activity in the presence of PCZ was evaluated *in vitro* using isolated testicular microsomes. In order to compare fetal

exposure to effects on fetal testosterone production, amniotic fluid concentrations of PCZ were measured to determine if they negatively associated with testosterone production. Measurement of amniotic PCZ levels also enabled us to evaluate the relative contribution of each anti-androgen mechanism by comparing calculated K_i values.

The research presented in the final chapter examined the effects of triazoles, the other subclass of conazoles, on steroidogenesis and male development to determine if they acted similarly to PCZ. It was hypothesized that myclobutanil and triadimefon (figure 4B, 4C) inhibit steroidogenesis *in vitro*. Additionally, it was hypothesized that maternal exposure to myclobutanil would decrease fetal testosterone production to determine if the chemical could disrupt steroidogenesis *in vivo*. Based upon reports that these chemicals increase serum testosterone (FAO/WHO 1985; Tully *et al.* 2006) in adult animals, myclobutanil, triadimefon, and a third triazole, propiconazole (figure 4D) were predicted to disrupt testosterone homeostasis after an exposure that started during gestation and ended in adulthood. The case may be that the “wiring” of the hypo-pituitary-gonadal axis during development was altered, resulting in elevated testosterone. To test this hypothesis, rats were exposed to a high dose of triadimefon during different life stages and serum testosterone was measured. Furthermore, the source of the increased circulating levels of testosterone was investigated. Testosterone levels within the testis were measured after each exposure to test the hypothesis that increased testosterone production within the testis contributed to the elevated serum levels.

These experiments will be the first to demonstrate the effects of a steroid synthesis inhibitor pesticide on pubertal development. They will also expand understanding of PCZ’s fetal anti-androgenic effects and the mechanism behind the inhibition of testosterone production.

Furthermore, these studies will demonstrate whether or not the conazoles tested act similarly *in vitro* and *in vivo*. Together the work in this dissertation will aid in understanding and predicting the effects of conazoles and steroid synthesis inhibitors on male reproductive development.

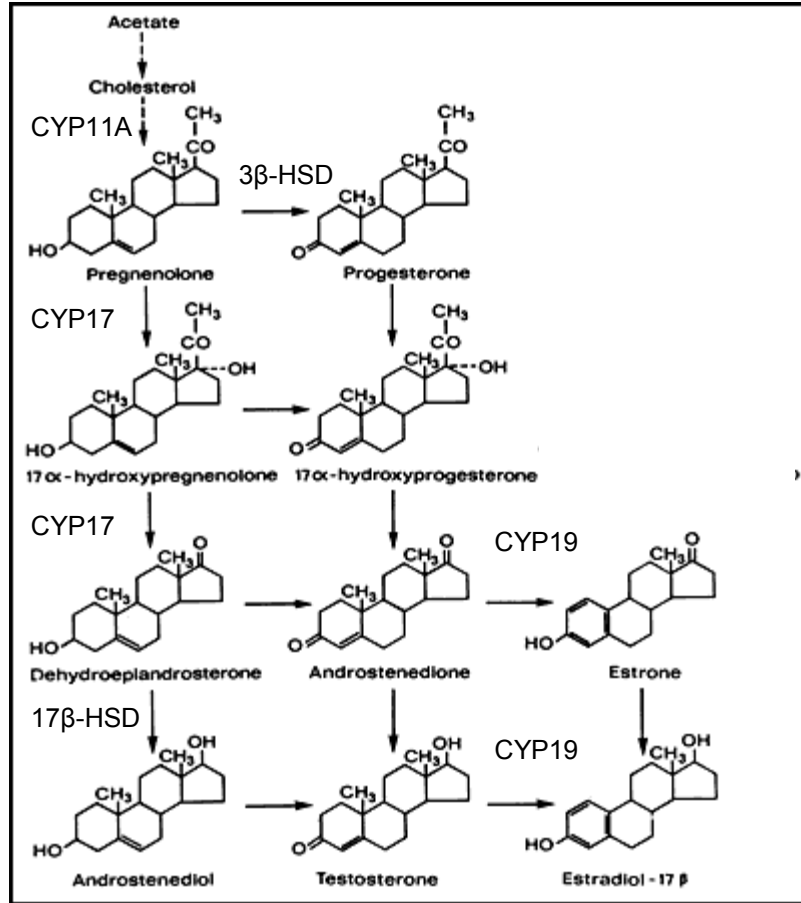


Figure 1. The pathway of gonadal steroidogenesis starting with pregnenolone (modified from (Kretser 2002). 3β- or 17β-HSD refers to the hydroxysteroid dehydrogenase enzymes.

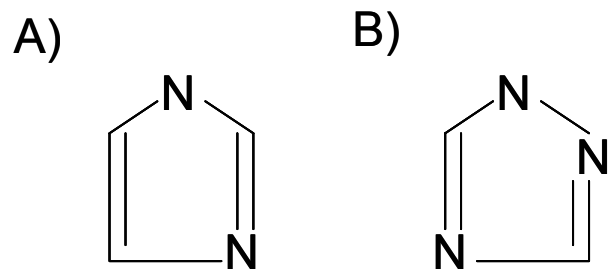
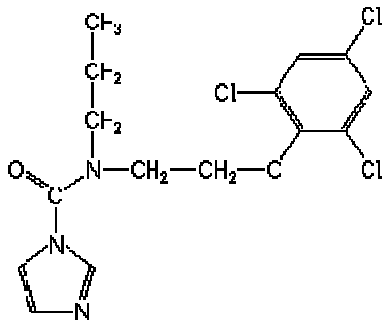
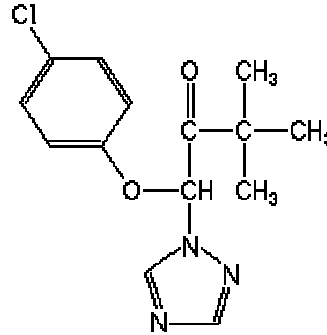


Figure 3. Common chemical structure of an imidazole (A) and triazole (B) fungicides.

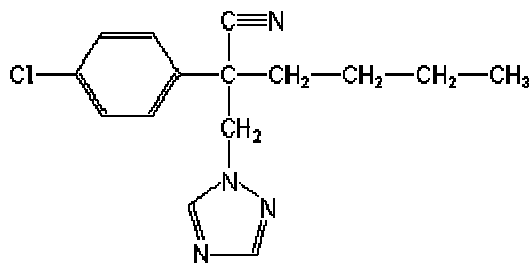
A) Prochloraz



B) Triadimefon



C) Myclobutanil



D) Propiconazole

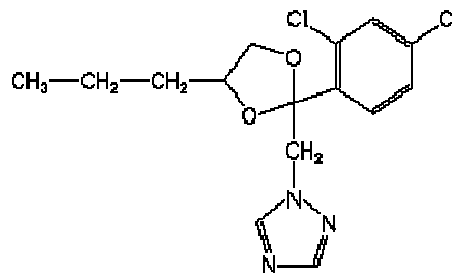


Figure 4. Chemical structures (Wood 2006) of prochloraz (A), an imidazole, and the triazoles: triadimefon (B), myclobutanil (C), and propiconazole (D).

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CHAPTER I

Prochloraz inhibits testosterone production at dosage below those that affect androgen-dependent organ weights or the onset of puberty in the male Sprague Dawley rat

This chapter is the format as submitted to the journal Toxicological Sciences.

ABSTRACT

Prochloraz (PCZ) is an imidazole fungicide that inhibits gonadal steroidogenesis and antagonizes the androgen receptor (AR). We hypothesized that pubertal exposure to PCZ would delay male rat reproductive development. Sprague Dawley rats were dosed by gavage with 0, 31.3, 62.5, 125 mg/kg/day of PCZ from postnatal day (PND) 23 to 42 or 51. There was a significant delay in preputial separation (PPS) by 125mg/kg/day PCZ and several of the androgen-dependent organ weights were decreased significantly, but the effects varied depending on the age at necropsy (42 vs 51). At both ages serum levels and *ex vivo* testosterone release from the testis were significantly decreased whereas serum progesterone and 17 α -hydroxyprogesterone levels were significantly increased at dose levels below those that affected PPS or reproductive organ weights. The hormone results suggested that PCZ was inhibiting CYP17 activity. In a second pubertal study that included doses down to 7.8 mg/kg/day, serum and *ex vivo* testosterone production were significantly reduced by 15.6 mg/kg/day PCZ. In order to examine the anti-androgenic effects of PCZ, independent of its effects on testosterone synthesis, castrated-immature male rats were dosed with androgen and 0, 15.6, 31.3, 62.5, 125 mg/kg/day for 10-11 days (Hershberger assay). In this assay, androgen-sensitive organ weights were only significantly decreased by 125 mg/kg/day PCZ suggesting that AR antagonism was weaker relative to the inhibition of steroidogenesis. These data from the pubertal assays demonstrate that PCZ decreases testosterone levels and delays rat pubertal development, as hypothesized. However, the fact that hormone levels were affected at dosage eight fold below that delayed the onset of puberty suggests that rather large reductions in serum testosterone may be required to delay puberty and consistently reduce androgen-dependent tissue weights.

INTRODUCTION

Evidence from wildlife studies and laboratory animal studies has raised concern that environmental chemicals might alter reproductive development in the human population (Colborn *et al.* 1993). Environmental chemicals that reduce androgen function have been shown to alter male development in laboratory rats (Gray *et al.* 2006; Sharpe 2006). The effects of anti-androgenic chemicals are dependant on the timing of exposure and the periods of gestational and pubertal development are particularly susceptible. Some chemicals can inhibit androgen function through multiple mechanisms and understanding the relevant mechanism of action is important to predict the subsequent consequences of exposure to gestational and pubertal reproductive development in humans and wildlife.

Prochloraz (PCZ) is an imidazole fungicide used in crop protection that can interfere with androgen signaling through at least two mechanisms. First, PCZ has been shown to antagonize the androgen receptor (AR) in transcription activation assays (Andersen *et al.* 2002; Vinggaard *et al.* 2002; Noriega *et al.* 2005) and *in vivo* it reduced the weights of androgen-sensitive tissues in the Hershberger assay (Vinggaard *et al.* 2002; Vinggaard *et al.* 2005b). In addition to being an AR antagonist, PCZ also inhibits steroidogenesis. Gestational exposure to PCZ reduced fetal testosterone levels and increased progesterone levels *ex vivo* (Wilson *et al.* 2004; Vinggaard *et al.* 2005a; Laier *et al.* 2006). Exposure to PCZ during the gestational period of sexual differentiation resulted in reproductive alterations in male offspring such as phallus abnormalities, reduced reproductive organ weights, and increased retention of nipples/areolas (Noriega *et al.* 2005; Vinggaard *et al.* 2005a; Laier *et al.* 2006). AR antagonism and/or reduced androgen levels are likely the primary mechanism of these effects on male reproductive development.

The effects of PCZ exposure during the period of pubertal development are not known. During this period, androgen action is necessary for development of the male reproductive system. Pesticides that alter androgen function have been shown to disrupt or delay this development. AR antagonists, such as vinclozolin and p,p'-DDE, significantly delay preputial separation (PPS) and development of androgen-sensitive organs (Kelce *et al.* 1995; Monosson *et al.* 1999). The purpose of this study was to test the hypothesis that pubertal exposure to PCZ delays pubertal development and to gain insight into the mechanism by which PCZ elicits these effects. Effects of PCZ on pubertal development were evaluated using the male pubertal rat assay that has been proposed as an alternative *in vivo* method for detecting chemicals that alter androgen or thyroid function (EDSTAC 1998). This protocol calls for a peripubertal exposure during which PPS is monitored and effects on androgen sensitive organ weights are recorded. We modified this protocol (Stoker *et al.* 2000) by including measurement of *in vivo* and *ex vivo* testosterone and adding a necropsy at mid-puberty (PND42/43), which is a time of rapid increases in androgen steroids (Monosson *et al.* 1999) and close to the day PPS is completed in the rat. Since PCZ might disrupt pubertal development by dual mechanisms of action, we compared the dose response relationships between the effects of PCZ on steroid hormone levels in the pubertal male with the effects in the Hershberger Assay. This Hershberger assay detects *in vivo* AR antagonism in a castrated-immature androgen treated male rat model (Gray *et al.* 2004; Owens *et al.* 2006) and therefore may allow us to assess the relative contribution between the two potential mechanisms of action.

MATERIALS AND METHODS

Animals and Dosing Solution. Immature and immature-castrated Sprague Dawley rats were delivered from Charles River Laboratories (Raleigh, NC) and housed in the Environmental

Protection Agency's Reproductive Toxicology Division animal facility on PND23 and PND44, respectively. Animals were provided with Purina Rat Chow 5001 and watered *ad libitum*. Environmental conditions were 21-24 °C, 40-55% humidity, and a 12L:12D light cycle (lights on at 0600 h). Animals in the Hershberger experiment were maintained on a 14L:10D cycle (lights on at 2100 h). Prior to dosing, animals were weight ranked and assigned to dose groups to minimize differences in means and variance among treatment groups. The animal use protocol for this study was approved by the National Health and Environmental Effects Research Laboratory's Institutional Animal Care and Use Committee.

PCZ (CAS#: 67747-09-5, 99.5 % purity by HPLC; Riedel-de Haën; Lot# 2226x) was administered by oral gavage in corn oil (CAS# 8001-30-7, Sigma Aldrich, St. Louis, MO) for a final volume of either 2.5ml/kg or 5.0ml/kg body weight. PCZ dissolved in the solution at the doses administered. In the pubertal experiments, rats were given a dose volume of 5.0ml/kg body weight from post-natal day (PND) 23-30 due to the small size of the animal and then a dose volume of 2.5ml/kg for the remainder of the study. In the Hershberger experiment, a 2.5ml/kg volume was used throughout dosing. Animals were given their final dose one to two hours before necropsy in all studies and necropsies were not more than 2.5 hours long.

Pubertal Experiments. Immature Sprague Dawley rats were dosed daily by gavage with 0, 31.3, 62.5, or 125 mg/kg PCZ (n = 8-10/dose) from PND 23-42/43 or PND 23-51/52. This experimental design of two dosing periods allowed for measurement of chemical effects on hormonal and organ weight endpoints at mid- and post-puberty. The progression of PPS was inspected daily from PND 37 to necropsy at PND 42/43 or PND 51/52. The prepuce was gently retracted far enough to note the presence of either a constriction at the base of the glans penis, or

connective tissue that prevented the full retraction of the prepuce. Complete PPS was distinguished from incomplete PPS in which portions of the prepuce remained attached to the glans, typically via a midline thread located on the ventral side of the phallus.

Organ weights were recorded at necropsy and trunk blood was collected by decapitation for serum measurements of testosterone, progesterone, 17α -hydroxprogesterone, androstenedione, estradiol, and luteinizing hormone (LH). In addition to serum measurements, *ex vivo* hormone production by the right testis of each animal was assessed. The right testis was removed, decapsulated, weighed, and then sectioned into 50-100mg pieces. From each rat, two of the 50-100mg pieces were incubated in oxygenated (95% O₂: 5% CO₂) Gibco M199 media (Invitrogen, Carlsbad, CA) with 100 mIU/ml human chorionic gonadotropin (hCG; Sigma Aldrich, St. Louis, MO) and another two pieces were incubated in the same media, but without hCG. Each piece was incubated in a 2.0ml siliconized micro-centrifuge tube for 1.5 hr. in a 34°C water bath shaker. The media was then collected by centrifuging the tubes at 4°C for five minutes at 1240 x g using a Beckman GS-6KR centrifuge. Collected media was stored at -70°C for later quantification of progesterone, 17α -hydroxyprogesterone, androstenedione, and testosterone. Samples were rethawed once for hormone measurements.

A second pubertal experiment was conducted with a wider dose range of 0, 3.9, 7.8, 15.6, 31.2, 62.5 mg/kg (n = 6/dose) in attempt to determine a no-effect level for serum testosterone and *ex vivo* testosterone production. The same methods and endpoints were used as in the first pubertal experiment except animals were dosed from PND 23-42/43 and not PND 23-50/51.

Hershberger Experiment: Male rats, castrated on PND 42, were dosed daily by gavage with PCZ (0, 15.6, 31.2, 62.5, 125mg/kg, n = 6/dose) from PND 49 to 58/59. Each rat received a 100ug testosterone propionate (TP) (Sigma Aldrich, St. Louis, MO) by sc injection in 0.1ml corn oil immediately after the gavage dose. A glass syringe and 25G 5/8" needle was used for TP injection. Animals were anesthetized with halothane on the day of necropsy and blood was collected via cardiac puncture to avoid contamination of blood with subcutaneous TP deposition. Testosterone and LH levels were measured in the serum and organ weights were recorded.

Hormones. Testosterone, androstenedione, 17 α -hydroxprogesterone, and progesterone were measured in serum and media using Diagnostic Products Corporation's Coat-A-Count kit (Los Angeles, CA). The 3rd Generation estradiol RIA by Diagnostic Systems Laboratories (Webster, TX) was used for measuring serum estradiol. Serum LH was measured by radioimmunoassay as previously described (Goldman *et al.* 1986) using materials supplied by the National Hormone and Pituitary Agency: iodination preparation I-6; reference preparation RP-3; and antisera S-11. Iodination material was radiolabeled with ¹²⁵I (Dupont/New England Nuclear) by a modification of the chloramine-T method (Greenwood *et al.* 1963). All samples within a study were run together to avoid interassay variation.

Statistical Analysis. All data were analyzed using the PROC GLM procedure from SAS (SAS v8, Cary, NC). Significant effects ($p < 0.05$) were further analyzed using LSMEANS to determine significance between the control and treatment groups. In the pubertal studies, all PPS timing and weight data (except liver weight) were analyzed using the initial body weight (PND 23 weight before dosing) as a covariate to adjust for the fact that larger weanling male rats attain puberty at an earlier age than smaller rats. In the first pubertal study, organ weights and

hormone data from the two time points were analyzed by two-way ANOVA to determine if there was an age by treatment interaction. Organ weight data in the Hershberger study were analyzed using ANOVA. Analysis of liver weights in both the pubertal and Hershberger assays included necropsy weight as a covariate. Heterogeneous data were \log_{10} transformed for analysis to normalize variance. If the hormone level of an individual sample was below the limit of detection, the lowest value on the standard curve was used for the statistical analysis.

RESULTS

Pubertal Experiment I.

The first pubertal exposure was conducted to determine if PCZ delayed pubertal development and reduced testosterone levels. PCZ significantly delayed the initiation and completion of PPS in the highest dose group, 125mg/kg/day (Table 1) by 1.1 and 1.8 days respectively. Necropsies performed at mid- and post-puberty were used to compare effects on steroidogenesis and organ weights between these two time points. The levator ani plus bulbocavernosus muscle (LABC) and epididymides were decreased in weight at the mid-puberty time point with greater sensitivity (i.e. affected at 62.5mg/kg) than at the post-puberty time point when the seminal vesicle and ventral prostate were affected only at 125mg/kg (Table 2). PCZ had some overt toxicity which was apparent mostly at the mid-puberty time point. PCZ significantly reduce body weight up to 7% at mid-puberty, but this effect was not seen at the post-puberty necropsy. Kidney weights were significantly decreased in the higher doses, liver weights were increased, but the adrenal weight was only marginally affected by PCZ (Table 3).

Serum testosterone was decreased dramatically by PCZ while treatment increased serum progesterone and 17α -hydroxyprogesterone compared to the controls (Figure 1A and 1B).

Serum androstenedione levels were generally below or close to limit of detection (0.15 ng/ml) so were not analyzed. Serum LH was decreased in the 125mg/kg/day dose group at the post-puberty time point, but this effect was not evident at mid-puberty (Figure 1D). Serum estradiol was unaffected by treatment (data not shown). In the *ex vivo* testis incubations, testosterone and androstenedione were significantly decreased and progesterone and 17 α -hydroxyprogesterone were increased compared to controls with and without hCG stimulation (Figure 2). The steroid profile was the same at the mid- and post-puberty time points. Two-way ANOVA analysis found no treatment and age interaction in either the weight or hormone data. With the combined data from both time points, the ventral prostate, seminal vesicle, LABC, and epididymides were all significantly affected in the 125mg/kg/day dose group, but not in the mid-dose group of 62.5mg/kg/day (data not shown).

Pubertal Experiment II.

A wider dose range with lower levels of PCZ was tested in the second pubertal experiment to define the dose response and no observable effect level (NOEL) of testosterone inhibition. Since the longer exposure period did not provide any enhanced sensitivity to PCZ, only the PND 23 – 42/43 dosing period was used. There were no significant effects on reproductive organ weights at 62.5mg/kg/day unlike in the first pubertal experiment (Table 2). The body weight at necropsy, LABC, and epididymides weight data from the first and second pubertal experiments were combined for a two-way ANOVA to determine if the combined data showed significant treatment effect and if there was an interaction between study and the organ weight data. The 62.5 mg/kg/day treatment had a marginal statistical effect ($p = 0.0834$) on the combined epididymides weight and a statistically significant ($p = 0.0199$) effect on combined LABC weight, but there was no treatment effect on body weight. There was a significant study effect

on LABC and body weight, but the interaction between study and organ weights was not significant. This suggests that the mean body weight and LABC differed between studies, but the treatment effect was not different between the two studies. The lack of no statistical significance at 62.5 mg/kg/day in the second study might be due to lower animal numbers and suggests that the organ weight effects at this dose are not robust. Liver weights increased significantly in the 62.5 mg/kg/day dose group, similar to the first pubertal exposure (Table 3). Serum progesterone and 17 α -hydroxyprogesterone did not increase in a similar fashion as in the first pubertal exposure. PCZ significantly reduced serum testosterone at lower doses than in the first pubertal experiment (Figure 1C). A NOEL of 7.8 mg/kg/day was determined for testosterone in the serum and *ex vivo* testis incubations. As in the first pubertal study, regardless of stimulation, testosterone and androstenedione levels were decreased in the *ex vivo* testis incubations while progesterone and 17 α -hydroxyprogesterone increased (Figure 3A-D). The initiation of PPS was not delayed (data not shown), which is consistent with the first pubertal study. Data of completed PPS were not collected since animal necropsy was at PND 42/43.

Hershberger Experiment.

Castrated male rats were treated with PCZ and TP to define the degree to which PCZ acted as an AR antagonist *in vivo*. At the highest dose group PCZ significantly or marginally reduced the weights of several androgen sensitive organs (Table 4) indicating that PCZ acts as an AR antagonist *in vivo* at this dose, but not at lower dose levels. Serum testosterone levels were not significantly different among the treatments. Serum LH was significantly decreased in the 125mg/kg/day dose group which is similar to the post-puberty time point of the first pubertal experiment. There was little overt toxicity in this assay. Body weight was not affected by treatment, but liver weights were significantly increased in the 31.3mg/kg/day and greater doses.

Kidney weights decreased similar to the first pubertal exposure, but this decrease was not statistically significant and there were no effects on adrenal weights (data not shown).

