

Abstract

MODIC, WALKER MATTHEW. The role of testicular aromatase in the atrazine mediated changes of estrone and estradiol in the male wistar rat. (Under the direction of William Miller and Susan Laws)

The impetus for this study were previous reports that the chloro-s-triazine herbicide, atrazine, alters the serum concentrations of estrone and estradiol in rats after *in vivo* exposure and increases aromatase activity and the levels of the aromatase gene transcript CYP19 mRNA in the H295R cell line following *in vitro* exposure. Serum steroids (progesterone, corticosterone, androstenedione, testosterone, estrone and estradiol) were measured in 60-day-old, male Wistar rats following a single (3, 6, and 24 hrs after dosing) or multiple (2, 3, 4, or 21 days) doses of atrazine [0 (vehicle), 50 or 200 mg/kg, oral]. Hypothalamic and testicular CYP19 mRNA were evaluated by real time RT-PCR for each time point. In addition, aromatase activity was measured using the $^3\text{H}_2\text{O}$ assay in testicular microsomes following *in vitro* and *in vivo* exposure to atrazine. These studies show that atrazine not only altered serum estrogens, but also caused significant increases in serum androgens, progesterone, and corticosterone. Estrogen precursors, androstenedione and testosterone, were increased in both atrazine groups as early as 6 hrs after a single dose. Within 24 hrs, the changes in androgen concentrations dissipated. Serum corticosterone was significantly elevated in the highest treatment group at all but two of the six time points. Elevated estrone and estradiol were consistently observed as early as the 3rd day of exposure, but no changes in CYP19 mRNA were observed at any time point in either tissue. Nor did atrazine affect the levels of aromatase activity in testicular microsomes following *in vitro* or *in vivo* exposure. A subsequent study using castrated male rats demonstrated that the effect on serum estrogens is not due to testicular steroidogenesis. Rather, these data suggest that an atrazine mediated

stress-induced adrenal response and/or a change in steroid synthesis or elimination may be responsible for the increase in serum steroids observed following exposure to atrazine.

**THE ROLE OF TESTICULAR AROMATASE IN THE ATRAZINE MEDIATED
CHANGES OF ESTRONE AND ESTRADIOL IN THE MALE WISTAR RAT**

by,

WALKER MATTHEW MODIC

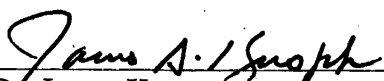
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MOLECULAR & STRUCTURAL BIOCHEMISTRY

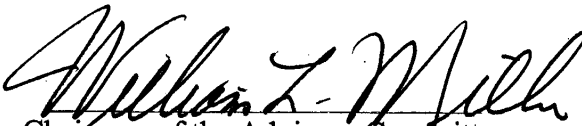
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DEDICATION

I would like to dedicate this work to my parents, whose love and continued willingness to place my best interests before their own has made this and most of my accomplishments past, present, and future possible.

BIOGRAPHY

Born November 27th 1977, in Sewickley Pennsylvania I was the oldest son of Dr. Chris and Terri Modic and the first of four brothers. My education began at an early age in the Montessori classrooms in which my mother taught. At age seven I entered the first grade at Sewickley Academy, where my education would continue until graduating from high school in the spring of 1996. During my tenure at Sewickley Academy, the smaller class sizes afforded me the attention needed to excel in spite of learning disabilities, as well as the chance to participate in numerous extracurricular activities including soccer, lacrosse, and Key Club, a student community service group. It would be these same academic qualities that would lead me to the small town of Granville, Ohio, to attend Denison University. There I completed a Bachelor of Science in Biology with a minor in Chemistry while playing varsity soccer.

Denison University not only offered the academic and extracurricular rigors I desired, but it also provided me the opportunity to study abroad, a life-changing event. In the spring of 1999, I spent a semester studying at the School for Field Studies outside of Nairobi, Kenya. It was there that my passion for the aesthetic value of the environment would find direction. There I encountered not only the most striking environmental landscape I may ever enjoy, but also the most pronounced economic depression and human depravation I had ever imagined. I realized that to deny the people who existed within this environment the opportunity to reap its fiscal benefits was unjust, and that any approaches used for environmental conservation should not come at a cost to those indigenous to the environment. It was then intuitive that conservational designs must yield equal if not greater

financial rewards than their alternatives to be considered feasible. Upon returning to the United States, I focused my academic efforts on biochemistry, a tool I believe will assist me in the design of such economically responsible conservational designs in our first world economy.

In the fall of 2000, I was fortunate enough to meet Dr. Susan Laws, a scientist at the U.S. Environmental Protection Agency. I was given the opportunity to obtain a Masters of Science from the Molecular and Structural Biochemistry department at North Carolina State University while investigating the effects of potentially hazardous chemicals in the environment as part of a cooperative agreement between the U.S. Environmental Protection Agency and North Carolina State University. The following is the culmination of that agreement, and while the road to its completion was lined with more obstacles than I ever could have anticipated, those challenges have only made its completion that much more gratifying.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank those whom without, this project could not have possible: Drs. Ralph Cooper and Tammy Stoker, U.S. EPA, who were always readily available to provide a tremendous wealth of technical and scientific expertise; Dr. Thomas Sanderson, University of Utrecht, for his assistance with the standardization of the $^3\text{H}_2\text{O}$ aromatase assay in our laboratory and for providing technical and scientific advise throughout this study; Dr. Gary Held, U.S. EPA, for selecting the primers and providing technical and scientific advice for the RT-PCR methods; Carmen Wood, for providing the protocols and training for the RNA isolation and RT-PCR methods; Christopher Langdale, U.S. EPA/NCSU Cooperative Agreement Trainee, for assistance in learning the computer programs used for statistical analyses; and Keith McElroy, Ashley Murr, and Angela Buckalew, U.S. EPA, for their technical assistance during the animal studies. Also deserving of particular accreditation is Janet Ferrell, U.S. EPA, whose unwavering personal and professional support during the course of this project has been invaluable and irreplaceable.

Finally, I would like to thank the members of my advisory committee from NCSU, Dr. William Miller, Chairman, Dr. James Knopp, and Dr. Bob Rose, who have provided valuable advice and perspective on my work over the duration of this project; and Dr. Susan Laws, U.S. EPA, who has been not only my mentor, but an impeccable role model over the duration of our time together. Were it not for her passion and persistence this project could not have existed.

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Introduction

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Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is a member of the 2-chloro-s-triazine family of herbicides. As an inhibitor of photosynthesis (Gysin and Knuesli, 1960) it is used extensively to control the growth of broad leaved plants and grassy weeds in commercial agriculture. An estimated 76.4 million tons are used annually in the United States (EPA risk assessment summary 2002). Atrazine, as well as many of its metabolic by-products (Figure 1) are highly persistent in water, mobile in soil (Seiler *et al.*, 1992), and have been detected in ground and surface water in areas of major usage (Baker 1998, Rawn *et al.*, 1998). Recent monitoring of surface and ground water has detected levels of atrazine in the Midwestern river basins and in community water supplies that exceed the maximum containment level of 3 µg/L set by the U.S. Environmental Protection Agency (Kello, 1989, Thurman *et al.*, 1991). The highest levels of atrazine are typically observed in the Midwestern states during the seasonal months of farming (Balu *et al.*, 1998). Thus, because of the high annual usage of this herbicide and its persistence in the environment, there is a potential for human exposure and possible risk to human health.

During the past few years atrazine has been the subject of a considerable body of research in an effort to determine whether or not exposure can be a risk to human health. Although epidemiological studies have detected an association between human exposure to high levels of triazines and increased risk of ovarian (Donna *et al.*, 1989) and breast (Kettles *et al.*, 1997) cancers in females as well as increased occurrence of prostate cancers in males (MacLennan *et al.*, 2002), there are currently no data to support a causal relationship. Laboratory studies have shown that exposure to atrazine caused an earlier onset of mammary tumors and lengthening of the estrous cycle in Sprague-Dawley rats (Eldridge *et al.*, 1999a, 1999b.). Although these results indicated that atrazine might be carcinogenic, subsequent