DISCUSSION

In the present study, we found that PCZ administered to the weanling male Sprague Dawley rat reduced testis testosterone production and serum testosterone levels at doses eight fold lower than those that delayed puberty or robustly reduced androgen-dependent organ weights (i.e. 15.6 mg/kg/day versus 125 mg/kg/day). The magnitude of the testosterone decrease in the first pubertal study without effects on PPS or organ weights raises the question of how pubertal development could appear unaffected when testosterone levels were so low. In fact, the purpose of the second pubertal study was to determine if the alteration of hormones could be replicated. Taken together, these data suggest that a large decrease in testosterone is needed to reduce androgen-sensitive organ weights and delay PPS. Since many of the tissues are dependent upon dihydrotestosterone (DHT) (Blohm *et al.* 1986; George *et al.* 1989) produced locally from testosterone, it is possible that there was enough serum testosterone for conversion to DHT in all but the highest dose of PCZ. Measuring the amount of DHT and testosterone in the various reproductive tissues would help resolve the question of why particular organs were affected. Although the organ weights and PPS measured in this study were not affected by the same dose that decreased testosterone levels, other endpoints might be more sensitive to low testosterone (e.g. sperm production and behavior).

The overall pattern of changes in hormone levels in the pubertal male rat suggests that the inhibition of activity associated with CYP17 is specifically responsible for the effects of reduced testosterone. In some instances, 17α -hydroxyprogesterone levels were not elevated or

marginally elevated at the high dose of 125mg/kg/day. At this high dose, PCZ inhibition of CYP17's hydroxylase activity may be stronger than the inhibition of lyase activity. It is unlikely that PCZ inhibits the LH stimulation pathway since the pattern of inhibition in the *ex vivo* testis incubations did not change when the tissue was stimulated with hCG. Presumably PCZ acts like other imidazoles, such as the pharmaceutical ketoconazole (Ayub and Levell 1987), which inhibit the CYP17 enzyme. Ketoconazole also has been reported to affect puberty, presumably through inhibition of steroidogenesis even though hormone measurements were not conducted (Ashby and Lefevre 2000) or showed no effect (Marty *et al.* 2001). The evidence for CYP17 inhibition is supported by our *ex vivo* measurements and may have been missed if only serum measurements were relied upon. Additionally androstenedione levels were generally undetectable in the serum, but the PCZ-induced alteration was also easily measured in the *ex vivo* testis incubations. Experiments are currently being conducted to determine if CYP17 activity is competitively inhibited by PCZ *in vitro* (Blystone *et al.*, *in prep*) and these data will be included in a second manuscript describing the effects of PCZ on fetal hormone levels and expression of steroidogenic genes during sexual differentiation.

The ability of PCZ to antagonize the AR was assessed using the Hershberger assay. Androgen sensitive weights were only affected at the highest dose (125mg/kg/day). Our results differ from those that showed effects as low as 50mg/kg/day in another Hershberger assay with Wistar rats (Vinggaard *et al.* 2002). The reason for the discrepancy is unknown, but may be due to differences in the two rat strains. Since PCZ and other conazole fungicides are known to affect the liver to varying degrees, it is possible that PCZ might decrease testosterone levels by increasing testosterone metabolism through induction of metabolic enzymes. However, this does

not appear to be the case since serum testosterone levels were unaffected in the Hershberger assay.

The question of which “antiandrogenic” mechanism is responsible for delaying pubertal development is difficult to answer with these data. In fact, both mechanisms of action may be operative *in vivo*. A comparison of lowest observable effect levels (LOEL) among the present experiments (Table 5) shows that there is an overlap in effective dose of AR antagonism, testosterone inhibition, and PPS. The Hershberger assay provided insight into the AR antagonism and there were clear effects at the 125mg/kg dose. In the non-castrated animal, there may be a cumulative effect between the two mechanisms with the weak AR antagonism and strong steroidogenic inhibition of PCZ working together to delay pubertal development.

PCZ demasculinized the male rat offspring when pregnant rats received PCZ by gavage from gestational day 14 to 18 at doses of 31.3, 62.5, 125, and 250 mg/kg/day (Noriega *et al.* 2005). Males displayed female-like nipples at 13 days of age at frequencies of 31%, 43%, 41%, and 71% in the lowest-dose to highest-dose groups, respectively. In addition, when necropsied as adults, F1 males displayed reduced weights and malformations in androgen-dependent tissues (125 and 250 mg/kg/day). Together these results indicate that PCZ alters sexual differentiation and delays puberty in an anti-androgenic manner at equivalent dosage levels. This suggests that male development, as measured by PPS and organ weights in the current study, may be similarly sensitive to PCZ during gestation and puberty. The ability of PCZ to alter fetal male rat hormone production levels is currently being evaluated in our laboratory to determine if the profile of effects is similar to that seen in the pubertal male rat herein and to determine if T production is more sensitive in one developmental period than the other (Blystone *et al.*, *in prep*).

In addition to its effects on androgen synthesis, PCZ is also a potent inhibitor of aromatase *in vitro* (Mason *et al.* 1987; Vinggaard *et al.* 2000) and the delayed delivery seen in PCZ treated dams (Noriega *et al.* 2005) suggests that it also inhibits estradiol synthesis *in vivo*. However, we were not able to detect a decrease in serum estradiol levels of PCZ treated male rats in the pubertal studies. The lack of effect on estradiol herein may be due to the fact that the male normally produces such low levels of estradiol (pg vs. ng of the substrate, testosterone) and the abundance of testosterone may out-compete PCZ for the aromatase enzyme.

One endocrine effect in the current study was contrary to our expectation. We expected that PCZ would increase serum LH, possibly dramatically since it was expected to reduce serum testosterone levels and act an AR antagonist. Either reducing T dramatically or acting as an AR antagonist alone would be expected to elicit an increase in LH by eliminating the negative feedback of T on the hypothalamic-pituitary axis. However, LH levels were unaffected in most PCZ doses in either the pubertal or the Hershberger experiments with the exception of decreased LH in the 125 mg PCZ/kg/day dose group. Other reports found that doses of PCZ that decreased androgen-dependent organ weights only increased LH at the highest dose of 250mg/kg/day (Vinggaard *et al.* 2002) while in a second Hershberger study, LH did increase at 100mg/kg/day (Vinggaard *et al.* 2005b). The data from our studies and previous studies indicate that in the LH response to PCZ is highly variable and further investigation is warranted if this is to be clarified.

In summary, PCZ delayed pubertal development and reduced reproductive organ weights in the Hershberger assay at a higher dosage than what dramatically reduced testosterone levels *in vivo* and *ex vivo*. These data provide the first example of how a pesticide that is a steroid synthesis inhibitor affects PPS, organ weights, and testosterone endpoints in the pubertal male rat, an assay

that is being considered for inclusion in the USEPA's Tier 1 screening battery for endocrine disruptors. Clearly, the sensitivity of the assay is enhanced by including the measurement of serum and *ex vivo* testis hormone production. In addition, we were able to detect the changes in a shorter time period (20 versus 30 days) (Stoker *et al.* 2000) and our assay required fewer animals per dose group (6 to 8 versus 15) than recommended (EDSTAC 1998). Based on these data we suggest that these testosterone measurements be included to detect steroid synthesis inhibitors like PCZ.

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Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in the fetal rat testis. *Toxicol. Lett.* 146, 207-215.

Table 1: Mean age and weight (\pm SEM) at the initiation and completion of PPS during the first pubertal PCZ exposure.

| Dose(mg/kg) | <i>Post-Natal Day of PPS</i> | | <i>Rat Weight (g) at PPS</i> | |
|--------------------|-------------------------------------|-------------------|-------------------------------------|-------------------|
| | Initiation | Completion | Initiation | Completion |
| 0 | 41.5 \pm 0.4 | 42.2 \pm 0.4 | 200.1 \pm 4.2 | 208.9 \pm 4.4 |
| 31.3 | 41.8 \pm 0.7 | 42.2 \pm 0.4 | 207.2 \pm 4.5 | 213.7 \pm 4.9 |
| 62.5 | 41.3 \pm 0.2 | 42.6 \pm 0.2 | 192.1 \pm 2.5 | 207.9 \pm 4.6 |
| 125 | 42.6 \pm 0.6* | 44.0 \pm 0.7** | 197.9 \pm 6.9 | 210.8 \pm 7.4 |

n = 9-14/dose group. * p < 0.05, ** p < 0.01.

Table 2: Mean reproductive organ weights (\pm SEM) at different time points (mid- and post-puberty) from the first pubertal PCZ exposure (Pub I) and in the second pubertal exposure (Pub II).

| <i>Dose</i> (mg/kg) | <i>Age</i> (PND) | <i>GP</i> (mg) | <i>VP</i> (mg) | <i>SV</i> (mg) | <i>LABC</i> (mg) | <i>Cowpers</i> (mg) | <i>Testes</i> (g) | <i>Epididymis</i> (mg) |
|---------------------------|---------------------|-------------------|-------------------|-------------------|---------------------|------------------------|----------------------|---------------------------|
| Pub I^a | | | | | | | | |
| 0 | 42/43 | 74.2 \pm 4.6 | 105.4 \pm 9.7 | 153.3 \pm 21.6 | 349.8 \pm 27.9 | 22.3 \pm 3.2 | 1.819 \pm 0.058 | 246.7 \pm 10.9 |
| 31.3 | 42/43 | 70.1 \pm 2.9 | 108.5 \pm 13.0 | 139.0 \pm 14.6 | 311.5 \pm 21.8 | 23.8 \pm 3.0 | 1.915 \pm 0.066 | 245.4 \pm 11.4 |
| 62.5 | 42/43 | 71.3 \pm 3.9 | 104.5 \pm 10.4 | 144.5 \pm 27.4 | 267.9 \pm 25.5** | 18.7 \pm 3.1 | 1.911 \pm 0.062 | 222.3 \pm 11.2* |
| 125 | 42/43 | 68.5 \pm 2.7 | 86.9 \pm 12.3 | 113.1 \pm 11.0 | 233.4 \pm 20.0*** | 22.4 \pm 3.0 | 1.731 \pm 0.084 | 207.4 \pm 9.0*** |
| Pub I^b | | | | | | | | |
| 0 | 50/51 | 82.4 \pm 4.0 | 173.1 \pm 15.5 | 443.6 \pm 37.8 | 514.0 \pm 28.7 | 37.7 \pm 4.3 | 2.470 \pm 0.060 | 363.9 \pm 15.3 |
| 31.3 | 50/51 | 81.3 \pm 2.5 | 174.0 \pm 17.2 | 469.8 \pm 55.3 | 580.2 \pm 50.0 | 41.3 \pm 4.5 | 2.608 \pm 0.136 | 376.0 \pm 16.2 |
| 62.5 | 50/51 | 81.6 \pm 3.2 | 166.3 \pm 6.6 | 455.4 \pm 33.5 | 560.5 \pm 16.0 | 36.9 \pm 2.3 | 2.504 \pm 0.106 | 372.7 \pm 6.7 |
| 125 | 50/51 | 78.6 \pm 2.7 | 118.4 \pm 18.6* | 329.1 \pm 34.0* | 490.9 \pm 17.2 | 36.2 \pm 3.2 | 2.501 \pm 0.069 | 352.8 \pm 13.4 |
| Pub II^c | | | | | | | | |
| 0 | 42/43 | 65.2 \pm 2.6 | 154.6 \pm 7.3 | 208.0 \pm 16.0 | 381.5 \pm 21.7 | 28.1 \pm 3.7 | 1.930 \pm 0.057 | 265.5 \pm 15.9 |
| 3.9 | 42/43 | 77.9 \pm 4.2** | 167.2 \pm 8.0 | 279.2 \pm 33.4* | 433.8 \pm 22.1 | 33.7 \pm 2.7 | 2.042 \pm 0.033 | 298.8 \pm 14.2 |
| 7.8 | 42/43 | 64.2 \pm 1.7 | 166.0 \pm 20.0 | 201.2 \pm 24.1 | 366.5 \pm 26.2 | 28.1 \pm 4.6 | 1.905 \pm 0.060 | 243.6 \pm 16.8 |
| 15.6 | 42/43 | 66.4 \pm 2.5 | 128.8 \pm 21.1 | 242.9 \pm 21.9 | 364.3 \pm 24.5 | 33.7 \pm 4.4 | 1.956 \pm 0.097 | 256.6 \pm 14.5 |
| 31.3 | 42/43 | 65.7 \pm 3.9 | 131.2 \pm 18.9 | 147.7 \pm 12.9 | 332.8 \pm 11.2 | 24.5 \pm 2.7 | 1.853 \pm 0.030 | 240.8 \pm 8.7 |
| 62.5 | 42/43 | 66.7 \pm 1.4 | 133.2 \pm 9.5 | 197.1 \pm 18.8 | 351.1 \pm 23.0 | 26.5 \pm 2.8 | 1.908 \pm 0.087 | 246.8 \pm 10.2 |

^a n=8/dose, ^b n=8-10/dose, ^c n=6/dose.

* p<0.05, ** p<0.01, *** p<0.001.

GP=glans penis, VP=Ventral Prostate, SV=Seminal Vesicle, LABC=Levator ani + Bulbocavernosus muscle.

Table 3: Mean body and non-reproductive organ weights (\pm SEM) at different time points (mid- and post-puberty) from the first pubertal PCZ exposure (Pub I) and second pubertal exposure (Pub II).

| <i>Dose (mg/kg)</i> | <i>Age (PND)</i> | <i>Body Wt. (g)</i> | <i>Liver^d (g)</i> | <i>Adrenals (mg)</i> | <i>Kidneys (g)</i> |
|---------------------------|----------------------|-------------------------|----------------------------------|--------------------------|------------------------|
| Pub I^a | | | | | |
| 0 | 42/43 | 210.5 \pm 6.0 | 10.81 \pm 0.41 | 31.5 \pm 2.5 | 1.956 \pm 0.070 |
| 31.3 | 42/43 | 213.7 \pm 6.5 | 11.42 \pm 0.57 | 29.3 \pm 1.7 | 1.978 \pm 0.060 |
| 62.5 | 42/43 | 195.7 \pm 6.3* | 10.40 \pm 0.38* | 30.6 \pm 1.5 | 1.755 \pm 0.072** |
| 125 | 42/43 | 197.7 \pm 7.0* | 10.94 \pm 0.52** | 28.7 \pm 1.7 | 1.770 \pm 0.065* |
| Pub I^b | | | | | |
| 0 | 50/51 | 281.2 \pm 7.0 | 14.00 \pm 0.50 | 37.9 \pm 1.1 | 2.488 \pm 0.062 |
| 31.3 | 50/51 | 277.5 \pm 11.9 | 14.06 \pm 0.77 | 34.6 \pm 2.2 | 2.414 \pm 0.133 |
| 62.5 | 50/51 | 276.3 \pm 6.4 | 14.27 \pm 0.34 | 35.1 \pm 2.2 | 2.425 \pm 0.061 |
| 125 | 50/51 | 265.0 \pm 10.7 | 14.82 \pm 0.68**** | 34.1 \pm 1.6 | 2.270 \pm 0.079* |
| Pub II^c | | | | | |
| 0 | 42/43 | 221.9 \pm 6.0 | 10.567 \pm 0.408 | 28.8 \pm 2.3 | 2.085 \pm 0.090 |
| 3.9 | 42/43 | 238.4 \pm 5.2* | 11.593 \pm 0.217 | 29.8 \pm 1.6 | 2.172 \pm 0.053 |
| 7.8 | 42/43 | 221.7 \pm 6.8 | 10.876 \pm 0.403 | 30.9 \pm 2.3 | 1.996 \pm 0.093 |
| 15.6 | 42/43 | 219.6 \pm 3.3 | 11.090 \pm 0.312 | 25.8 \pm 1.4 | 1.980 \pm 0.040 |
| 31.3 | 42/43 | 216.0 \pm 5.0 | 10.614 \pm 0.522 | 22.9 \pm 2.4* | 2.002 \pm 0.077 |
| 62.5 | 42/43 | 221.8 \pm 7.5 | 11.649 \pm 0.615** | 29.8 \pm 1.5 | 2.028 \pm 0.048 |

^a n=8/dose, ^b n=8-10/dose, ^c n=6/dose,

^d Liver weight analyzed with necropsy weight as a covariate. * p<0.05, ** p<0.01, ***

p<0.001.

Table 4: Mean body and organ weights (\pm SEM) from the Hershberger assay (castrated male + testosterone propionate) after 10-11 day exposure to PCZ.

| <i>PCZ Dose^a</i> <i>(mg/kg/day)</i> | <i>Body</i> <i>(g)</i> | <i>GP</i> <i>(mg)</i> | <i>VP</i> <i>(mg)</i> | <i>SV</i> <i>(mg)</i> | <i>LABC</i> <i>(mg)</i> | <i>Cowper's</i> <i>(mg)</i> | <i>Liver</i> <i>(g)</i> | <i>LH</i> <i>(ng/ml)</i> | <i>Testosterone</i> <i>(ng/ml)</i> |
|---|---------------------------|--------------------------|--------------------------|--------------------------|----------------------------|--------------------------------|----------------------------|-----------------------------|---------------------------------------|
| 0 | 280.7 ± 6.6 | 93.5 ± 5.9 | 158.2 ± 9.5 | 601.2 ± 38.1 | 545.5 ± 14.6 | 41.6 ± 2.1 | 10.54 ± 0.47 | 2.783 ± 0.891 | 1.668 ± 0.171 |
| 15.6 | 283.1 ± 7.5 | 77.9* ± 2.5 | 141.8 ± 12.1 | 559.2 ± 26.0 | 549.5 ± 17.9 | 41.5 ± 2.9 | 11.22 ± 0.48 | 3.709 ± 0.507 | 1.362 ± 0.089 |
| 31.3 | 285.1 ± 6.6 | 88.0 ± 3.4 | 156.9 ± 8.7 | 498.6 ± 42.1 | 509.2 ± 21.7 | 39.3 ± 3.2 | 11.91** ± 0.59 | 3.317 ± 1.251 | 1.649 ± 0.131 |
| 62.5 | 286.6 ± 6.8 | 98.2 ± 4.4 | 140.9 ± 12.4 | 550.3 ± 47.0 | 554.8 ± 10.0 | 43.1 ± 3.6 | 12.65*** ± 0.59 | 4.135 ± 0.871 | 1.703 ± 0.294 |
| 125 | 272.0 ± 6.6 | 88.9 ± 4.9 | 127.1 ± 11.4 | 437.7** ± 28.3 | 446.8*** ± 9.8 | 33.7 ± 2.2 | 13.02*** ± 0.67 | 1.023* ± 0.526 | 1.446 ± 0.188 |

^a n = 6 per dose.

* p<0.05, ** p<0.01, *** p<0.001

Table 5: The LOEL (mg/kg/day) for various endpoints in each experiment*.

| <i>Endpoint</i> | <i>1st Pubertal Study (PND 42/43)</i> | <i>1st Pubertal Study (PND50/51)</i> | <i>2nd Pubertal Study (PND42/43)</i> | <i>Hershberger</i> |
|--|--|---|---|--------------------|
| PPS | 125 | 125 | N.E. | - |
| Repro Organs | 62.5 | 125 | N.E. | 125 |
| <u>Testosterone</u> | | | | |
| Serum | 31.3 | 31.3 | 15.6 | N.E. |
| Ex Vivo (-hCG) | 31.3 | 31.3 | 15.6 | - |
| Ex Vivo (+hCG) | 31.3 | 31.3 | 15.6 | - |
| <u>Androstenedione</u> | | | | |
| Serum | - | - | - | - |
| Ex Vivo (-hCG) | 31.3 | 31.3 | 15.6 | - |
| Ex Vivo (+hCG) | 31.3 | 31.3 | 7.8 | - |
| <u>17α-hydroxyprogesterone</u> | | | | |
| Serum | 31.3 | 31.3 | N.E. | - |
| Ex Vivo (-hCG) | 31.3 | 31.3 | 15.6 | - |
| Ex Vivo (+hCG) | 31.3 | 31.3 | 15.6 | - |
| <u>Progesterone</u> | | | | |
| Serum | 31.3 | 62.5 | N.E. | - |
| Ex Vivo (-hCG) | 62.5 | 31.3 | 62.5 | - |
| Ex Vivo (+hCG) | 31.3 | 31.3 | 31.3 | - |

* The lowest dose in the first pubertal study was 31.3 mg/kg/day whereas it was 3.9 in the second study. The endpoints included in the proposed USEPA/EDSP pubertal male rat protocol and OECD Hershberger assays are shaded. The NOEL for standard endpoints in the pubertal male assay is 62.5 mg/kg/day versus 3.9 for hCG stimulated *ex vivo* androstenedione production and 7.8 for the other serum and *ex vivo* androgens.

N.E. = no significant dose related effect in treatments.

(-) = endpoint was not evaluated.