studies indicated that a central nervous system (CNS) mode of action was responsible for the earlier onset of mammary tumors in the rats. Cooper *et al.* (1998, 2000) demonstrated that atrazine could alter the hypothalamic concentrations of norepinephrine and dopamine and block the estrogen-induced surge of luteinizing hormone (LH) and prolactin (PRL) in ovariectomized females. However, this blockade could be overcome with an intravenous injection of Gonadotropin Releasing Hormone (GnRH). These observations suggested that atrazine, by altering neural hypothalamic-pituitary regulation of ovarian function, induced premature reproductive aging, or constant estrus in the rat. Female rats experiencing constant estrus secrete an appreciable concentration of estradiol (Huang and Meites, 1975, Huang *et al.*, 1978, Cooper *et al.*, 1986, Everett 1989). It is this endocrine milieu that occurs during reproductive senescence in the female rat, which is conducive to the development of mammary gland tumors. Reproductive senescence in humans is markedly different. Menopause occurs as the result of the depletion of primordial follicles and an absence of estradiol (Gosden and Faddy 1994, Burger 1996). Thus, the mode of action by which atrazine induces the premature development of mammary gland tumors does not appear to be relevant to humans.

Although the role of atrazine in female rodent tumorigenesis is not applicable to humans, it does demonstrate disruptive effects on the endocrine and reproductive systems in rodents. These disruptive effects were determined by a 1998 EPA Scientific Advisory Panel to have the potential to affect pubertal development. Laws *et al.*, (2000) and Stoker *et al.*, (2000) demonstrated that exposure to atrazine resulted in the delayed onset of puberty in both male and female Wistar rats. In the male rat, serum estradiol and estrone levels were increased following 30 days of exposure (Stoker *et al.*, 2000). Despite the ability of atrazine

to increase serum estrogens in rats, neither atrazine nor its metabolites bind to the estrogen receptor or induce estrogen receptor mediated responses *in vivo* or *in vitro* (Connor *et al.*, 1996). In the male, estrogens act only in a paracrine or intercrine fashion (Simpson *et al.*, 2000). Therefore, altered levels of serum estrogens in males are the consequence of site specific alterations in the synthesis or clearance of estrogens within the tissues by which they are produced. The project reported here was designed to investigate whether or not atrazine alters the synthesis of estrogens through an alteration in testicular aromatase activity.

The synthesis of estrone and estradiol from androstenedione and testosterone, respectively, is catalyzed by aromatase (Figure 2), a member of the cytochrome P450 gene super family, in concert with the ubiquitous NADPH cytochrome P450 reductase (Simpson *et al.*, 1994). Recent *in vitro* studies have suggested that the atrazine induced changes in serum estrogen concentrations may be mediated by changes in aromatase transcription and/or activity. Exposure of human adrenocortical carcinoma H295R cells to atrazine resulted in elevated levels of CYP19 (aromatase) mRNA and aromatase activity (Sanderson *et al.*, 2000, Sanderson *et al.*, 2002a, Sanderson *et al.*, 2002b, Heneweer *et al.*, 2004). Analogous induction profiles were observed for the human placental carcinoma cell line JEG-3 (Sanderson *et al.*, 2002b). However, atrazine was unable to propagate similar increases in either the human breast cancer cell line MCF-7 (Sanderson *et al.*, 2002b), the rat leydig cell carcinoma R2C (Heneweer *et al.*, 2004), or the human ovarian granulose-like tumor KGN cell line (Morinaga *et al.*, 2004).

While the *in vitro* results from the H295R and JEG-3 cell lines suggest a logical mechanism for altered estrogen levels, their biological significance remains in question because of the complex, tissue specific manner through which aromatase is regulated. The