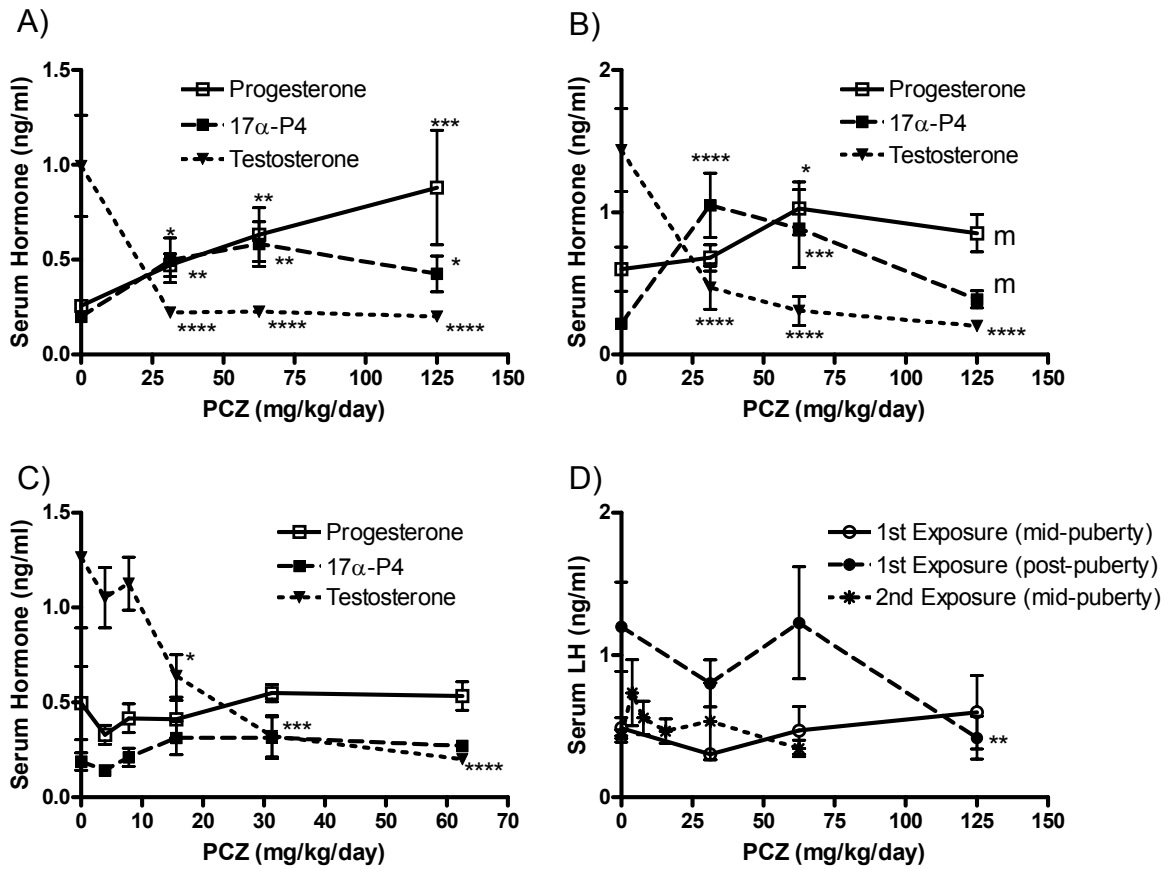


Figure 1: Mean serum hormones (\pm SEM) from mid-puberty (A) and post-puberty (B) of first peripubertal exposure and mid-puberty (C) of second peripubertal exposure. Mean serum LH (\pm SEM) from all three pubertal exposures (D). m = $p < 0.07$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

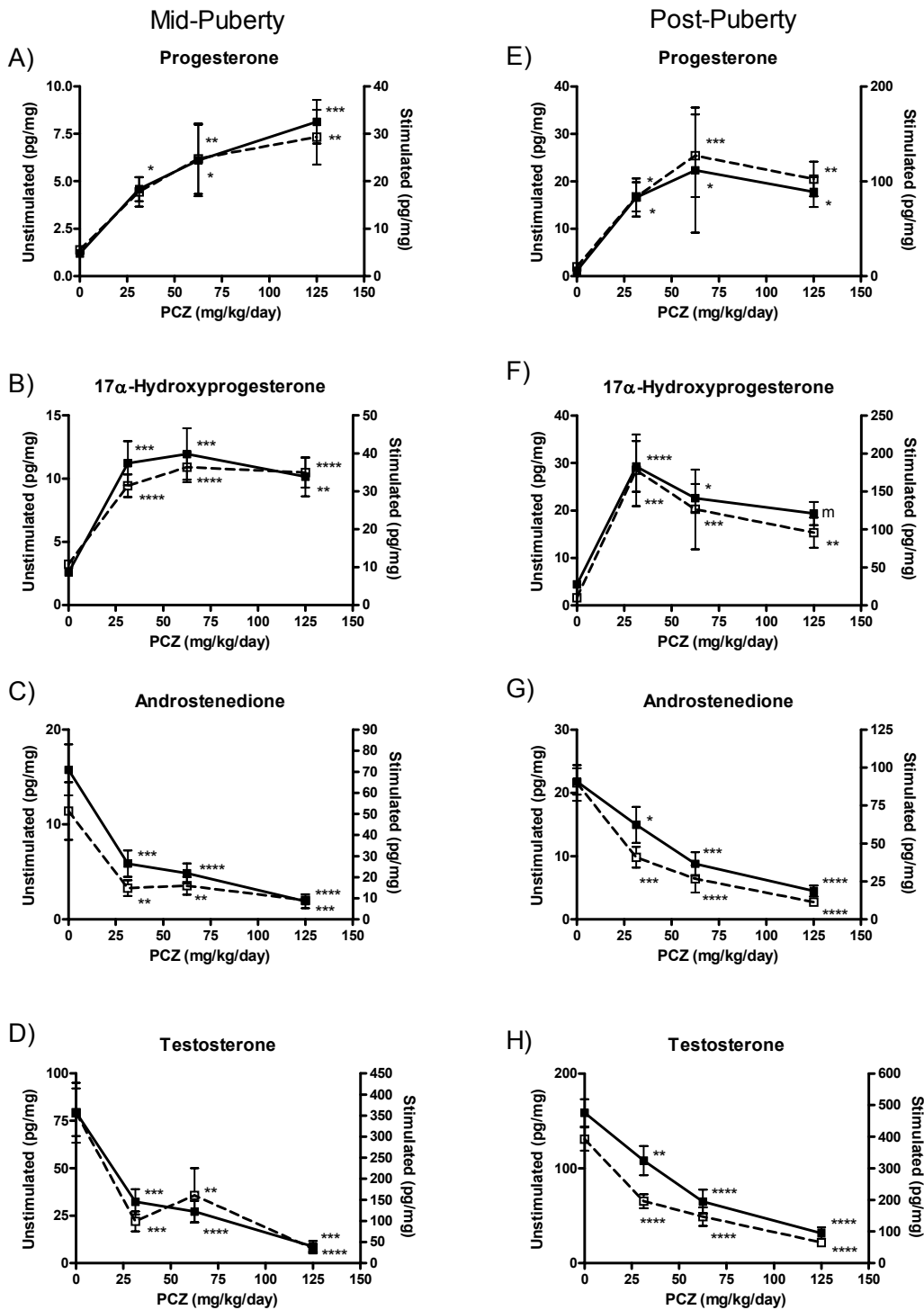


Figure 2: Mean *ex vivo* hormone levels (\pm SEM) at mid-puberty (A-D) and post-puberty (E-H) from the first peripubertal exposure. Solid line (\blacksquare) is from tissue stimulated with hCG and dashed line (\square) is from un-stimulated tissue. m = $p < 0.07$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

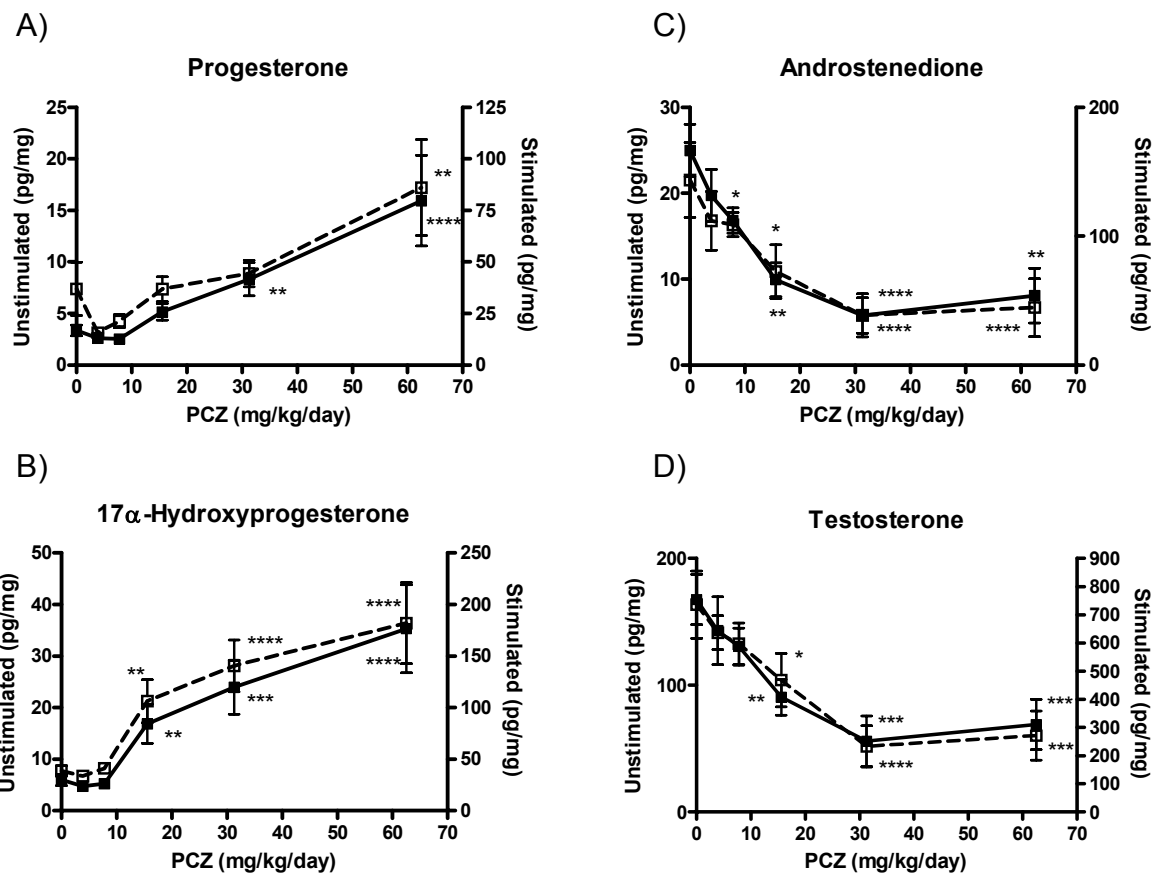


Figure 3: Mean *ex vivo* hormone levels (±SEM) at mid-puberty (A-D) from the second peripubertal exposure. Solid line (■) is from tissue stimulated with hCG and dashed line (□) is from un-stimulated tissue. m = p<0.07, * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

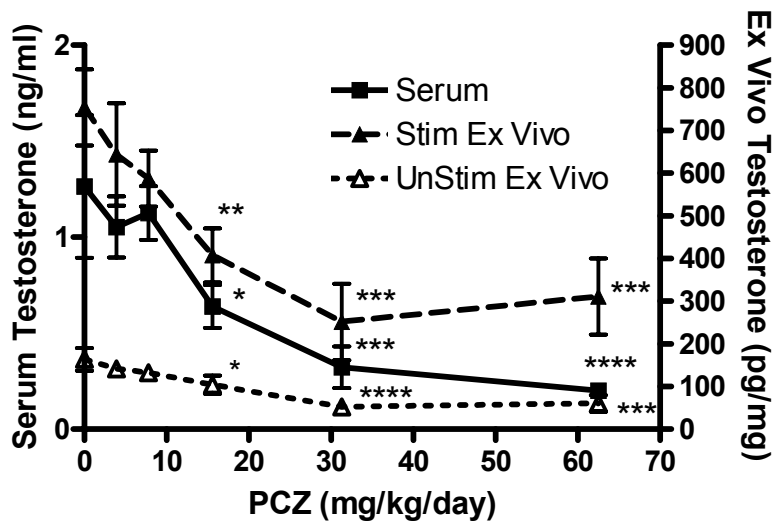


Figure 4: The relationship of mean (\pm SEM) serum testosterone (\blacktriangle) levels and *ex vivo* testosterone levels with hCG stimulation (\blacksquare) and without (\square) hCG stimulation from the second peripubertal exposure. $m = p < 0.07$, $* = p < 0.05$, $** = p < 0.01$, $*** = p < 0.001$, $**** = p < 0.0001$.

CHAPTER II

Sensitivity of fetal rat testicular steroidogenesis to maternal prochloraz exposure and the underlying mechanism of inhibition

This chapter was formatted for submission to the journal *Biology of Reproduction*.

ABSTRACT

The fungicide prochloraz (PCZ) induces malformations in androgen-dependent tissues in male rats when administered during sex differentiation. The sensitivity of fetal testicular steroidogenesis to PCZ was investigated to test the hypothesis that the reported morphological effects from maternal exposure were due to reduced testosterone. Pregnant Sprague Dawley rats were dosed by gavage with 0, 7.8, 15.6, 31.3, 62.5, 125 mg/kg/day (n = 8) of PCZ from gestational day (GD) 14 to 18. On GD 18 the effects of PCZ on fetal steroidogenesis were assessed by measuring hormone production from ex vivo fetal testes after three hours incubation. Lastly, PCZ levels in amniotic fluid and maternal serum were measured by HPLC/MS and correlated to the inhibition of steroidogenesis. Fetal progesterone and 17 α -hydroxyprogesterone production levels were increased significantly at every PCZ dose, whereas testosterone levels were significantly decreased only at the two high doses. These results suggest that PCZ inhibits the conversion of progesterone to testosterone through the inhibition of CYP17. To test this hypothesis, PCZ effects on CYP17 gene expression and CYP17 hydroxylase activity were evaluated. PCZ had no effect on testicular CYP17 mRNA levels as measured by qRT-PCR. However, microsomal CYP17 hydroxylase activity was significantly inhibited by the fungicide (K_i = 865 nM). Fetal testosterone production was reduced when PCZ levels in amniotic fluid reached ~500 ppb which compares favorably with the determined hydroxylase activity K_i for PCZ. These results demonstrate that PCZ lowers testicular testosterone synthesis by inhibiting CYP17 activity which likely contributes to the induced malformations in androgen-dependant tissues during this time period.

INTRODUCTION

Development of the male reproductive system during gestation is dependant on androgen stimulation, and environmental chemicals that reduce androgen function have been shown to alter this development [1, 2]. Some phthalates such as di(n-butyl) phthalate and di(ethylhexyl) phthalate disrupt male rat development by reducing fetal testosterone and insl3 production in the fetal testis [3-5]. It is unknown whether similar effects might be occurring in the human population. However, maternal levels of certain phthalates have been associated with reduced ano-genital distance (AGD) in male children, a marker of androgen function, which suggests these chemicals might affect development [6].

The conazole pesticide prochloraz (PCZ) also disrupts male rat differentiation. Maternal exposure to PCZ during the gestational period of sexual differentiation resulted in phallus abnormalities, reduced reproductive organ weights, and increased retention of nipple/areolas in male rat offspring [7-9]. There are two anti-androgen mechanisms identified for PCZ that may contribute to the altered development. First, PCZ is reported to reduce fetal testosterone production in vivo and ex vivo [5, 8, 9]. Second, PCZ is reported to be an androgen receptor (AR) antagonist in vitro and in vivo [7, 10, 11]. It is unclear whether PCZ action as an androgen receptor antagonist or as an inhibitor of testosterone synthesis are predominant modes of action.

Studies examining the effects of PCZ on fetal testosterone production used one or two doses, which precludes assessment of the PCZ-testosterone production dose response relationship [5, 8, 9]. Additionally, the relationship between reduced fetal testosterone production and morphological effects in adult males has not been investigated. This uncertainty makes it

difficult to assess whether the changes in androgen-dependent tissues in adult males due to maternal PCZ exposure are the result of decreased testosterone. Therefore, this study first examined the sensitivity of fetal steroidogenesis to PCZ treatment by defining, with five PCZ doses, a dose-response curve of ex vivo fetal testosterone production. These data were compared to the reported dose-response of morphological effects in adult male rats [7] to assess the correlation between them. Then we investigated the effects of PCZ on fetal CYP17 mRNA expression and CYP17 hydroxylase activity since this enzyme was viewed as a likely target for the anti-androgen activity of PCZ. StAR and CYP11A mRNA were also evaluated as potential targets of PCZ. Lastly, we measured amniotic fluid levels of PCZ to determine whether the PCZ concentrations negatively correlated with testosterone production.

MATERIALS AND METHODS

Animals and Dosing

Timed pregnant Sprague Dawley rats were delivered on GD 2-3 (GD 1 = day after mating) from Charles River Laboratories (Raleigh, NC) and housed in the Environmental Protection Agency's Reproductive Toxicology Division animal facility. Animals were fed Purina Rat Chow 5008 and watered *ad libitum*. Environmental conditions were 22-23°C, 50-60% humidity, and a 14L:10D light cycle (lights on at 9pm). Prior to dosing, animals were weight ranked and assigned to dose groups to minimize differences in means and variance among treatment groups. PCZ (CAS# 67747-09-5, 99.5 % purity by HPLC; Riedel-de Haën; Lot# 2226x) was delivered to rats in corn oil (CAS# 8001-30-7, Sigma Aldrich, St. Louis, MO) in a volume of 2.5ml/kg body weight. Dams were dosed daily from GD 14 to 18 with 0, 7.8, 15.6, 31.2, 62.5, or 125 mg/kg PCZ (n = 8/dose). Calculated dose was determined by daily weight measurements. For microsome

preparations, immature male Sprague Dawley rats delivered from Charles River Laboratories (Raleigh, NC) on PND 23 were housed in the Environmental Protection Agency's Reproductive Toxicology Division animal facility. Animals were fed Purina Rat Chow 5001 and watered *ad libitum*. Environmental conditions were 21-24°C, 40-55% humidity, and a 12L:12D light cycle (lights on at 6am). The animal use protocol for this study was approved by the National Health and Environmental Effects Research Laboratory's Institutional Animal Care and Use Committee.

Ex Vivo Testis Incubations

On GD18 dams were anesthetized with CO₂ then decapitated between 8:00-9:45 h. Trunk blood was collected for serum progesterone and estradiol measurement. Fetal testis testosterone production was evaluated using a method described previously [5]. Briefly, the uterine tract was removed and each fetus was quickly removed and placed in a tissue culture dish on ice. Each fetus was sexed under a dissecting microscope. The right and left testis were removed from the first three males of each litter and incubated separately for 3 hrs with gentle rocking at a humidified 37 °C in a 24-well plate containing 500 ul of Gibco M199 media (no phenol red) in each well (Invitrogen, Carlsbad, CA). Media was supplemented with 10% dextran-charcoal stripped fetal bovine serum (Hyclone, Logan, UT). After the 3 hrs, media was removed and frozen at -80°C in silicon treated micro-centrifuge tubes until hormone measurement. All hormones were measured using Diagnostic Products Corporation's Coat-A-Count kit (Los Angeles, CA).

Fetal Testis Gene Expression

The testes from the remaining males of each litter were removed for quantitative real time RT-PCR. The testes pooled by litter were immediately homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) and frozen at -80°C. Total RNA was extracted within 2-3 weeks using the method published by the manufacture. Extracted RNA was digested for 30 min at 37°C with 1 IU DNase I (Promega, Madison, WI) and stopped by heat inactivation (65°C for 5 min). Samples were quantified first using the Nanodrop spectrophotometer (Nanodrop technologies Inc., Wilmington, DE) then by fluorescence with Ribogreen reagent (Invitrogen, Carlsbad, CA) using the manufacturer's method. DNAsed RNA (1 ug) was reverse transcribed using 4µl of Promega 5X Improm buffer (Madison, WI), 2.4 µl of MgCl₂ (25 mM), 1µl dNTP (10 mM), 0.5 µl RNAsin, 1 µl Promega Improm-II reverse transcriptase, and 1µg random hexamer primers. Following reverse transcription, aliquots equivalent to 100 ng of cDNA were used for real time PCR. Primer sequences for StAR, CYP11A, and CYP17 and a dual labeled fluorescent probe (Fam-Black Hole Quencher 1) (Table 1) synthesized by IDT (Intergrated DNA Technologies, Coralville, IA) were used. PCR reactions of 50 µl contained 2µl dNTP (10mM), 10 pmol forward primer, 10 pmol reverse primer, and 1.25 pmol Taqman probe, 1µl Taq DNA polymerase (5 U/µl) (Invitrogen, Carlsbad, CA), 5µl Promega Improm 10x buffer, DEPC treated water, and varying amounts of 25 mM MgCl₂ (12 µl for StAR reaction, 12 µl for CYP11A, and 5 µl for CYP17). All samples were run in duplicate on a single plate using a Bio-Rad Icyler (Hercules, CA) and cycles of 95 °C for 15 seconds, 56 °C for 20 seconds, and 72 °C for 10 seconds were repeated 40 times. The threshold cycle was chosen to ensure all reactions were in exponential amplification. Copy number was determined from a standard curve generated by amplifying known cDNA quantities of each gene.

CYP17 Hydroxylase Activity

PCZ inhibition of CYP17 hydroxylase activity was assessed using microsomes prepared from testes of adult Sprague Dawley rats. On PND 106, animals were anesthetized with CO₂ then decapitated. The testes from each animal were removed and put into a cold 100 mM potassium phosphate buffer solution (pH 7.4) containing 250 mM sucrose and 1 mM EDTA after removing the tunica. Testes were then homogenized using a Wheaten glass homogenizer and centrifuged for 10 min at 10,000 x g in a Beckman XL-90 centrifuge with Type 90Ti Rotor. The supernatant was removed and spun at 105,000 x g for 1 hr. The resulting pellet was washed with fresh buffer and spun again at 105,000 x g for 1 hr. The final pellet was re-suspended in 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. Protein concentration was determined by Bradford assay using BioRad Bradford Reagent (Hercules, CA). Enzymatic reactions with 0, 0.5, 1.0, 2.0, 4.0 μM PCZ were conducted in duplicate and the experiment was repeated (n = 2). Reaction solutions consisted of 100 mM potassium phosphate buffer (pH 7.4), 50 μg protein, and NADPH regenerating system (BD Gentest, Bedford, MA) for a total volume of 500 μL. The multiple concentrations of progesterone substrate included 1.1 μM of [4-¹⁴C]progesterone (53 mCi/mmol activity, American Radiolabeled Chemicals, St. Louis, MO). Reactions were stopped with 2 ml ethyl acetate at 10 minutes and placed in ice. Progesterone and its metabolites were extracted twice from the reaction mixture with 2 ml ethyl acetate, dried under nitrogen, and then spotted with a small volume of ethyl acetate onto a Whatman polyester-backed silica gel medium thin layer chromatography plate (Fisher Scientific, Pittsburgh, PA). Progesterone and metabolites were resolved using a 3:1 mixture of chloroform and ethyl acetate. Plates were then read using a Packard Instant Imager (Downers Grove, IL). Hydroxylase activity

was calculated as the sum of the produced progesterone metabolites resolved on the TLC plates: 17 α -hydroxyprogesterone, androstenedione, testosterone, and a minor metabolite believed to be 16 α -hydroxyprogesterone [12-14]. The sum was used under the assumption that all progesterone metabolites were derived from CYP17 hydroxylase activity (e.g. androstenedione was produced only after hydroxylation of progesterone occurred). Michaelis constant (K_m) and maximum velocity (V_{max}) were determined from the double reciprocal plot (Lineweaver-Burke plot) of velocity⁻¹ ($1/V_o$) at each substrate concentration⁻¹ ($1/[S]$). The inhibitor binding constant (K_i) was then calculated from the negative x-intercept of the line produced from linear regression of K_m/V_{max} at each PCZ concentration using Graphpad Prism 4.0 software (San Diego, CA). The values for K_m , V_{max} , and K_i were the average ($n = 2$) of the experimental runs.

Dosimetry

Maternal serum and amniotic fluid (pooled by litter) were collected from half of the treated dams ($n = 4$) to determine PCZ concentration. Samples were frozen at -80 °C and then shipped overnight on dry ice to the EPA Mid-Continent Ecology Division in Duluth, MN for measurement. Serum and amniotic fluid samples were weighed in the 2 ml centrifuge tubes in which they were received, and then transferred (with acetonitrile rinse) to 15 ml polypropylene centrifuge tubes and diluted to 5 ml with acetonitrile. The empty 2 ml tubes were re-weighed to determine sample weights. Samples were vortexed, sonicated for 30 minutes, vortexed again, and then placed in a refrigerator overnight. Samples were then centrifuged at 5 °C and 3000 rpm for 20 minutes, and the liquid portion carefully poured into clean tubes and concentrated to approximately 0.5 ml in a warm water bath under a nitrogen stream. Each concentrate was diluted to 1.0 ml with 50/50 acetonitrile/water, vortexed, placed in ice for two hours, and then

centrifuged as before. Samples were transferred to 0.45 um PTFE spin filters and centrifuged at 10,000 rcf for 5 minutes. The filtrate was adjusted to 1.0 ml with acetonitrile/water and transferred to HPLC vials.

PCZ standards were prepared in 10% methanol/water, and spiked matrix samples were prepared by adding an aliquot of a PCZ solution (prepared in methanol) into control serum or control amniotic fluid (resulting in a nominal concentration of 953 ppb). PCZ was obtained from Sigma (St Louis, MO) and all solvents were chromatography and HPLC grade or better.

The processed samples were placed into crimp top amber vials and analyzed for PCZ by reversed phase HPLC followed by mass spectrometry. The Agilent model 1100 HPLC (Wilmington, DE, USA) consisted of a capillary pump, chilled auto sampler (4 °C), heated column compartment (25 °C), and a diode-array detector. An aliquot of sample (30 µl) was injected onto a Zorbax SB-C18 column (2.1 x 150 mm) (Agilent, Wilmington, DE) and eluted isocratically with 80% methanol/water at a flow rate of 0.25 ml/min. PCZ concentrations were determined using the response at wavelength 220 nm or 230nm and an external standard method of quantitation. The Agilent mass spectrometer (MSD) was used to confirm the presence of PCZ in the samples using an atmospheric pressure electrospray interface and SIM acquisition of the molecular ion (positive polarity).

Statistical Analysis

Data were analyzed using the PROC GLM procedure from SAS (SAS v8, Cary, NC).

Significant effects ($p < 0.05$) were further analyzed using LSMEANS to determine significance

between the control and treatment groups. Hormone data from each right and left fetal testis were averaged and then a litter mean was generated for analysis. Since testes were pooled by litter for RNA extraction, the individual data points were litter means. Heterogeneous data (variance increasing with treatment) were log₁₀ transformed for analysis to normalize variance. Correlation between amniotic and serum PCZ concentrations were analyzed by PROC CORR (Spearman) procedure from SAS.

RESULTS

PCZ treatment had no effect on dam body weight at necropsy, but did significantly decrease maternal weight gain (Table 2), which suggests some maternal toxicity. Post-implantation loss (i.e. fetal loss) was not increased, indicating that PCZ did not increase fetal mortality at these doses. Maternal serum progesterone levels were unaffected at all doses. Estradiol levels were significantly reduced at the 125 mg/kg/day treatment (Figure 1), which supports that PCZ might inhibit maternal aromatase activity in vivo. PCZ significantly affected ex vivo fetal hormone production. Progesterone and 17 α -hydroxyprogesterone levels increased significantly at all doses and a no-observed effect level could not be determined (Figure 2A and 2B). PCZ decreased testosterone and androstenedione production, but only at doses of 31.3 mg/kg/day and higher (Figure 2C and 2D). The decrease in the androgens by PCZ was correlated with reported fetal morphological changes (Table 3).

The pattern of hormone production suggests that PCZ may inhibit the conversion of progesterone to testosterone via CYP17. CYP17 mRNA levels were not significantly affected by PCZ treatment (Figure 3). StAR and CYP11A mRNA levels were also unaffected by PCZ, which

suggests that the reduced testosterone production is not a result of altered gene expression (Figure 3). While PCZ did not alter the expression levels of these genes, it did inhibit the CYP17 hydroxylase activity in a dose-dependant manner (Figure 4A). The K_m and V_{max} values determined from the Lineweaver-Burke plot were $1.71 \mu\text{M}$ ($\pm 0.60 \text{ SEM}$) and $0.833 \text{ pmol/min/ug}$ ($\pm 149 \text{ SEM}$) respectively (Figure 4B). The linear regressions generated for each concentration of PCZ intersected before the y-axis ($1/[S] = 0.08$ or $[S] = 12.5 \mu\text{M}$). Increasing PCZ concentrations increased $K_{m(\text{app})}$ and increased $V_{max(\text{app})}$. The K_i from the K_m/V_{max} x-intercept was 865 nM ($\pm 240 \text{ SEM}$).

Although variable within the treatment group, PCZ concentration within amniotic fluid and maternal serum generally increased with dose and correlated well with each other. The average recovery from six spiked samples was 102.7% ($\pm 35.1 \text{ S. D.}$) and the limit of detection was 20 ppb . PCZ was not detected in any of the control serum or amniotic fluid samples. The variability within treatment groups did not correlate with the order of necropsy suggesting the variability was not due to time between dosing and necropsy. The correlation between the amniotic fluid and maternal serum was highly significant (Spearman, $R_s = 0.9611$, $p < 0.0001$). The PCZ concentration within the amniotic fluid was generally about half that in the maternal serum except at the highest dose implying that placenta might have been a barrier to PCZ distribution (Figure 5A). The lowest concentrations of PCZ in amniotic fluid that were associated with suppressed testosterone synthesis ($\sim 500 \text{ ppb}$) (Figure 5B) compare favorably with the K_i of CYP17 hydroxylase activity for PCZ (326 ppb). These observations support the hypothesis that PCZ disrupts fetal steroidogenesis by inhibiting CYP17 enzyme activity. This

hypothesis was further supported by the negative correlation between ex vivo testosterone production and amniotic fluid levels (Spearman, $R_s = -0.4933$, $p < 0.0143$).

DISCUSSION

We hypothesize that PCZ suppression of testosterone production contributes to the developmental effects of PCZ on the male fetus. Consistent with this hypothesis, we show that the dose-response relationships for the effects of PCZ on testosterone production and developmental abnormalities are similar (Table 3). The reported morphological effects from gestational exposure to PCZ that lasted into adulthood occurred at doses of 62.5 mg/kg/day and higher [7] which is similar to the doses that reduced ex vivo fetal testosterone production in this study. Furthermore, doses of PCZ that had been previously shown to increase nipple retention and decrease Cowper's gland weight in male neonates also correspond to doses that reduced testosterone production [7-9]. The effects of PCZ on fetal testosterone production versus testosterone production in the pubertal male were quite different: a 24% versus 80-90% reduction respectively at the 125 mg/kg/day dose [15]. This observation combined with the reported morphological effects suggests that the fetal male is more sensitive to reduced testosterone. More apparent than effects on androgen production were the increased levels of progesterone and 17 α -hydroxyprogesterone at all doses. We do not know if these elevated progestin levels contribute towards any fetal pathology.

In the present study, PCZ did not affect expression of CYP17, StAR, and CYP11A mRNA. These results are different from the mRNA expression alterations by maternal di(n-butyl) phthalate exposure, a compound which also produces anti-androgen effects in male offspring

[16, 17]. These findings are consistent with those of a previous study which found that these genes, among others, were not affected at GD 21 or PND 16 after maternal PCZ exposure [9].

The enzymatic activity of CYP17 was significantly affected in the present study. CYP17 hydroxylase converts progesterone to 17α -hydroxyprogesterone, an intermediate which can disassociate from the enzyme or be further converted to androstenedione through CYP17 lyase activity [18]. PCZ inhibited the hydroxylase activity (progesterone to 17α -hydroxyprogesterone conversion) and the K_i is consistent with the K_i of several imidazole therapeutics [19]. The increasing $V_{\max(\text{app})}$ was not expected and suggests that PCZ is not a pure competitive inhibitor. Lower or higher substrate concentrations were excluded from analysis to determine if they contributed to the increased $V_{\max(\text{app})}$ by affecting the slope, but removing them did not affect the result. Additionally, when hydroxylase activity was calculated using only the 17α -hydroxyprogesterone metabolite, the increasing $V_{\max(\text{app})}$ result remained, which suggests that the other metabolites are not altering the slope. We do not know how PCZ increased the $V_{\max(\text{app})}$, but it might have to do with the structure of CYP17. CYP17 may contain a bi-lobed active site [20, 21] instead of a single active site [22] and PCZ interaction with an unoccupied site might increase the $V_{\max(\text{app})}$. PCZ inhibition of CYP17 appears to be different from other imidazoles [19, 23] and further work is needed to clarify how PCZ interferes with CYP17's hydroxylase activity and the lyase activity, which was not evaluated separately.

The amniotic fluid concentrations of PCZ were favorable to the CYP17 hydroxylase inhibition mechanism of testosterone reduction since they were above the hydroxylase K_i reported here. The AR antagonist activity of PCZ might also contribute to the anti-androgen effects in male

offspring. In the pubertal male, the AR antagonism of PCZ was weaker relative to its inhibition of steroidogenesis, but did contribute to the delay in pubertal development [15]. The IC_{50} for 1.0 nM R1881 binding to the AR was 60 μ M PCZ using cytosolic preparations from the rat prostate [7]. The estimated K_i of 20 μ M ($K_i = IC_{50}/(1+[ligand]/K_d)$ [24], $K_d = 0.5$ nM [25]) using the reported IC_{50} is considerably higher than the 4.0 μ M amniotic fluid concentration of PCZ in the 125 mg/kg/day treatment group. Together these K_i values suggest that PCZ AR antagonism is weaker than its CYP17 inhibition in the fetal male. However, PCZ reduction in testosterone levels likely strengthens PCZ AR antagonism in vivo by reducing testosterone competition for the AR and may result in a cumulative effect between the two anti-androgen mechanisms.

The maternal toxicity, evident by reduced weight gain, has been previously reported [9].

Delayed parturition by PCZ has also been reported [7, 8]. Although these data were collected several days before parturition, the reduced serum estradiol levels at GD 18 may be indicative of PCZ affecting the estradiol increase leading up to parturition [26]. Previous findings of aromatase inhibition in vitro [11, 27] have not been substantiated in vivo for either the male or female rat and these data are the first to report a decrease of estradiol in vivo. This indicates that PCZ could delay female pubertal development by inhibiting estradiol production, but it is not clear if the reduced estradiol is due to aromatase (CYP 19) or CYP17 inhibition.

In conclusion, the results of this study suggest that PCZ inhibition of testosterone production contributes to the reproductive malformations in the adult male after maternal exposure. The mechanism of reduced testosterone production does not appear to be the down regulation of genes involved in steroidogenesis, but instead the inhibition of CYP17 enzyme activity.

Amniotic levels of PCZ were supportive of both CYP17 inhibition and PCZ AR binding. Additionally, the CYP17 hydroxylase K_i was considerably lower than the estimated K_i for AR binding, which suggests that the CYP17 inhibition may be more prevalent in the fetal male at lower doses.

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Table 1: Primer set sequences for quantitative real-time RT-PCR.

| <i>Gene</i> | <i>Primer Set</i> |
|---------------------|---|
| StAR | Probe: 5'-Fam-TAG ACC AGC CCA TGG ACA GAC TCT ATG AAG AAC T -BHQ-3' Forward: 5'-AGA AGG AAA GCC AGC AGG AGA-3' Reverse: 5'-TCT CCC ATG GCC TCC ATG-3' |
| CYP11A (P450scc) | Probe: 5'-Fam-AGT ACC CTG GTG TCC TTT ATA GCC TCC TGG G- BHQ-3' Forward: 5'-GGG ACT TAA GGC AGA AGC GA-3' Reverse: 5'-ATG TTC TTG AAG GGC AGC TTG-3' |
| CYP17 (P450c17) | Probe: 5'-Fam-TCC TGG CTT TCC TGG TGC ACA ATC C-BHQ-3' Forward: 5'-ACG GTG GGA GAC ATC TTT GG-3' Reverse: 5'-TGG TCA ATC TCC TTT TGG ATC TTC-3' |

Table 2. Maternal weight and post-implantation loss (mean \pm SEM) among PCZ treatments.

| <i>Dose (mg/kg/day)</i> | <i>Necropsy Wt. (g)</i> | <i>Weight Gain^a(g)</i> | <i>Post-implantation Loss^b(%)</i> |
|-----------------------------|-----------------------------|---------------------------------------|--|
| 0 | 349.4 \pm 10.5 | 33.5 \pm 3.0 | 5.4% \pm 2.0 |
| 7.8 | 341.2 \pm 8.9 | 28.3 \pm 1.4 | 3.7% \pm 1.6 |
| 15.6 | 329.2 \pm 14.2 | 29.2 \pm 2.4 | 1.4% \pm 1.4 |
| 31.3 | 331.2 \pm 9.7 | 25.7 \pm 2.4* | 1.4% \pm 1.4 |
| 62.5 | 319.5 \pm 13.4 | 19.8 \pm 1.8**** | 8.3% \pm 8.3 |
| 125 | 333.1 \pm 10.2 | 20.1 \pm 2.4*** | 7.0% \pm 1.9 |

^a GD14 dam wt. – GD18 wt. (necropsy wt.).

^b resorptions/(fetuses + resorptions).

* p<0.05, *** p<0.001, **** p<0.0001.

Table 3. Summary of PCZ effects on fetal testosterone production from this study and reported incidence of altered development in adult males after maternal exposure.

| Dose (mg/kg/day) | <i>Present Study</i> | <i>Noriega et al. 2005 (PND 125+ Necropsy)</i> | | | |
|---------------------|----------------------------|--|-----------------------|-------------------------------------|--------------------------------------|
| | Testosterone Production | Nipple Retention (%) | Incomplete PPS (%) | Hypospadias or Cleft Phallus (%) | Repro. Organ Effects ^a |
| 0 | - | | | | |
| 7.8 | - | | | | |
| 15.6 | - | | | | |
| 31.3 | (N.S.) | 1.9 | - | - | - |
| 62.5 | ↓ | 2.2 | 2.2 | - | * |
| 125 | ↓ | 15.6 | 18.8 | 12.5 | * |

- No effect at dose tested, (N. S.) decrease not statistically significant, PPS preputial separation.

^a Effects in testis histology (62.5 and 125 mg/kg/day) and reduced ventral prostate, epididymides, and testis weights (125mg/kg/day).

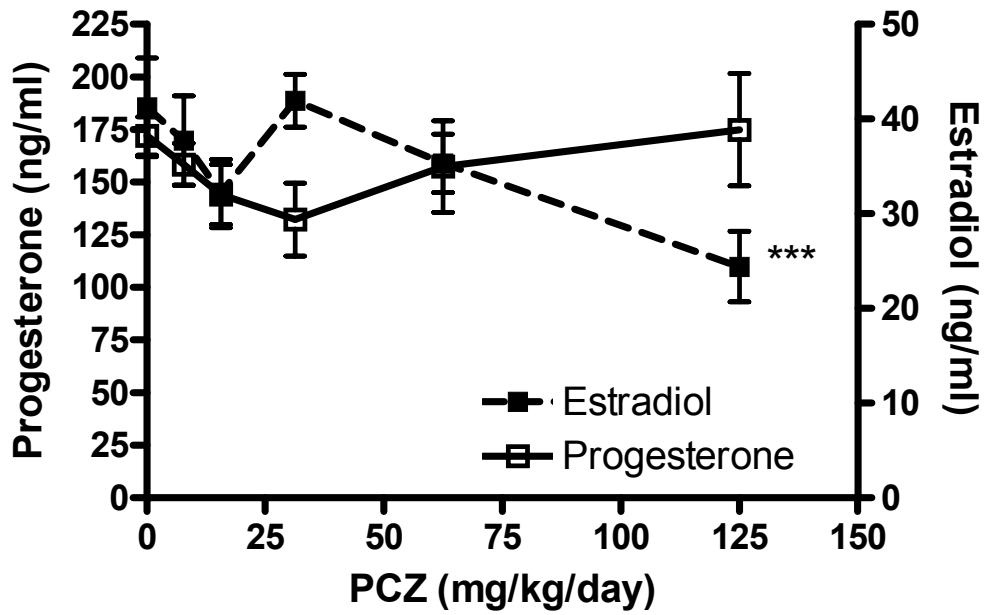


Figure 1. Mean (\pm SEM, $n = 8$) maternal serum estradiol (■) and progesterone (□) levels at GD18 after PCZ treatment (GD14 to 18). *** $p < 0.001$.

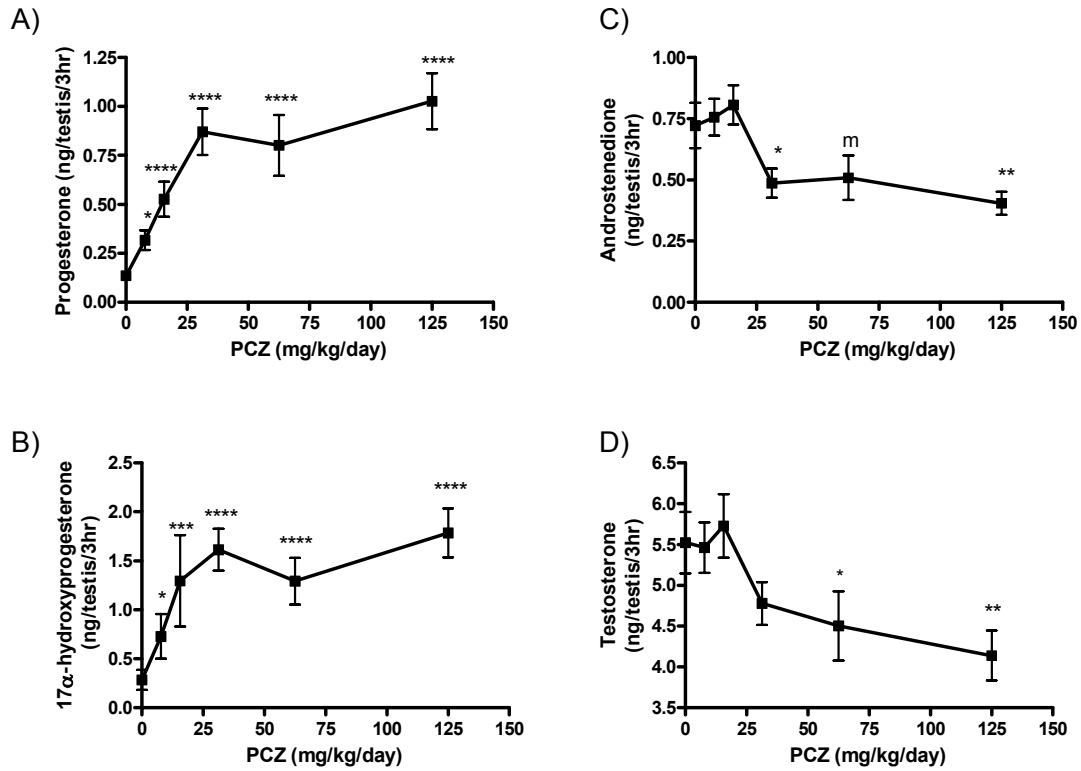


Figure 2. Litter mean (\pm SEM, $n = 7-8$) of ex vivo fetal testis production of progesterone (A), 17 α -hydroxyprogesterone (B), androstenedione (C), and testosterone (D) at GD 18 after a 3 hr incubation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, m: $p=0.0546$.

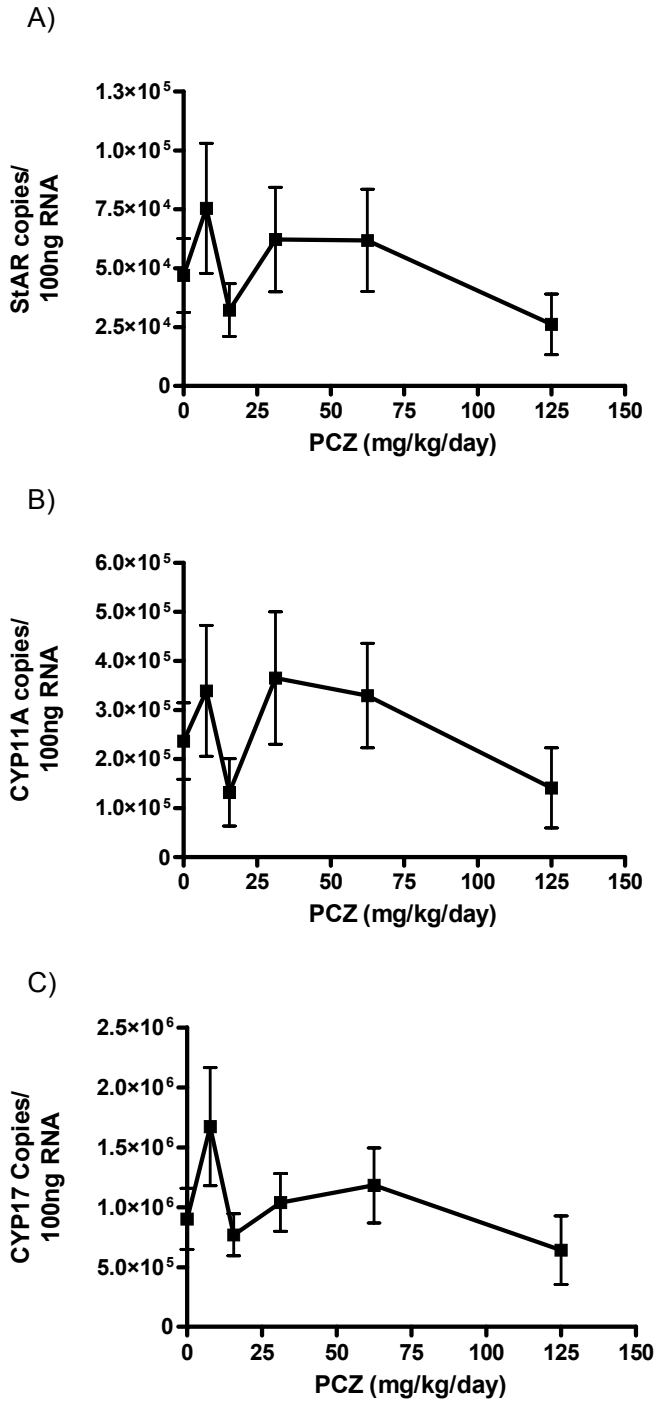


Figure 3. Litter mean (\pm SEM, $n = 5-8$) mRNA levels of StAR (A), CYP 11A (B), and CYP17 (C) in the fetal testes.

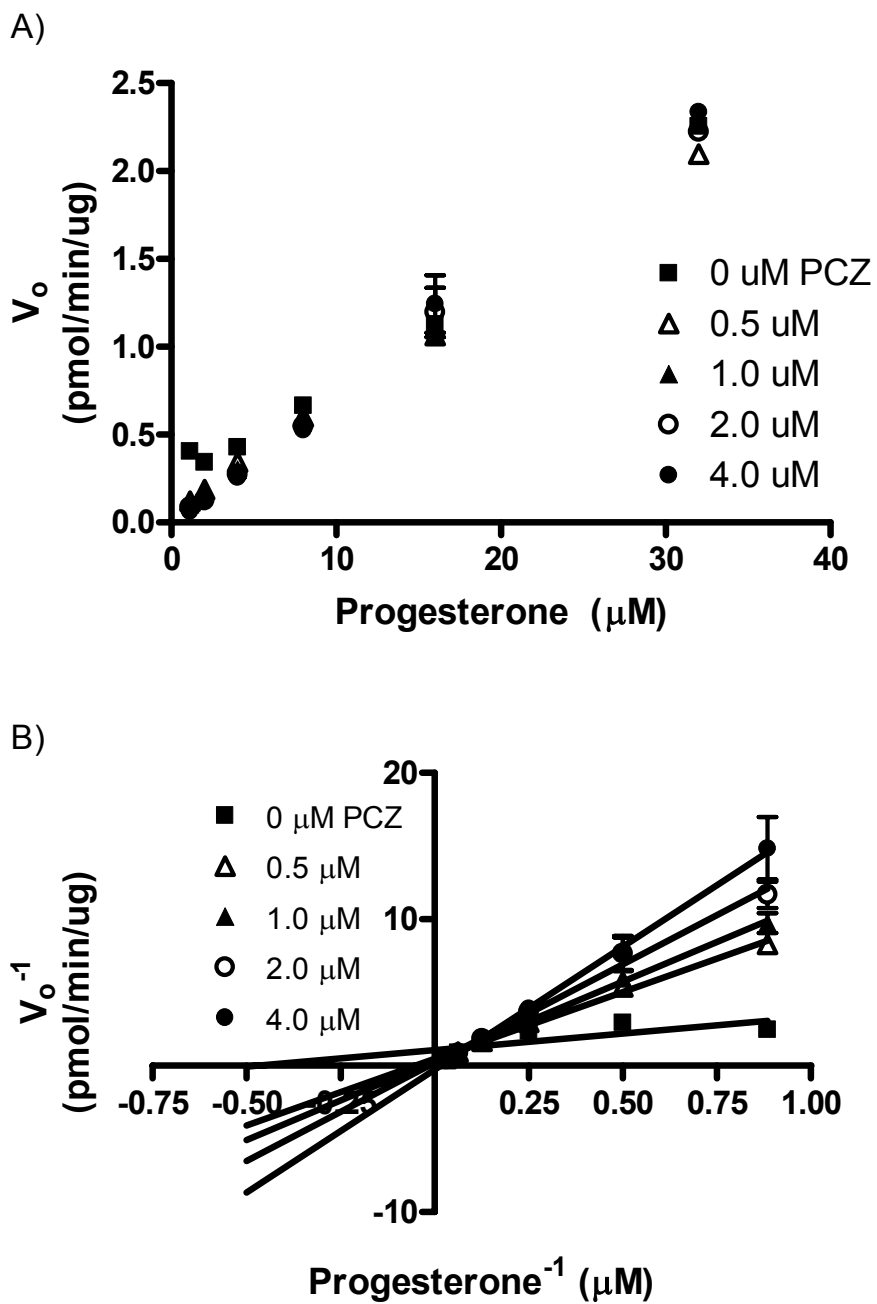


Figure 4. Mean (\pm SEM, $n = 2$) hydroxylase activity of CYP17 at various progesterone (substrate) and PCZ concentrations (A). Lineweaver-Burke (double reciprocal) plot of the same data (B).

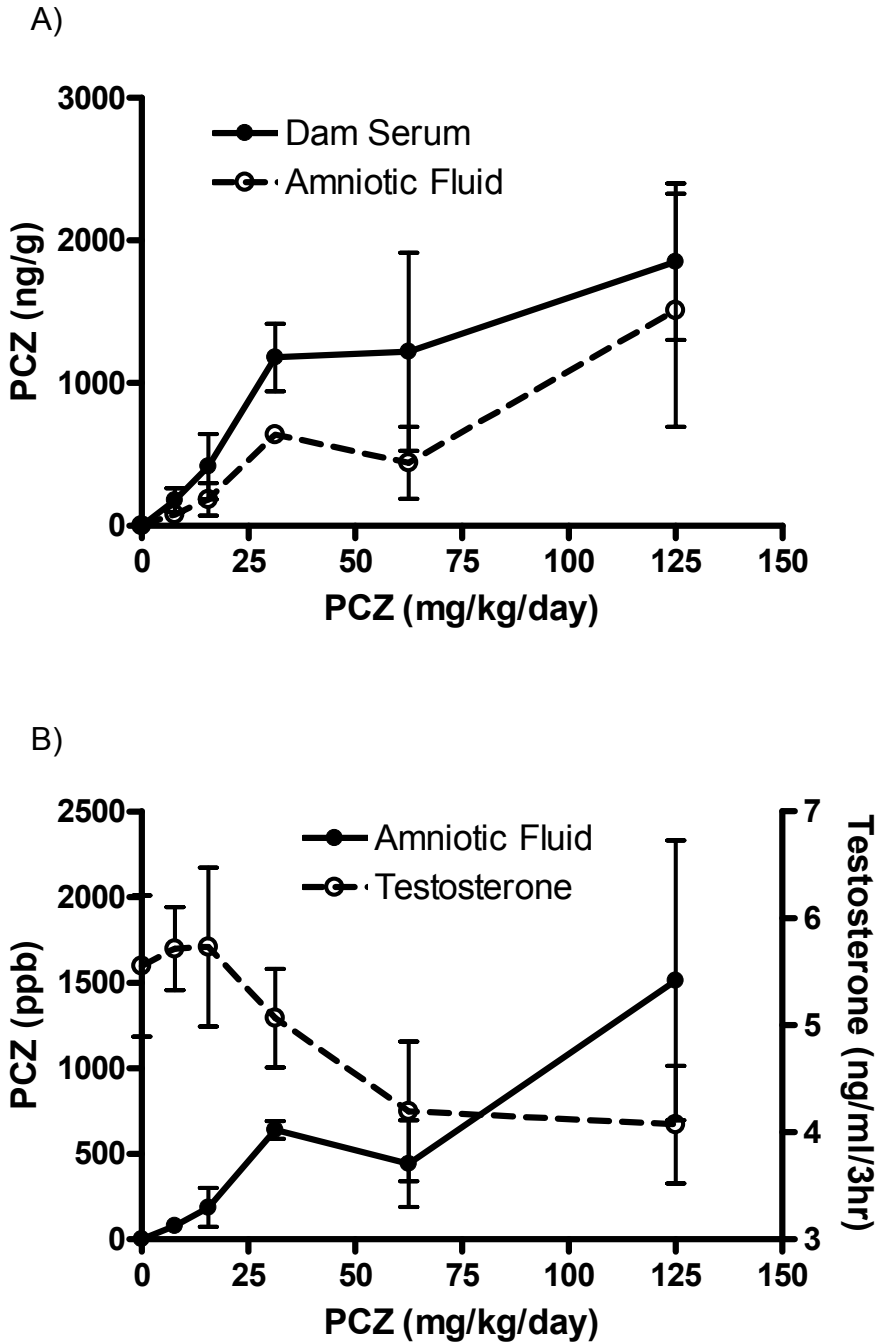


Figure 5. Mean PCZ (\pm SEM, $n = 4$) concentration in maternal serum and amniotic fluid (A). The PCZ concentration (\pm SEM, $n = 4$) within the amniotic fluid and corresponding ex vivo testosterone production from the fetal testes of the same litters (B).

CHAPTER III

The *In Vitro* and *In Vivo* Effects of Triazole Fungicides on Male Rat Gonadal Steroidogenesis

ABSTRACT

Triazole pesticides are related to the imidazole compounds which some are known to inhibit steroidogenesis. The triazoles triadimefon and myclobutanil were hypothesized to inhibit steroidogenesis within a testis culture system and reduce *ex vivo* fetal testosterone production after maternal exposure. Hormone production from adult and neonatal rat testes was measured in the presence of 0, 1, 10, 100 μ M myclobutanil or triadimefon. In addition, *ex vivo* testosterone production from fetal testes was measured after maternal exposure to 10, 50, 100 mg/kg/day myclobutanil from gestational day (GD) 12 – 19. Both chemicals were weak inhibitors of steroidogenesis *in vitro* and the hormone data suggested that they specifically targeted CYP17. However, maternal exposure to myclobutanil did not significantly affect *ex vivo* fetal testosterone production.

Based upon the finding that these chemicals increased serum testosterone in rats, developmental exposure GD 6 – post natal day (PND) 90 to each chemical was hypothesized to disrupt steroid homeostasis and lead to reproductive abnormalities. Time pregnant dams were given feed laced with myclobutanil (100, 500, 2000ppm), propiconazole (100, 500, 2500ppm), and triadimefon (100, 500, 1800ppm) starting on GD 6 and continued in F₁ male offspring. At PND 1, 22, 50, and 92, one male from each litter was necropsied to measure effects on organ weights and hormones. Triadimefon increased serum testosterone at PND 50 and all three chemicals increased serum testosterone at PND 92.

The increased testosterone was hypothesized to be due to increased production via LH stimulation of the testes. Furthermore, this effect was hypothesized not to require exposure during an important organizational event such as sexual differentiation or maturation. To test these new hypotheses, rats were exposed to triadimefon 1800ppm during different life stages (GD6–PND 90, PND 23–53, and PND 60-90). Following exposure, serum testosterone, luteinizing hormone (LH), and intratesticular testosterone were measured. Neither developmental (GD 6–PND 90) nor pubertal (PND 23-53) exposures resulted in elevated testosterone, but the adult (PND 60-90) exposure did increase serum testosterone and intratesticular testosterone. Together these data demonstrate that triadimefon and myclobutanil are weak inhibitors of testicular steroidogenesis *in vitro*, but increase testosterone production *in vivo*, which for triadimefon, is due to increase testis testosterone production.

INTRODUCTION

Conazoles are a family of triazole- or imidazole-based fungicides used for crop protection and pharmaceutical treatment of fungal infections. They inhibit cytochrome P450 (CYP) 51 by competitively binding to the heme component of the enzyme (Ghannoum and Rice 1999). In fungal cells this binding depletes ergosterol, which disrupts membrane function, resulting in cytotoxicity. Conazoles also inhibit other CYPs, and consequently there is concern that inadvertent exposure to agricultural conazoles might inhibit steroidogenesis and adversely affect normal reproduction in humans and other mammalian species (Zarn *et al.* 2003).

The triazoles are similar to the imidazoles which are known to decrease testosterone production (e.g. prochloraz). Previous studies have shown that triazole conazoles can inhibit the CYP19 aromatase conversion of testosterone to estrogen (Andersen *et al.* 2002b; Sanderson *et al.* 2002). However, there are few reports concerning the effects of triazoles on testosterone synthesis. The triazoles hexaconazole and flusilazole inhibit testosterone synthesis in Leydig cell culture and increase the incidence of Leydig cell tumors in rats (FAO/WHO 1990, 1995), but effects of other triazoles on rat steroidogenesis are not known. Understanding how the triazoles myclobutanil and triadimefon affect steroidogenesis would aid the risk assessment for these chemicals.

The study described herein was designed to investigate the effects of myclobutanil and triadimefon on testosterone synthesis. In the first experiment, the effects of mylobutanil and triadimefon on *in vitro* testosterone production from neonatal and adult rat testes were

measured to test the hypothesis that these chemicals inhibit testosterone production. *In vitro* endocrine testing allows for evaluation of a chemical's ability to inhibit the synthesis of steroids, even though the absorption, distribution, metabolism, and elimination (ADME) of the chemical is not accounted for in the testing (Gray *et al.* 1997). A second experiment then tested the hypothesis that maternal exposure to myclobutanil would reduce fetal testosterone production.

Based upon the finding that myclobutanil and triadimefon increased serum testosterone levels in adult male rats after a 14 day exposure (Tully *et al.* 2006), it was hypothesized that developmental exposure to these two chemicals plus another triazole, propiconazole, would increase serum testosterone production and alter reproductive development. To test this hypothesis, serum testosterone levels and reproductive organ weights were measured at various time points during a gestational day (GD) 6 – post-natal day (PND) 90 exposure to three doses of each chemical. Following this experiment, the mechanism behind the increase in serum testosterone was investigated. This effect was hypothesized to be a non-developmental or organizational effect; for example, the increase in testosterone was not due to triadimefon disruption of neuroendocrine programming as the animal develops. Additionally, exposure to triadimefon was hypothesized to increase testosterone levels by increasing testosterone production in the testes via luteinizing hormone (LH) stimulation. These hypotheses were tested by measuring serum hormone and intratesticular testosterone levels after triadimefon exposure during different life stages.

MATERIALS AND METHODS

In Vitro Myclobutanil and Triadimefon Steroidogenesis Study

Sliced ~100mg pieces of adult testes (PND 90 - 100, n = 5-8) and intact neonatal testes (PND 1, n = 5 litters) from Sprague Dawley rats were incubated in 1.5 ml of M199 media (Gibco, Grand Island, NY) supplemented with 0.2% bovine serum albumin (Sigma, St. Louis, MO) and 10% charcoal/dextran treated fetal bovine serum (Hyclone, Logan, UT). Technical grade (>95% purity) myclobutanil (CAS# 88671-89-0, LKT Laboratories Inc., St. Paul, MN) or triadimefon (CAS# 43121-43-3, Bayer CropScience, Kansas City, KS) was added to the incubation media for a final concentration of 1, 10, or 100 μ M. Each of these test chemicals was premixed with ethanol to aid in dilution, such that the final ethanol volume was 0.05% of total culture volume. Human chorionic gonadotropin (hCG) (Sigma, St. Louis, MO) was added at 100 mU/ml (hCG, an LH receptor agonist) to stimulate testosterone production. Positive control medium (hCG minus test chemical) and negative control medium (minus hCG and test chemical) both contained 0.05% ethanol. Tissue was incubated at 34°C in 2.0 ml siliconized tubes rotated at ~10 rpm. At three time points, 0.5, 1.5, and 2.5 h., the media was removed and replenished with fresh media containing the appropriate chemical. All treatments were replicated three times from each adult rat testes and the average of the three replicates was used for analysis. The testes from each litter were pooled and each testis was then assigned a specific treatment such that the entire litter was exposed to all the treatments. Treatments were not replicated within each litter due to litter and testis size constraints. Data were analyzed using the SAS GLM procedure (SAS Institute Inc., Cary, NC). Since each adult or litter was exposed to all eight treatments within the testis culture assay, animal or litter was used as a blocking factor within analyses of variance.

Myclobutanil Gestational Exposure Study

Time pregnant Sprague Dawley rats were received on GD 1 - 5 (GD 0 = day after mating) from Charles River Laboratories (Raleigh, NC) and housed in the Environmental Protection Agency's Reproductive Toxicology Division animal facility. Animals were fed Purina Rat Chow 5002 and watered *ad libitum*. Environmental conditions were 22-23 °C, 50-60% humidity, and a 12L:12D light cycle. Prior to dosing, animals were weight ranked and assigned to dose groups to minimize differences in means and variance among treatment groups. The animal use protocol for this study was approved by the National Health and Environmental Effects Research Laboratory's institutional animal use and care committee. Myclobutanil (CAS#: 88671-89-0) was delivered to rats by gavage dissolved (suspended at 100 mg/kg/day) in 15% Alkamuls EL-620 in a volume of 5.0 ml/kg body weight. Dams were dosed daily from GD 12 to 19 (n = 9/dose) with 0, 10, 50, 100 mg/kg/day myclobutanil.

On GD19 dams were anesthetized with CO₂ and then decapitated. Trunk blood was collected for estradiol measurement to determine if treatment affected aromatase activity.

The uterine tract was removed and each fetus was quickly dissected out, weighed, and placed in a tissue culture dish on ice. Each fetus was then sexed under a dissecting microscope. The right testis was removed from three males of each litter and incubated separately for 3 hr at a humidified 37 °C in a 24-well plate containing 500 ul of Gibco M199 media in each well (Invitrogen, Carlsbad, CA). Media was supplemented with 10% dextran-charcoal stripped fetal bovine serum (Hyclone, Logan, UT) and 100 mU/ml hCG (Sigma, St. Louis, MO). The

plate was rocked during incubation and after 3 hrs the media was removed and frozen at -90 °C for hormone measurement.

Multi-Triazole Developmental Exposure Study

Time pregnant Wistar Hans IGS rats were received from Charles River laboratories between GD 1 – 3. Animals were weight ranked and divided into treatment groups to equalize mean weights and variance. Starting on GD 6, a Purina rat chow 5002 diet laced with different chemicals was provided to the appropriate treatment group: control feed containing the acetone solvent, myclobutanil (100, 500, 2000ppm), propiconazole (100, 500, 2500ppm), and triadimefon (100, 500, 1800ppm). Animals were weighed weekly and the amount of feed consumed was measured to calculate an average dose. The F₁ males were weaned on PND 21 and continued to be fed the appropriate chemical. On PND 1, 22, 50, and 92, one male from each litter was necropsied for measurement of selected organ weights. After anestizing each animal with CO₂, blood was collected via cardiac puncture for hormone measurement on PND 50 and 92. Additionally a subset of animals at PND 50 and 99 were tail bled to determine if the method of blood collection affected results. AGD was measured in PND 0 males and the progression of PPS in immature males was measured daily by Amber Goetz to determine if treatment affected these androgen sensitive endpoints. Insemination success of young adult males was evaluated by Amber Goetz to determine if the triazoles affected this hormone sensitive event.

Triadimefon Multi-Exposure Study

Developmental and Pubertal Exposure. Sixty-five timed pregnant Wistar rats were delivered from Charles River Laboratories between gestational day (GD) 0 and 2 (sperm positive = GD 0). Starting on GD 6, the dams were provided Purina rat chow 5002 laced with 1800 ppm triadimefon (preprepared feed was a gift from Bayer CropScience, Kansas City, KS).

Triadimefon was used because it has a stronger effect than myclobutanil on serum testosterone levels in the multi-triazole study, and the effect has been identified in several strains, including Sprague Dawley (Tully *et al.* 2006) and Wistar (FAO/WHO 1985).

Twenty-two dams were given control feed containing vehicle (acetone) and forty-three dams were given feed laced with 1800ppm of triadimefon. Animals were weighed on a weekly basis and dose was determined by measuring food consumption. On the day of parturition, pups were sexed, weighed, and tattooed for later identification. Anogenital distance was measured in male pups to determine if the chemical acted in an anti-androgenic manner.

Dams that did not deliver were necropsied for measuring post-implantation loss. The uterine horn was removed and stained with a 2% solution of ammonium sulfide to quantify implantation sites. All pups were weighed again on PND 8 and then the litters were culled to no more than 10 pups/dam in order to equalize growth rates. Male pups were weighed and weaned to two to a cage on PND 21. Immature males from half of the remaining control litters (9 out of 19 litters, 43 pups total) were then started on T1800 feed on PND 23. These animals were given a pubertal triadimefon exposure (PND 23 – 53) to determine chemical effects on pubertal development. All animals were decapitated on PND 90 for organ weight and serum and testicular hormone measurements.

In the triadimefon exposure group of the multi-triazole study insemination success was significantly reduced (Goetz *et al.* 2006). To test if this effect was due to altered pubertal development six adult male rats (PND 76-78) from each exposure (Control, T1800 PND 23 – 53, and T1800 GD 6 – PND 90) were housed with a young adult female at proestrus for four hours in wire mesh cages. At the end of the four hours, plugs underneath the cages were counted and sperm in the uterine horn was measured. Another 8 - 9 male rats from the control and pubertal exposure treatment groups were housed with young adult females (unexposed to chemical) overnight (15 hrs) to determine if treatment of males affected pre- and post-implantation success. Females were necropsied on GD 15 and the number of fetuses, implants (stained with 2% solution of ammonium sulfide), and corpus luteum (CL) were counted.

Adult Triadimefon Exposure. A third triadimefon exposure was conducted in adult rats to determine if the effect of elevated testosterone required exposure during development. Starting on PND 60, male Wistar Han IGS rats (n = 15/treatment) were fed Purina rat chow 5002 containing 1800 ppm triadimefon plus vehicle acetone (preprepared feed was a gift from Bayer CropScience, Kansas City, KS) or vehicle control alone. Treatment lasted 30 days, during which time feed was weighed on a weekly basis to determine dose. Animals were tail bled 14 days into dosing for testosterone measurements. On day 30 (PND 90) animals were decapitated between 08:30 and 10:30. Trunk blood was collected for serum measurements of testosterone, estradiol, LH, and prolactin. Liver, epididymis, ventral prostate, seminal vesicle, and pituitary were weighed, and the testes were weighed and frozen. The frozen right testis was homogenized in cold Dulbecco's PBS (Gibco, Grand

Island, NY) with an Ultra-Turrax T25 homogenizer (Janke-Kunkel IKA, Boutersem, Belgium), and centrifuged at 4°C for 10 min at 4000 RCF using a Beckman J2-21M centrifuge. Supernatant was then centrifuged with a 5417R centrifuge (Eppendorf, Westbury, NY) at 20,000 RCF for 10 min at 4°C. Supernatant was collected and stored at -80°C for intratesticular testosterone measurements.

Hormone Measurements. Levels of testosterone, androstenedione, 17 α -hydroprogesterone, and progesterone were measured in the media using coat-a-count ¹²⁵I radioimmunoassay kits (Diagnostic Products Corporation, Los Angeles, CA). Serum LH was measured using the rat disassociation enhanced lanthanide fluorimetric immunoassay (DELPHIA) (Haavisto *et al.* 1993). Serum PRL was measured by radioimmunoassay using materials supplied by the National Hormone and Pituitary Agency for LH and PRL: iodination preparation I-6; reference preparation RP-3; and antisera S-9. Iodination material was radiolabeled with ¹²⁵I (Dupont/New England Nuclear) by a modification of the chloramine-T method (Greenwood *et al.* 1963). Con6 control standards (Diagnostic Products Corporation, Los Angeles, CA) were used to verify assay quality for steroid hormone measurements. Lactate dehydrogenase (LDH) levels in the media, an indicator of cytotoxicity, were measured using an LDH detection kit (Roche Diagnostics, Indianapolis, IN).

Statistical analysis. Data were analyzed using the SAS Proc GLM procedure (SAS Institute Inc., Cary, NC). Data were log₁₀ transformed to normalize heterogeneity of variance. AGD and liver weights were analyzed using body weight as a covariate. PPS was analyzed using

the initial body weight (PND 23) as a covariate. Statistical significance between control and treatment was set at $p < 0.05$.

RESULTS

In Vitro Myclobutanil and Triadimefon Steroidogenesis Study

This study tested the hypothesis that these two triazoles are inhibitors of testis steroidogenesis. At the highest chemical concentration in the adult testis incubation, inhibition was less than 50% for both chemicals at the first time point and increased to 63-68% by triadimefon and slightly over 50% by myclobutanil (Figures 1A). The inhibition of testosterone production within the neonatal testis culture generally decreased after each successive time point for both chemicals. The hormone variability among the time points at the three chemical concentrations was higher in the neonatal testis culture, which is most likely due to the use of the litter vs. an adult individual for statistical analysis. The decrease in androstenedione production (Figures 2A, 2B) and increase in 17α -hydroxyprogesterone (Figures 3A, 3B) and progesterone (Figures 4A, 4B) production implies that CYP17 was inhibited. LDH or this method of LDH detection was not a good marker for cell toxicity due to the high variability in LDH levels in the adult testis incubations and LDH not detected in the neonatal testis culture media. No significant difference in LDH among the treatment groups was detected, with the exception of 100 μ M triadimefon at the 2.5 h time point, when LDH levels were significantly lower than the control ($p < 0.011$, data not shown).

Myclobutanil Gestational Exposure Study

Myclobutanil had no significant effect on fetal weights or hormone measurements (Table 1). The decrease in litter size was almost statistical significant at 100mg/kg/day myclobutanil suggesting that it might be decreasing post-implantation survival at this dose. Testosterone production was slightly reduced, but this effect was not statistically significant (Table 1). Since testosterone production was unaffected, the hypothesis that myclobutanil affects fetal steroidogenesis through maternal exposure was not supported.

Multi-Triazole Developmental Exposure Study

The quantification of dose is detailed in (Goetz *et al.* 2006) and was observed to vary over the exposure period. Maternal exposure was elevated due to high consumption. Dose was initially elevated in the offspring, but decreased as the offspring aged. Serum testosterone from necropsied animals was unaffected (Figure 5A), but serum testosterone was significantly elevated (nearly three fold) in tail bled triadimefon 500ppm treated animals (Figure 5B). When the data from the tail bleed and necropsy were combined and analyzed in a two-way ANOVA, there was no significant interaction effect, which suggests that the method of collection did not affect treatment results. In the combined data there was no statistically significant effect, which indicates that with higher animal numbers the testosterone effect in the triadimefon 500ppm treatment may not be robust (Figure 5C). Since there was high mortality in the 1800ppm triadimefon and 2000ppm myclobutanil dose groups, a second study was conducted with these dose groups to gather data. In this second study, serum testosterone was not statistically affected at PND 50 although triadimefon

1800ppm elevated serum levels (Figure 5D). PPS was not delayed except in the triadimefon 1800ppm group (analyzed without initial body weight as a covariate).

At PND 92 all three chemicals significantly increased serum testosterone levels in the necropsied animals (Figure 6A), but effects in the PND99 tail bled animals were statistically marginal (Figure 6B). PND 92 data and PND 99 data were combined for a two-way ANOVA and again there was no significant interaction between method of collection and hormone results. The elevated testosterone by treatment remained statistically significant in the combined data (Figure 6C), suggesting the effect is more robust in older animals. There was no significant effect on serum estradiol or LH levels at PND 92, PND 99, or when data was combined (data not shown). In the second study, serum testosterone was significantly decreased by 100ppm triadimefon and increased by 1800ppm triadimefon (Figure 6D). Serum LH was significantly increased in the 100ppm triadimefon group which corresponds to the significant decrease in testosterone (Figure D) and suggests a feedback response.

There were some unexpected increases in weights within the treatment groups. Myclobutanil and triadimefon increased body weights at PND 22 (Table 2). This effect did not last as the animals aged and animals in the high dose group of myclobutanil and triadimefon had a significantly reduced body weight at PND 50 and 92. The liver weights of the animals were significantly increased in the high dose groups of all three chemicals either at PND 50 or 92, but not at PND 22 (Table 3). This implies that chemical exposure during lactation was blunted due to maternal clearance. Testicular weight was increased in several treatment groups, but prevalence of the effect decreased as the animals aged (Table 4). The ventral

prostate and seminal vesicle were decreased in the triadimefon 1800ppm treatment group in the 1st study ($p = 0.0021$ and $p = 0.0057$ respectively), but this effect was not repeated in the 2nd study (data not shown).

Triadimefon Multi-Exposure Study

Developmental and Pubertal Triadimefon Exposure. The purpose of this study was to test the hypotheses that triadimefon exposure would increase testosterone production within the testis via LH stimulation and this effect did not require exposure during a critical period of development. The triadimefon dose during the different exposures varied depending on the age of the animal. Average maternal dose was 114.4 mg/kg/day (± 12.7 S.D.) during gestation and rose to 267.8 mg/kg/day (± 57.1 SD) during lactation (Figure 7A). The increased food consumption during lactation to meet energy requirements probably contributed to the elevated dose. Following weaning, there were three exposure groups assigned. A control group of F₁ males, F₁ males which were exposed to triadimefon starting on GD 6, and a new group of control F₁ males that were exposed to triadimefon from PND 23 – 53 (pubertal exposure group). The average dose for the pubertal exposure group was 216.2 (± 26.1 S.D.) mg/kg/day which was close to the 233.2 (± 34.1 S.D.) mg/kg/day dose during the same period for the animals exposed starting on GD 6 (developmental exposure) (Figure 7C). The dose was high for both groups after weaning due to high consumption and low body weight of the immature males, but clearly drops as the animals aged (Figure 7C).

Maternal exposure to triadimefon 1800ppm had profound toxicity on the dam and F₁ offspring. Triadimefon reduced maternal body weight (Figure 1B), greatly increased litter

mortality, and reduced F₁ male body weights at PND 0 and 8 (Table 5). F₁ male ano-genital distance (AGD) was increased by triadimefon (Table 5). As the F₁ males aged, the triadimefon exposed animals grew at a slower rate (Figure 7D). The F₁ males that were only exposed from PND 21 – 53 (pubertal exposure) lost weight during exposure, but slowly recovered once they were back on control feed (Figure 7D). Preputial separation was significantly delayed in both triadimefon exposures, and weight at PPS was decreased although this was not statistically significant (Table 5). The reduced growth due to treatment (Figure 7D) probably delayed puberty.

F₁ Males exposed to triadimefon starting on GD 6 did not inseminate females, which replicates the previous observation. Males exposed to triadimefon during pubertal development had a reduced insemination rate (Table 6) suggesting that pubertal exposure might be contributing to this effect. Since developmentally exposed males were unable to inseminate females, pre- and post-implantation success was not evaluated for these animals. Pubertal exposure to triadimefon did not affect pre- and post-implantation success, suggesting the sperm quality was unaffected by treatment (Table 6).

Triadimefon treatment did affect the weights of several organs in the adult male, which implies a general and reproductive toxicity. At PND 90 liver weight was increased in males exposed throughout the study and males exposed only during pubertal development (Table 7). The ventral prostate, seminal vesicle, epididymides, and testes weights were reduced in males treated from GD 6 – PND 90. The weight of the ventral prostate and seminal vesicle was reduced in males treated during puberty, but there was no statistically significant effect.

The lack of a statistically significant effect on the reproductive organs might be due to the 36 days the animals had to recover from triadimefon exposure (PND 54 to PND 90). There was an expectation that serum testosterone levels would be increased after triadimefon treatment, but there was no treatment effect on serum testosterone at PND 53 or PND 90 (Figure 8A, 8B). Intratesticular testosterone levels were elevated in the developmentally exposed animals (GD 6 – PND 90) at PND 90, but this effect was not statistically significant (Figure 8C). Serum LH and estradiol were also unaffected by treatment (Figure 8D, 8E).

Adult Triadimefon Exposure. A third triadimefon exposure was conducted in adult males to determine if serum testosterone could be elevated through a non-developmental mechanism. In this study the average ingested dose was calculated to be 126.4mg/kg/day (\pm SD 7.1 mg/kg/day). Feed intake of treated males was on average 10% less than the control males over the course of dosing and body weight of treated animals was significantly decreased (8-10%) compared to the controls (data not shown). Four individuals (one control and three treated) were removed from the study due to factors not related to treatment. Body weights were reduced and liver weights were increased at PND 90 suggesting a general toxicity to the animal (Table 8). It is unclear if triadimefon acted in an anti-androgen manner.

Epididymides weight was reduced, but not ventral prostate or seminal vesicle weight. Serum testosterone levels at two weeks into dosing were unaffected (Figure 9A), but increased nearly three fold at PND 90 (Figure 9B) with a concomitant increase in intratesticular testosterone (Figure 9C). This finding supports the hypothesis that the treatment increases testosterone by increasing testicular testosterone production and the effect does not require exposure during a critical period of development. LH and estradiol levels were elevated, but

this effect was not statistically significant (Figure 9D, 9E) so it is not clear if these two hormones played any role in stimulating testosterone production. Prolactin was measured as an indicator of triadimefon induced disruption of dopamine controlled hypothalamic activity. Serum prolactin levels were expected to decrease, but instead were non-statistically significantly elevated (Table 8).

DISCUSSION

These *in vitro* data demonstrate that both myclobutanil and triadimefon inhibited testosterone production, albeit weakly. The *in vitro* pattern of hormone production indicates that CYP17 activity was inhibited by both chemicals in the neonatal and adult testis. This has been demonstrated with several imidazole compounds (Ayub and Levell 1987c; Engelhardt *et al.* 1991), and the triazoles hexaconazole (Lloyd 1991), and flusilazole (FAO/WHO 1995). This is also similar to the effects of prochloraz in the previous chapters. The inhibition of testosterone synthesis by hexaconazole and flusilazole was used to explain the increased incidence of Leydig cell tumors, and a similar hypothesis was made that ketoconazole would induce tumors due to the same mechanism if tested under a US EPA criteria for maximum tolerated dose and length of exposure (Cook *et al.* 1999b). Since triadimefon and myclobutanil also inhibit CYP17, but are not reported to induce Leydig cell tumors, it may be a matter of differences in the strength of inhibition that explains the production of tumors. There might have been chemical induced cytotoxicity although none was detected in the testis incubations. However, the continued increase in 17 α -hydroxyprogesterone and progesterone production over the time points implies cytotoxicity, if present, was minimal at these doses.

The lack of an effect on fetal testosterone production after maternal exposure to myclobutanil did not support the hypothesis that this chemical inhibits fetal testicular steroidogenesis *in vivo*. The *in vitro* data suggests that high levels would be needed to inhibit steroidogenesis, but these levels probably were not reached due to maternal clearance of myclobutanil. Furthermore, there was no *in vivo* evidence of inhibited fetal testosterone production since F₁ males AGD increased within the multi-triazole and multi-lifestage exposure studies.

The data from the multi-triazole experiment supported the hypothesis that developmental exposure would result in disrupted steroidogenesis. Testosterone levels were generally increased at PND 92/99 and not at PND 50 implying that this effect occurs in older animals. Similar results have been reported in adult Wistar rats after a developmental exposure to triadimefon (FAO/WHO 1985) and both myclobutanil and triadimefon were reported to increase testosterone in Sprague Dawley rats after a fourteen day exposure (Tully *et al.* 2006). This is the first report of propiconazole increasing serum testosterone, which was not found after the fourteen day exposure.

The lack of an effect on serum testosterone showed that pubertal exposure (PND 23- 53) to triadimefon did not alter any organization event that occurs during this time period. No effect on serum testosterone in the GD 6 – PND 90 triadimefon exposed animals was unexpected and did not support the hypothesis tested in the multi-triazole study that treatment disrupts homeostasis of testosterone production. It is unclear why an effect wasn't detected since there were more litters and offspring in the multi-exposure studies than in the

multi-triazole study and triadimefon exposure at a similar dose increased serum testosterone in previous studies (FAO/WHO 1985; Tully *et al.* 2006). The effects of triadimefon exposure on organ weights, insemination success, AGD, and PPS were similar to the multi-triazole study, which suggests that the observation triadimefon 1800ppm robustly increases testosterone might have been incorrect.

Unlike the other exposures, the thirty day adult exposure to triadimefon did produce an increase in serum testosterone. This finding supported the hypothesis that the serum testosterone increase by triadimefon is not due to an altered programming event. In the adult exposure there was also an increase in intra-testicular levels of testosterone revealing that increased production in the testis was driving the increase in serum testosterone. This supports the hypothesis that the testis is producing more testosterone, but it could not be determined if the increased production was through LH stimulation. It is possible that the elevated LH levels, although not statistically significant, might be driving the increased testosterone production. The unchanged serum LH levels does provide evidence for interference with the hypothalamic-pituitary-gonadal (HPG) axis, since if the latter were functioning correctly, then the elevated testosterone levels would be expected to reduce LH levels (negative feedback loop).

Based upon *in vitro* aromatase and receptor binding assays, triadimefon could be acting as an aromatase inhibitor (Trosken *et al.* 2004) or androgen receptor antagonist (Okubo *et al.* 2004) to disrupt testosterone regulation by blocking the negative feedback in the HPG axis. The imidazole prochloraz has similar dual actions in significantly inhibiting aromatase

activity and inhibiting the androgen receptor *in vitro* and *in vivo* (Andersen *et al.* 2002b; Vinggaard *et al.* 2002b). However, *in vivo* evidence for aromatase inhibition or androgen receptor antagonism by triadimefon is lacking. Serum estradiol increased slightly in the current study, and previous reports with males and female rats have not shown a decrease in estradiol by triadimefon or myclobutanil (Goetz *et al.* 2006; Rockett *et al.* 2006).

Additionally, it is unclear if the evidence from these studies supports the hypothesis that triadimefon acts as an androgen receptor antagonist. The general toxicity at the high dose of myclobutanil and triadimefon in which androgen sensitive organ weights were decreased makes any conclusion about an anti-androgen mechanism difficult. In addition, the increase in F₁ male AGD and the lack of an effect on PPS except in cases of great weight loss implies that these triazoles do not act as androgen receptor antagonists *in vivo*.

Triadimefon might be altering the balance of neurotransmitters or activating their receptors within the hypothalamus. Triadimefon exposure has been shown to affect behavior presumably by altering neurotransmitters within the brain (Crofton *et al.* 1988; Walker and Mailman 1996; Reeves *et al.* 2004b, a). If triadimefon is affecting neurotransmitters within the hypothalamus, then this mechanism could be disrupting the HPG axis. Although it does not appear that triadimefon is acting as an indirect dopamine agonist as suggested in the behavioral studies. Prolactin was slightly increased by treatment when it was expected to decrease (Waeber *et al.* 1983). Support for the altered neurotransmitter hypothesis is lacking for the other two triazoles tested. Myclobutanil has not been tested for behavioral effects, but propiconazole, which increased serum testosterone after developmental exposure (Goetz *et al.* 2006), does not induce behavioral changes (Crofton 1996). It could be the case that the

other triazoles affect the hypothalamic regulation of testosterone, but triadimefon is the only one potent enough to induce behavioral changes.

In summary, a high concentration of triadimefon or myclobutanil was needed to decrease testosterone production *in vitro*. Maternal exposure to myclobutanil did not affect fetal testosterone production and both chemicals increased F₁ male AGD which suggests that inhibition of fetal testosterone production does not occur *in vivo*. Increased serum testosterone in the adult male rat was observed in a multi-triazole study, but the effect was not repeated in a GD 6 – PND 90 or PND 23 – PND 53 1800ppm triadimefon exposure. Adult exposure to triadimefon 1800ppm did increase serum and intratesticular testosterone, which supports the hypothesis that this effect is driven by action on the testis and does not require exposure during a critical period of development (i.e. gestation, lactation, or puberty). The mechanism behind the increased testosterone production was not elucidated, but might involve a disruption of the HPG axis.

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Table 1: Summary of myclobutanil effects (average \pm SEM) on maternal and litter endpoints.

| <i>Myc (mg/kg/day)</i> | <i>Dam Body Wt. (g)</i> | <i>Dam Liver Wt. (g)</i> | <i>Fetal Male Wt (g)</i> | <i>Fetal Female Wt. (g)</i> | <i>Litter Size</i> | <i>Dam Estradiol (ng/ml)</i> | <i>Fetal Testosterone (ng/ml/3hr)</i> |
|----------------------------|---------------------------------|----------------------------------|----------------------------------|-------------------------------------|------------------------|--------------------------------------|---|
| 0 | 350.3 \pm 9.1 | 14.66 \pm 0.51 | 2.44 \pm 0.06 | 2.28 \pm 0.06 | 12.9 \pm 0.7 | 5.94 \pm 1.42 | 64.61 \pm 6.54 |
| 10 | 346.1 \pm 6.9 | 14.55 \pm 0.37 | 2.44 \pm 0.05 | 2.29 \pm 0.04 | 12.6 \pm 0.7 | 7.36 \pm 1.08 | 61.77 \pm 3.38 |
| 50 | 344.1 \pm 11.5 | 14.86 \pm 0.68 | 2.47 \pm 0.03 | 2.30 \pm 0.03 | 12.4 \pm 0.4 | 2.90 \pm 1.09 | 56.83 \pm 4.94 |
| 100 | 340.8 \pm 8.9 | 14.79 \pm 0.58 | 2.49 \pm 0.03 | 2.37 \pm 0.03 | 10.8m \pm 1.1 | 7.57 \pm 1.40 | 56.94 \pm 4.42 |

m: p=0.0509

Table 2: Summary of treatment effects on F₁ body weight (average ± SEM) at different ages within the multi-triazole study.

| <i>Treatments</i> | <i>PND 1</i> | <i>PND 22 (g)</i> | <i>PND 50 (g)</i> | <i>PND 92 (g)</i> |
|-----------------------------|--------------|-------------------|-------------------|-------------------|
| Control | N/A | 45.6 ± 1.3 | 214.2 ± 5.3 | 365.7 ± 7.4 |
| M100 | N/A | 50.9 ± 1.5* | 208.0 ± 5.4 | 365.3 ± 10.0 |
| M500 | N/A | 52.2 ± 2.1** | 201.4 ± 5.2 | 356.0 ± 8.8 |
| M2000 | N/A | 47.7 ± 1.5 | 196.0 ± 4.6* | 325.0 ± 16.0** |
| P100 | N/A | 50.8 ± 1.5* | 218.2 ± 4.8 | 369.2 ± 8.6 |
| P500 | N/A | 51.8 ± 1.7** | 206.7 ± 7.3 | 368.1 ± 10.0 |
| P2500 | N/A | 45.4 ± 1.7 | 199.8 ± 4.8 | 343.5 ± 5.1 |
| T100 | N/A | 48.8 ± 1.2 | 202.6 ± 9.8 | 366.0 ± 7.2 |
| T500 | N/A | 48.0 ± 0.9 | 201.3 ± 3.9 | 349.2 ± 8.7 |
| T1800 | N/A | N/A | N/A | 264.0 ± 10.0**** |
| 2nd Study | | | | |
| Control | N/A | N/A | N/A | 373.2 ± 17.2 |
| T1800 | N/A | N/A | N/A | 278.7 ± 16.5** |

* p < 0.05, ** p < 0.01, **** p < 0.0001

Table 3: Summary of treatment effects on F₁ male liver weight (average ± SEM) at different ages within the multi-triazole study^a.

| <i>Treatments</i> | <i>PND 1</i> | <i>PND 22 (g)</i> | <i>PND 50 (g)</i> | <i>PND 92 (g)</i> |
|-----------------------------|--------------|-------------------|-------------------|-------------------|
| Control | N/A | 1.89 ± 0.12 | 10.84 ± 0.30 | 15.24 ± 0.42 |
| M100 | N/A | 2.01 ± 0.10 | 10.43 ± 0.33 | 13.57 ± 0.56 |
| M500 | N/A | 2.28 ± 0.15 | 10.13 ± 0.27 | 14.59 ± 0.85 |
| M2000 | N/A | 2.04 ± 0.14 | 11.04 ± 0.32**** | 14.50 ± 0.83 |
| P100 | N/A | 2.13 ± 0.09 | 10.96 ± 0.39 | 14.90 ± 1.01 |
| P500 | N/A | 2.01 ± 0.13 | 10.73 ± 0.47 | 16.59 ± 0.50 |
| P2500 | N/A | 1.92 ± 0.12 | 12.67 ± 0.36**** | 15.43 ± 0.81**** |
| T100 | N/A | 2.01 ± 0.24 | 10.17 ± 0.64 | 14.12 ± 0.70 |
| T500 | N/A | 2.04 ± 0.06 | 10.74 ± 0.26** | 13.78 ± 0.42 |
| T1800 | N/A | N/A | N/A | 13.50 ± 0.52** |
| 2nd Study | | | | |
| Control | N/A | N/A | N/A | 13.68 ± 1.05 |
| T1800 | N/A | N/A | N/A | 13.64 ± 1.01** |

^a body weight at necropsy was used as a covariate for analysis.

** p < 0.01, **** p < 0.0001

Table 4: Summary of treatment effects on F₁ testes weight (average ± SEM) at different ages within the multi-triazole study.

| <i>Treatments</i> | <i>PND 1^a (mg)</i> | <i>PND 22 (mg)</i> | <i>PND 50 (g)</i> | <i>PND 92 (g)</i> |
|-----------------------------|-------------------------------|--------------------|-------------------|-------------------|
| Control | 3.07 ± 0.24 | 257.6 ± 18.0 | 2.247 ± 0.116 | 3.530 ± 0.124 |
| M100 | 3.93 ± 0.27** | 301.3 ± 8.5* | 2.448 ± 0.073 | 3.246 ± 0.227 |
| M500 | 3.46 ± 0.17 | 326.9 ± 16.7** | 2.320 ± 0.190 | 3.296 ± 0.142 |
| M2000 | 3.90 ± 0.80m | 312.0 ± 18.9* | 2.383 ± 0.142 | 3.418 ± 0.181 |
| P100 | 3.03 ± 0.12 | 294.9 ± 14.2m | 2.577 ± 0.113m | 3.385 ± 0.130 |
| P500 | 3.70 ± 0.22m | 301.7 ± 0.24* | 2.615 ± 0.144* | 3.747 ± 0.164 |
| P2500 | 3.14 ± 0.13 | 281.1 ± 16.7 | 2.455 ± 0.046 | 3.474 ± 0.114 |
| T100 | 3.85 ± 0.15m | 281.5 ± 11.4 | 2.612 ± 0.092* | 3.309 ± 0.070 |
| T500 | 3.43 ± 0.22 | 309.5 ± 11.7* | 2.541 ± 0.067 | 3.532 ± 0.115 |
| T1800 | N/A | N/A | N/A | 3.212 ± 0.193 |
| 2nd Study | | | | |
| Control | N/A | N/A | N/A | 3.533 ± 0.428 |
| T1800 | N/A | N/A | N/A | 3.468 ± 0.114 |

^a Weights of left testis were used for PND 1 analysis.

m p < 0.07, * p < 0.05, ** p < 0.01

Table 5: Mortality and parturition data after treatment to Triadimefon 1800ppm^a and litter average of F₁ male weight and AGD (average ± SEM).

| <i>Treatment</i> | <i>Control</i> | <i>Triadimefon 1800ppm</i> |
|-----------------------------------|----------------|----------------------------|
| # of Dams | 20 | 34 |
| GD 6 – 21 Mortality | 0% (0) | 2.9% (1) |
| Parturition | 100% (20) | 67.6% (23) |
| Post Implantation Loss | 0% (0) | 29.4% (10) |
| PND 0 – 8 Litter Mortality | 5% (1) | 47.8% (11) |
| Litters remaining | 95% (19) | 35.3% (12) |
| F ₁ Males (by litter): | | |
| PND 0 weight (g) | 6.25 ± 0.12 | 5.61 ± 0.12*** |
| PND 0 AGD (mm) | 3.67 ± 0.07 | 3.91 ± 0.09** |
| PND 8 weight (g) | 17.50 ± 0.61 | 11.05 ± 0.57**** |

a = Percent of total; litter numbers in parentheses.

AGD was significantly increased by 6.5% when weight was used as a covariate.

Table 6: Fertility indices of F₁ males (PND 76-78) after two different exposures to triadimefon 1800ppm.

| <i>Treatment</i> | <i>Control^a</i> | <i>Pubertal Exp.^b</i> | <i>Developmental Exp.^c</i> |
|--|----------------------------|----------------------------------|---------------------------------------|
| Insemination ^d | 100.0% (5/5) | 66.7%(4/6) | 0.0% (0/6) |
| Implant Success ^e | 90.4% ±2.3 | 87.7% ±2.5 | ND |
| Post-Implantation Success ^f | 94.1% ±4.1 | 95.0% ±5.0 | ND |

a = Control Exposure (GD 6 – PND 90)

b = Pubertal Exposure (PND 23 – 53)

c = Developmental Exposure (GD 6 – PND 90)

d = Percent success (number of successful inseminations by all males).

e = # of implantation sites/ # of corpus luteum

f = # of fetuses/ # of implantation sites; survival up to GD 15.

Table 7: Litter organ weights at PND 90 after two different exposures to triadimefon 1800ppm and treatment effects on preputial separation (PPS) (average \pm SEM).

| <i>Treatment</i> | <i>Control^a</i> | <i>Pubertal Exp.^b</i> | <i>Developmental Exp.^c</i> |
|------------------|----------------------------|----------------------------------|---------------------------------------|
| Weight (g) | 356.0 \pm 11.6 | 326.6 \pm 4.5* | 266.4 \pm 6.1**** |
| Liver (g) | 12.50 \pm 0.51 | 12.12 \pm 0.30* | 12.74 \pm 0.40**** |
| Testes (g) | 3.311 \pm 0.083 | 3.390 \pm 0.082 | 2.955 \pm 0.067** |
| Epididymides (g) | 1.083 \pm 0.033 | 1.077 \pm 0.033 | 0.910 \pm 0.023*** |
| V. Prostate (g) | 0.385 \pm 0.013 | 0.344 \pm 0.016m | 0.276 \pm 0.013**** |
| S. Vesicle (g) | 1.099 \pm 0.023 | 1.001 \pm 0.030m | 0.871 \pm 0.045**** |
| Pituitary (mg) | 9.51 \pm 0.22 | 9.27 \pm 0.19 | 7.04 \pm 0.40**** |
| PPS (PND) | 43.2 \pm 0.4 | 47.0 \pm 0.4* | 53.9 \pm 1.5*** |
| PPS Weight (g) | 172.4 \pm 4.7 | 166.5 \pm 2.6 | 164.6 \pm 5.0 |

a = Control Exposure (GD 6 – PND 90, n = 10 litters)

b = Pubertal Exposure (PND 23 – 53, n = 9 litters)

c = Developmental Exposure (GD 6 – PND 90, n = 11 litters)

VP m: p = 0.0513, SV m: p = 0.0669

* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001

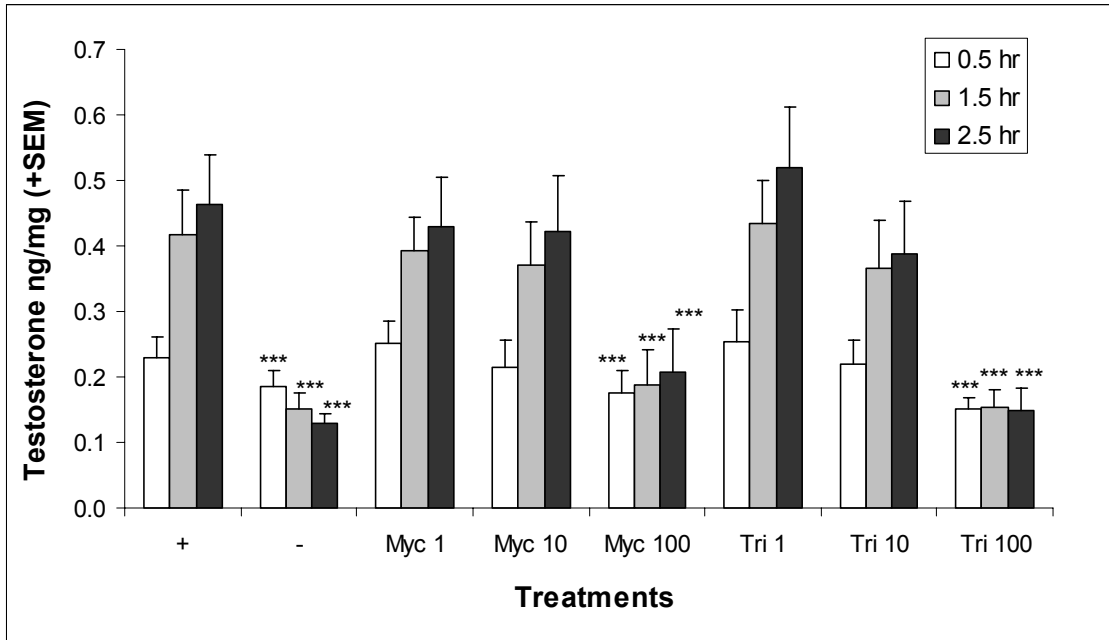
Table 8: Average weight and hormone measurements (average \pm SEM) from control and animals treated (PND 60 - 90) via feed to triadimefon 1800ppm.

| <i>Treatment^a</i> | <i>Control</i> | <i>Triadimefon 1800ppm</i> | <i>% Δ</i> |
|------------------------------|--------------------|----------------------------|------------------------------|
| Body Weight (g) | 366.08 \pm 5.63 | 338.25 \pm 5.84 ** | -7.6 |
| Liver Weight (g) | 12.977 \pm 0.282 | 16.499 \pm 0.447 **** | +27.1 |
| Testes (g) | 3.474 \pm 0.055 | 3.485 \pm 0.066 | - |
| Epididymides (g) | 1.162 \pm 0.016 | 1.102 \pm 0.020 * | -5.2 |
| Ventral Prostate (g) | 0.379 \pm 0.020 | 0.371 \pm 0.017 | - |
| Seminal Vesicle (g) | 1.148 \pm 0.044 | 1.133 \pm 0.052 | - |
| Pituitary (mg) | 10.2 \pm 0.02 | 9.2 \pm 0.02 * | -9.8 |
| Serum T (14 day) (ng/ml) | 2.63 \pm 0.60 | 2.72 \pm 0.46 | - |
| Serum T (30 day) (ng/ml) | 2.15 \pm 0.39 | 6.20 \pm 1.26 ** | +188.3 |
| Intra-testicular T (ng/ml) | 33.70 \pm 5.71 | 70.76 \pm 12.05 ** | +110.0 |
| LH (ng/ml) | 0.433 \pm 0.069 | 0.680 \pm 0.128 | - |
| Estradiol (pg/ml) | 15.04 \pm 1.30 | 18.08 \pm 1.10 | - |
| Prolactin (ng/ml) | 4.81 \pm 0.82 | 8.00 \pm 1.99 | - |

a=control animals (n = 14) and treated animals (n=12)

* = p < 0.05, ** = p < 0.01, **** = p < 0.0001.

A)



B)

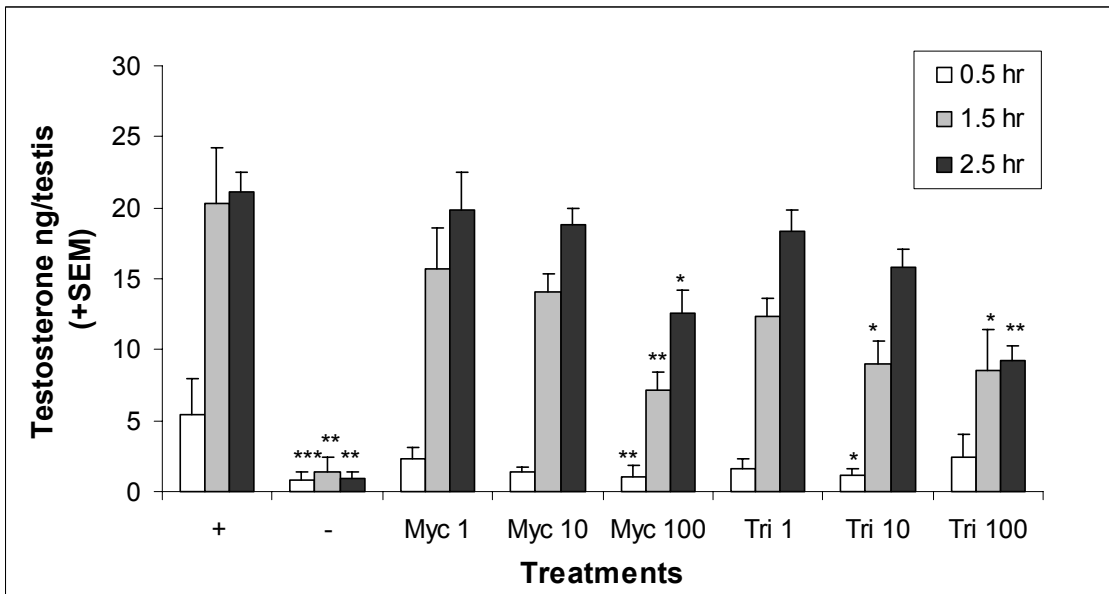
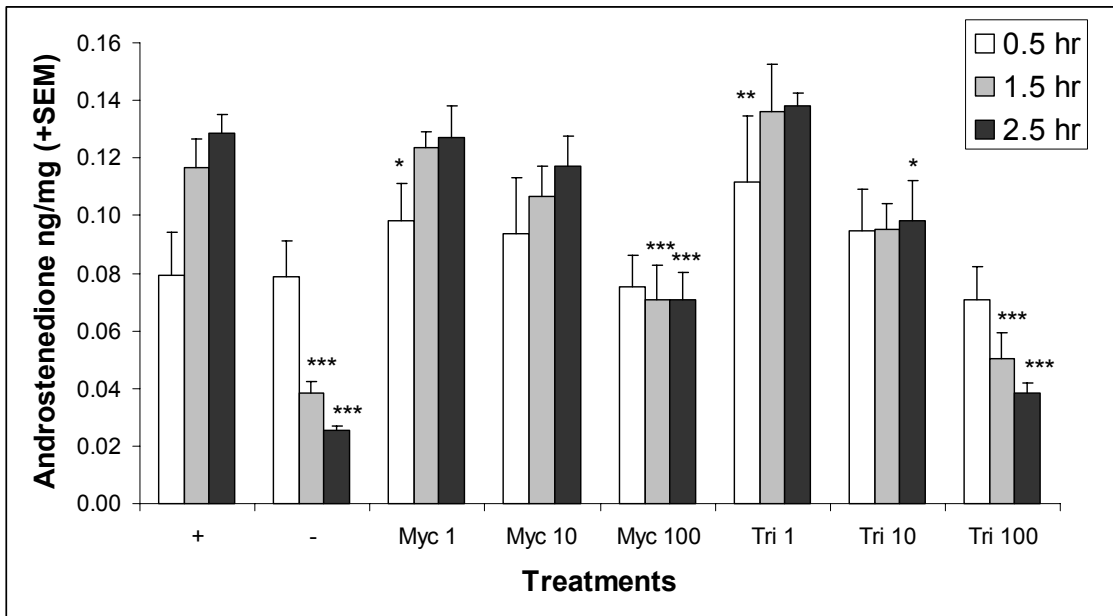


Figure 1: *In Vitro* testosterone production by the adult (A) and neonatal (B) testis after myclobutanil and triadimefon exposure. The control group (+) stimulated by hCG with no chemical; (-) no chemical and was not stimulated by hCG. Asterisks indicate a significant difference (* p < 0.05, ** p < 0.01, and *** p < 0.001) between the treatment group and control group at each time point.

A)



B)

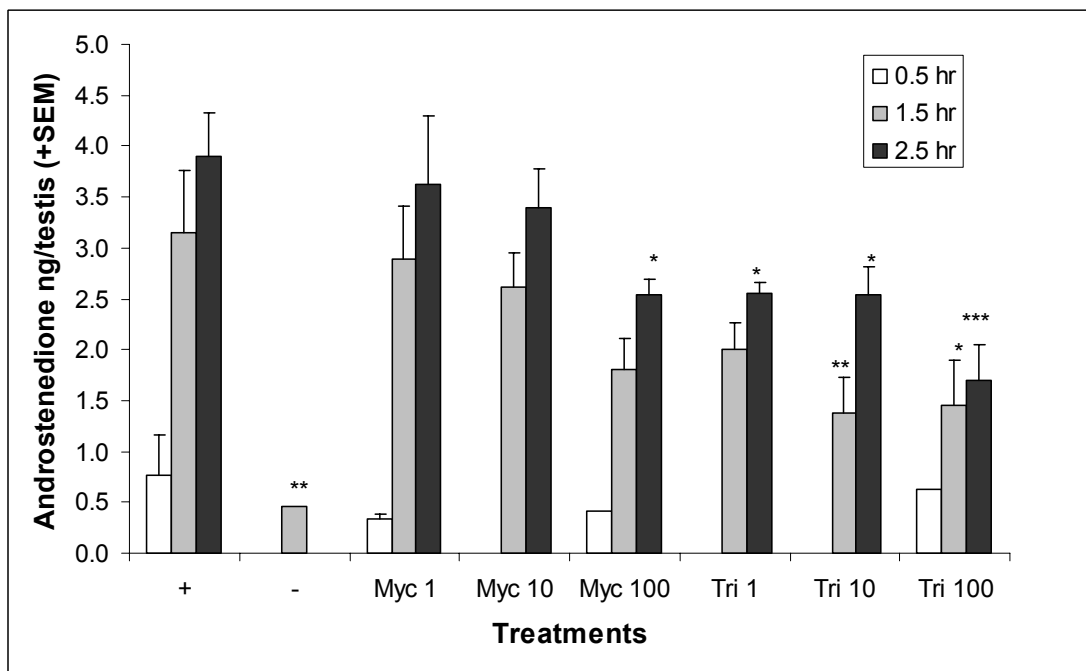
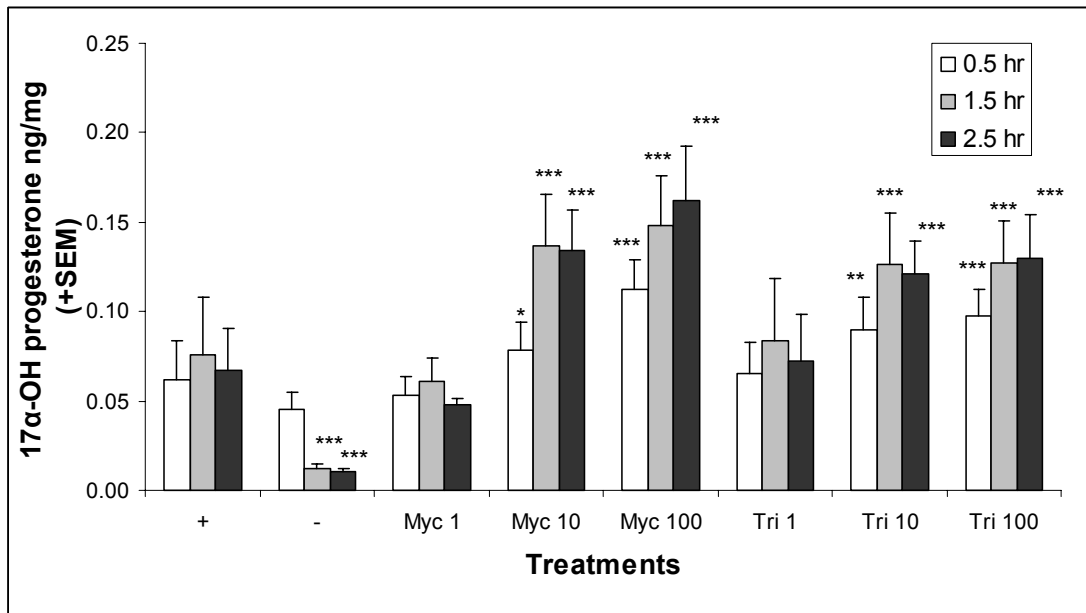


Figure 2: *In Vitro* androstenedione production by the adult (A) and neonatal (B) testis after myclobutanil and triadimefon exposure. Asterisks indicate a significant difference (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) between the treatment group and control group at each time point.

A)



B)

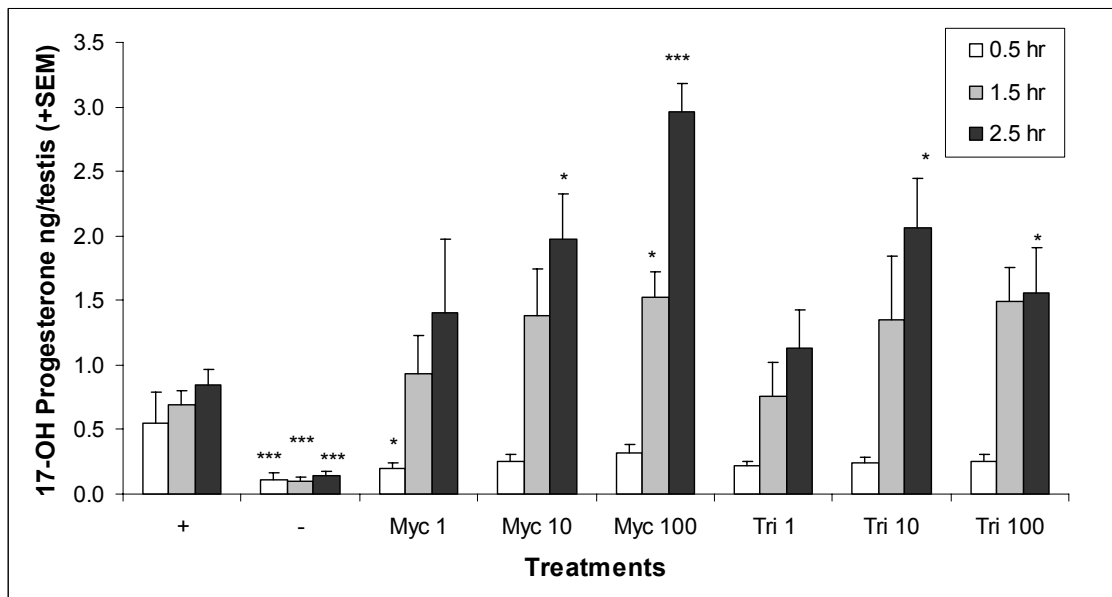
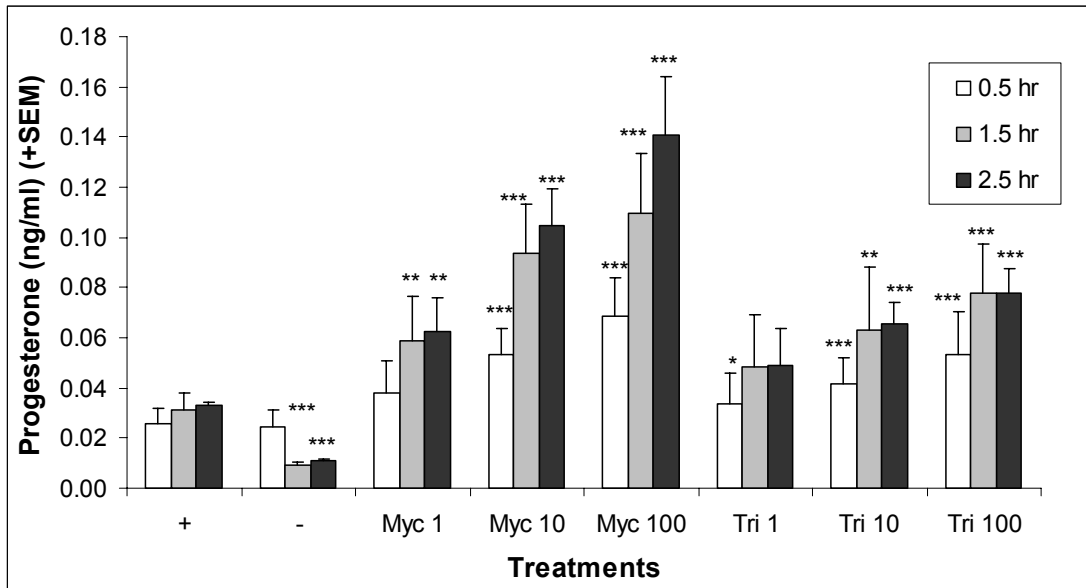


Figure 3: *In Vitro* 17 α -hydroxyprogesterone production by the adult (A) and neonatal (B) testis after myclobutanil and triadimefon exposure. The control group (+) stimulated by hCG with no chemical; (-) no chemical and was not stimulated by hCG. Asterisks indicate a significant difference (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) between the treatment group and control group at each time point.

A)



B)

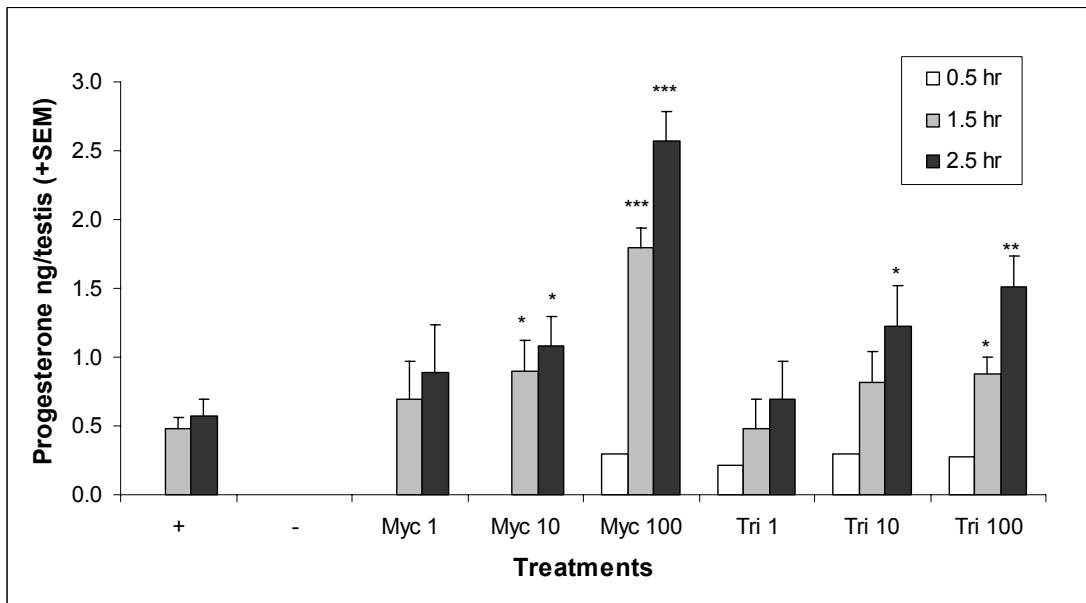


Figure 4: *In Vitro* progesterone production by the adult (A) and neonatal (B) testis after myclobutanil and triadimefon exposure. Asterisks indicate a significant difference (* p < 0.05, ** p < 0.01, and *** p < 0.001) between the treatment group and control group at each time point.

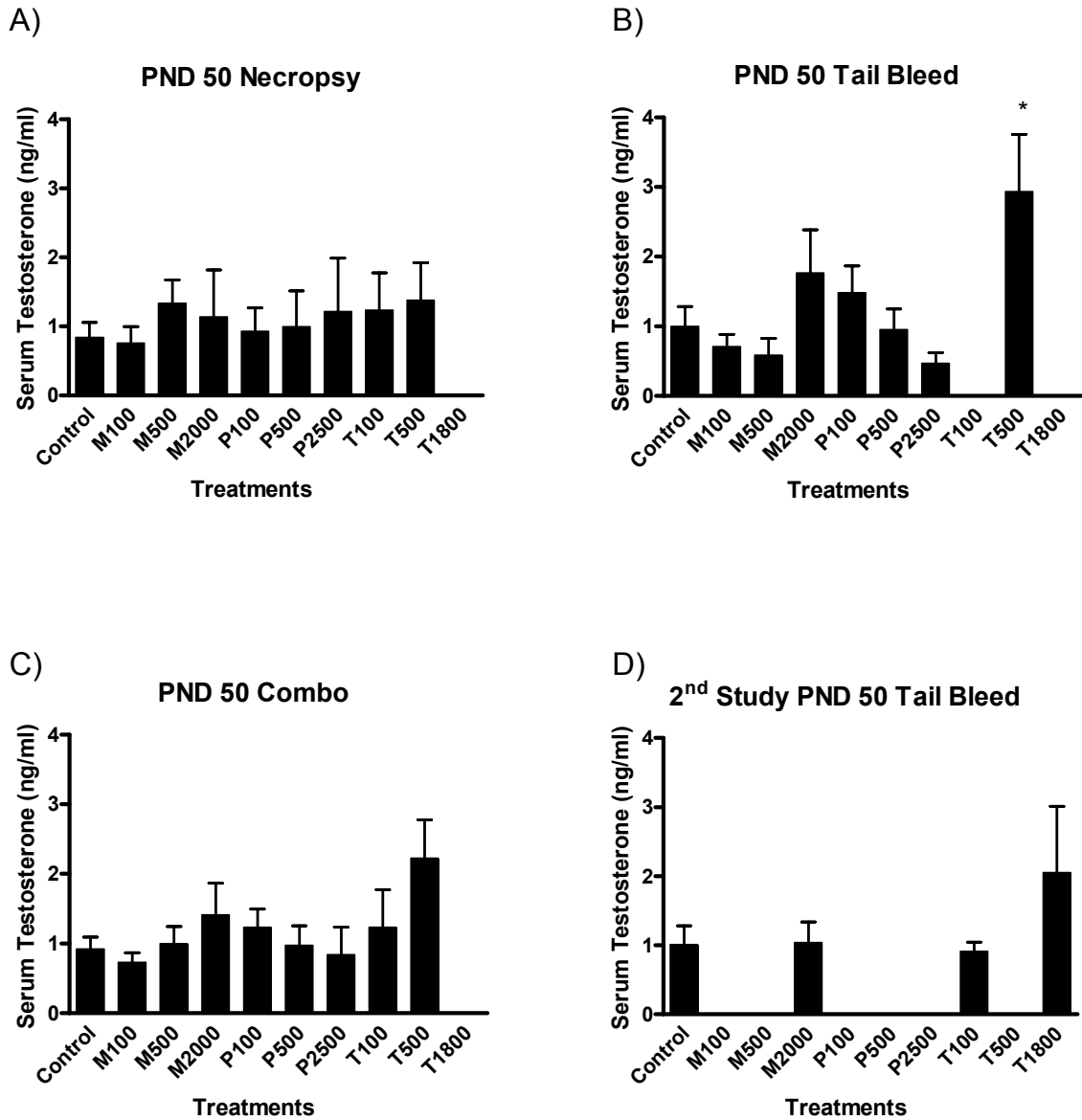


Figure 5: Average (\pm SEM) serum testosterone levels at PND 50 from the multi-triazole experiment. Blood collection was either through heart stick (A) or tail bleed (B). Data were combined for analysis (C) and a second study was conducted to gather more data in the high dose groups (D). * $p < 0.05$.

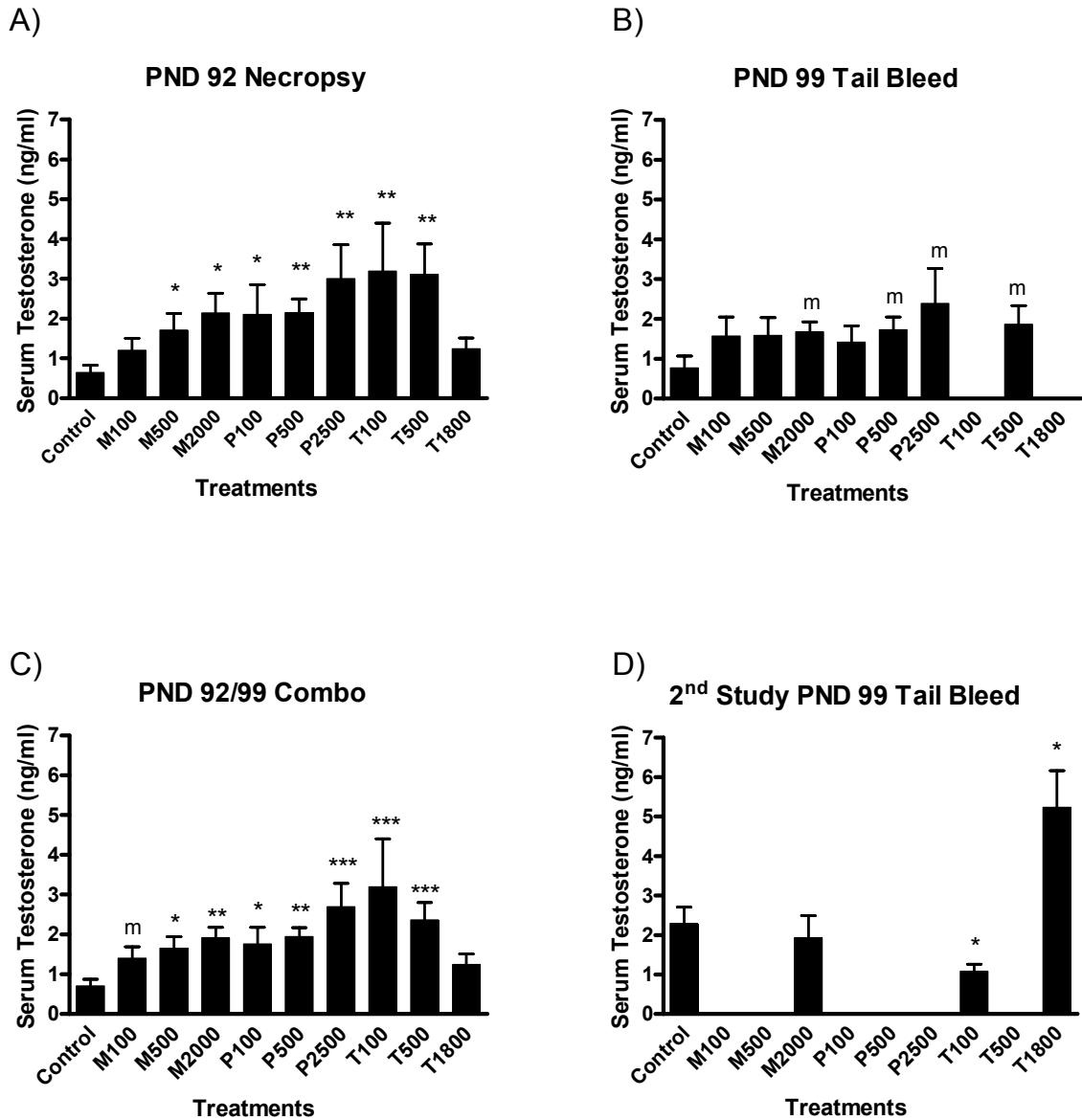


Figure 6: Average (\pm SEM) serum testosterone levels at PND 92 and PND 99 from the multi-triazole experiment. Blood collection was either through heart stick (A) or tail bleed (B). Data were combined for analysis (C) and a second study was conducted to gather more data in the high dose groups (D). m $p < 0.07$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

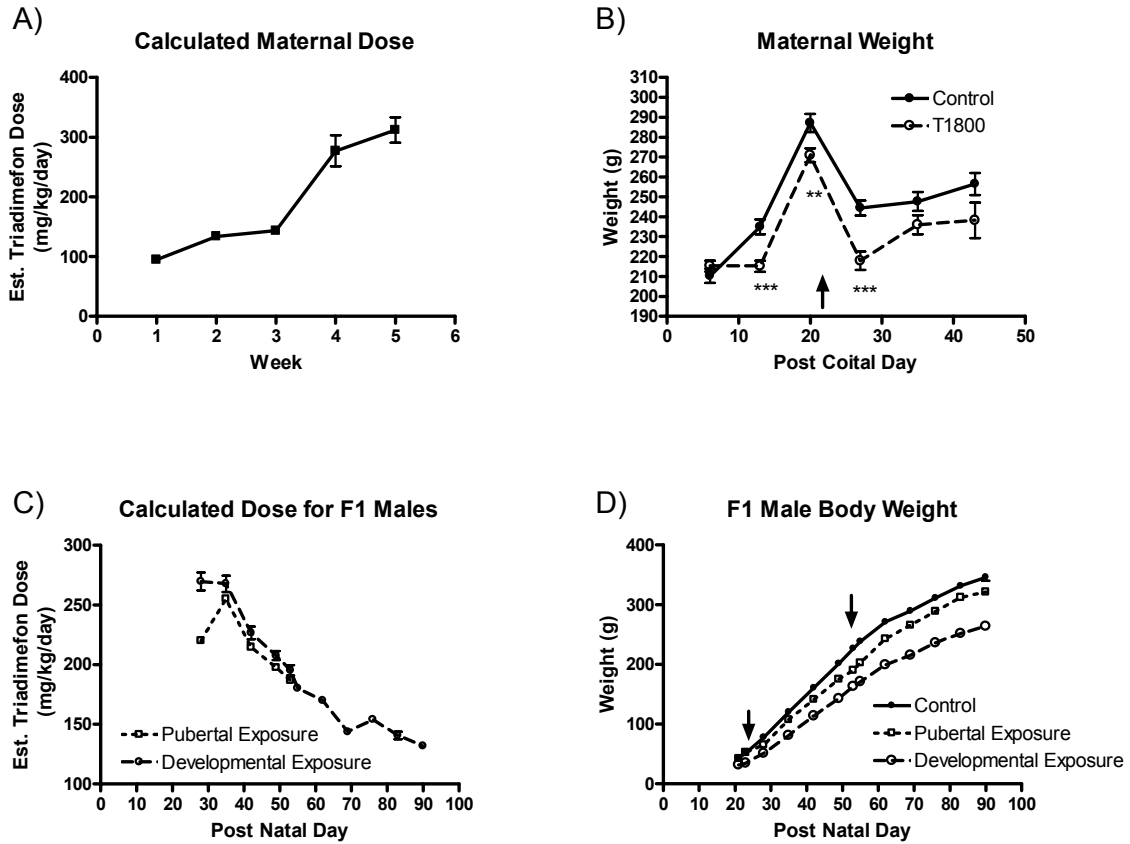


Figure 7: Average (\pm SEM) calculated maternal (A) and F₁ male dose (C) and the effects on maternal (B) and F₁ male (D) body weight. Arrows indicate either date of parturition (B) or period of pubertal dosing (D). ** $p < 0.01$, *** $p < 0.001$.

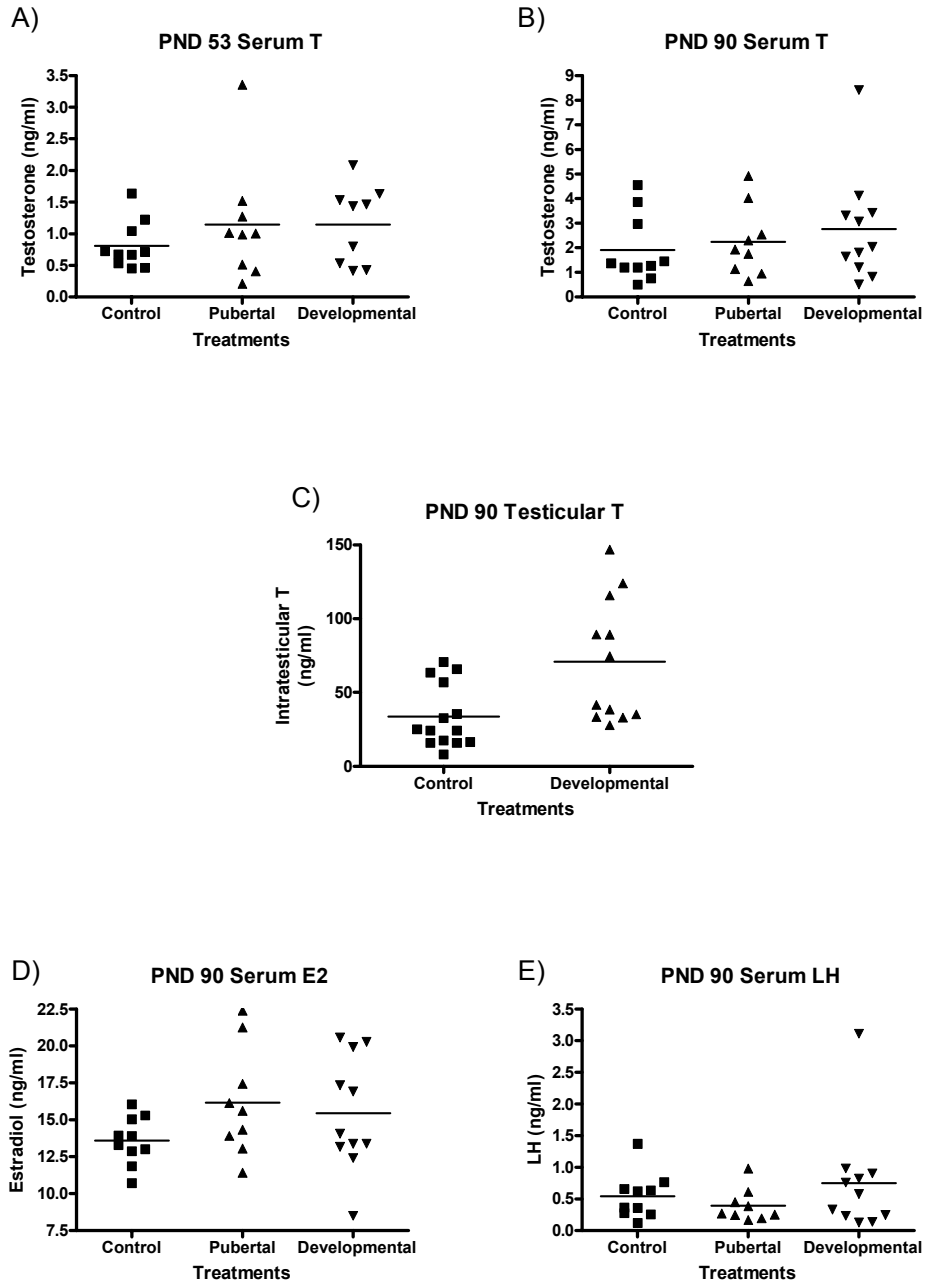


Figure 8: Serum (average indicated by bar) testosterone levels at PND 53 (A) and PND 90 (B), testicular testosterone at PND 90 (C), serum estradiol at PND 90 (D), and serum LH at PND 90 (E) after developmental (GD 6 – PND 90) or pubertal (PND 23 – 53) triadimefon 1800ppm exposure.

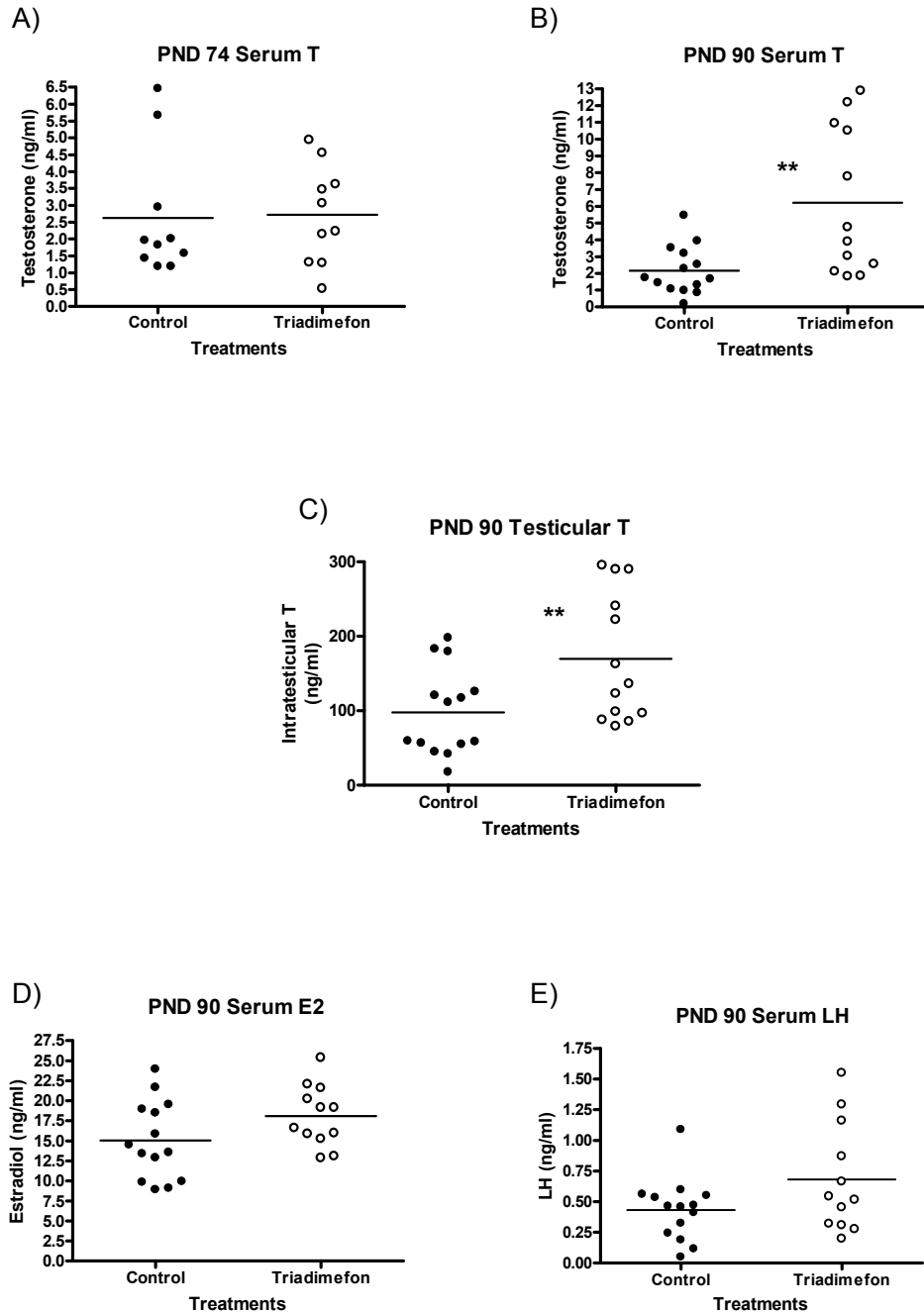


Figure 9: Serum (average indicated by bar) testosterone levels at PND 74 (A) and PND 90 (B), testicular testosterone at PND 90 (C), serum estradiol at PND 90 (D), and serum LH at PND 90 (E) after PND 60 – 90 exposure to triadimefon 1800ppm. ** $p < 0.01$.

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CONCLUSIONS

In the first chapter, we demonstrated that PCZ inhibits gonadal steroidogenesis within the pubertal male and delays pubertal development. The dosage that delayed pubertal development also dramatically reduced serum testosterone levels and testosterone production in the testis. Additionally at this dose PCZ AR antagonism was evident, as demonstrated in the Hershberger assay, and it is likely that both anti-androgen mechanisms contribute to the delay in pubertal development. The *ex vivo* hormone and Hershberger data suggested that PCZ inhibition of testosterone production was relatively stronger than its ability to antagonize the androgen receptor. The inclusion of the serum hormone and *ex vivo* hormone production data in the EDSP pubertal protocol helped identify the target of PCZ action (i.e. CYP17) and increased the sensitivity of the protocol to steroid synthesis inhibitors. Complimentary to these observations, the hormone data also demonstrated that the organ weight and preputial separation measurements were not highly sensitive to reduced testosterone production in the pubertal male. This finding was unexpected, but will aid in predicting and interpreting the results of other pesticides that inhibit testicular steroidogenesis.

In chapter two, the decrease in fetal testosterone production by PCZ corresponded to the reported effects in androgen-dependent tissues after maternal PCZ exposure, which suggested that this anti-androgen mechanism contributed to the reported effects. The inhibition of testosterone production by PCZ appears to be from direct inhibition of the CYP17 enzyme and not through reducing CYP17 gene expression. Amniotic levels of PCZ were sufficient to induce both PCZ inhibition of the CYP17 enzyme and PCZ AR

antagonism. In comparison to the pubertal studies, the reduction in fetal testosterone production *ex vivo* was not as dramatic.

The implications of these findings for the human population are that PCZ exposure might delay puberty or alter sexual differentiation. The male rat model used in these experiments has a similar physiology in androgen production and function to that of human physiology. However, there are a couple of differences between the rat and human endocrine physiology that could increase or decrease PCZ toxicity. The human CYP17 enzyme has a different substrate preference than rat CYP17 although Rat CYP17 is 69% similar to the human enzyme (Fevold *et al.* 1989) (Fevold *et al.* 1989; Flück *et al.* 2003). The amino acid differences which contribute to the difference in substrate preference (Koh *et al.* 1993) might also contribute to a higher or lower sensitivity to PCZ exposure.

Another important difference is the rat adrenal gland does not appear to have the CYP17 enzyme present (Le Goascogne *et al.* 1991; Pelletier *et al.* 2001) suggesting that androgens are not synthesized even though there is evidence of CYP17 RNA expression (Laier *et al.* 2006). The human adrenal gland expresses CYP17 protein and produces large amounts of the androgen dihydroepiandrosterone (DHEA) which contributes to pubertal development (Auchus and Miller 1999; Labrie *et al.* 2005). PCZ effects on adrenal androgen production, not detected in the rat model, might detrimentally affect human pubertal development. Additionally CYP17 has been identified in a number of other tissues, such as the liver (Katagiri *et al.* 1998; Pezzi *et al.* 2003) and brain (Shibuya *et al.* 2003), and it is not known if PCZ affects the physiological role of this enzyme in these tissues.

The results from the last chapter demonstrate that not all conazoles act in a similar manner *in vivo* although the inhibition of CYP17 *in vitro* seems to be a common effect among them. The increase in serum testosterone by the triazoles was opposite of PCZ's *in vivo* effects. The ramification of this increased testosterone on other reproductive endpoints (e.g. PPS, organ weights) was hard to decipher in the presence of extensive general toxicity. At least with the triazole triadimefon, the testis appears to be the source of the increased serum testosterone. The mechanism behind the increased testicular testosterone was not fully elucidated, but it was determined that the increase in testosterone production was not to be due to altered development.

In conclusion, this work was the first to show a steroid synthesis inhibiting pesticide delays puberty. In addition, these studies quantified the sensitivity of fetal testosterone production to maternal PCZ exposure and demonstrated that PCZ inhibits CYP17 hydroxylase activity. The comparison between the triazole and imidazole conazoles showed that they similarly inhibit CYP17, but the triazoles tested appear to increase serum testosterone through an unknown mechanism. Together this work will provide a basis from which to aid in understanding the conazole pesticide inhibition of testosterone production and the resulting effects on male development.

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