aromatase enzyme expressed in all tissue types is identical (Simpson *et al.*, 1997), but the promoters, signaling pathway, and proteins involved in initiation of transcription vary between tissue types (for review see Simpson *et al.*, 2002). In placental tissue CYP19 expression is driven by promoter I.1 (Means *et al.*, 1991, Mehendroo *et al.*, 1991), while healthy adipose tissue utilizes promoter I.4 (Mehendroo *et al.*, 1993), and in gonadal tissue the proximal promoter pII is the primary means of regulating CYP19 expression (Jenkins *et al.*, 1993, Lanzino *et al.*, 2001). Further complicating the regulation of CYP19 transcription is the fact that cancerous cells tend not to use the same promoter as healthy cells of the same tissue. In addition, cancerous cells are able to alter promoter utilization in adjacent cells. It has been demonstrated that in breast cancer cells, within and adjacent to the tumor, promoters I.3, PII (Harda *et al.*, 1993, Agarwal *et al.*, 1996, Zhou *et al.*, 1997) and I.7 (Sebastian *et al.*, 2002) are primarily responsible for CYP19 expression as opposed to the typical adipose promoter I.4. The possibility certainly exists that this promoter swapping event is not unique to adipose tissue. In fact, most reports on healthy human adrenal tissue indicate that it transcribes little, if any, CYP19 mRNA (Kimura *et al.*, 1995, Phornphutkul *et al.*, 2001, and Wantanabe *et al.*, 2000) while CYP19 mRNA can readily be detected in cancerous adrenal tissue (Sanderson *et al.*, 2000, Sanderson *et al.*, 2002a, Sanderson *et al.*, 2002b, Heneweef *et al.*, 2004 Bourmima *et al.*, 2003). However, there is evidence that normal human adrenals are able to synthesize estrone and estradiol (Wasada *et al.*, 1978, Wajchenberg *et al.*, 1980, McKenna *et al.*, 1990), and more recently that adrenal tissue does transcribe CYP19 mRNA (Bourmima *et al.*, 2003, Kimura *et al.*, 1995). Analysis of the 5'-untranslated regions of adrenal CYP19 mRNA indicate that, like adipose tissue, the normal promoter is different than the promoters found in cancerous tissue. The potential for promoter swapping events in

other cancerous tissue types, like those used in the aforementioned *in vitro* studies, emphasizes the need to further investigate the potential effect of atrazine on the regulation of CYP19 expression and aromatase activity *in vivo*.

The study reported here was designed to determine if the increased serum estrogens observed in male rats following exposure to atrazine results from a change in aromatase activity. To test this hypothesis, we evaluated the effect of atrazine on serum steroid concentrations, testicular aromatase activity, and testicular and hypothalamic CYP19 mRNA levels in male rats. Testicular tissue was selected because it (1) utilizes the PII promoter (Lanzino *et al.*, 2001) as does the H295R cell line (Heneweer *et al.*, 2004, Watanabe *et al.*, 2004); (2) is a major site of steroid synthesis; and (3) provides a large quantity of easily acquired tissue. The effect of atrazine on the expression of CYP19 mRNA was also evaluated in the hypothalamus because it is another known site of aromatase expression (Roselli *et al.*, 1985, Sanghera *et al.*, 1991, Abdelgadir *et al.*, 1994, Yamada-Mouri *et al.*, 1996, Kato *et al.*, 1997). Though hypothalamic tissue produces low levels of PII promoter transcripts, aromatase expression is primarily under the control of promoter I.f (Yamada-Mouri *et al.*, 1996, Kato *et al.*, 1997). As well, hypothalamic aromatase plays a central role in the sexual development and differentiation of the brain (MacLusky *et al.*, 1981) as well as in the maintenance of male sexual behavior (Baum and Wersinger 1993, Matsumoto *et al.*, 2003). Thus, the hypothalamus provided not only an opportunity to examine the effect of atrazine on an alternate promoter, but also a tissue of physiological significance.

To better characterize the effect of atrazine on serum estrogens and to clearly understand any role of aromatase in mediating those changes, this project was conducted in several parts. A series of time-course studies were conducted to identify the temporal

changes in serum steroid concentrations and CYP19 mRNA beginning with the first dose and continuing through 4 days of dosing. To this end, the effect of atrazine on steroids and testicular and hypothalamic CYP19 mRNA were examined at time points when and prior to, the changes in serum estrone and estradiol occurred. Secondly, to determine whether or not atrazine might cause any translational or post translational effects resulting in altered aromatase activity, microsomes were prepared from testicular tissue of animals treated with atrazine for three days and analyzed for aromatase activity. Finally, to evaluate the role of testicular aromatase in the alteration of serum estrogen levels, a study was conducted using castrated male rats.

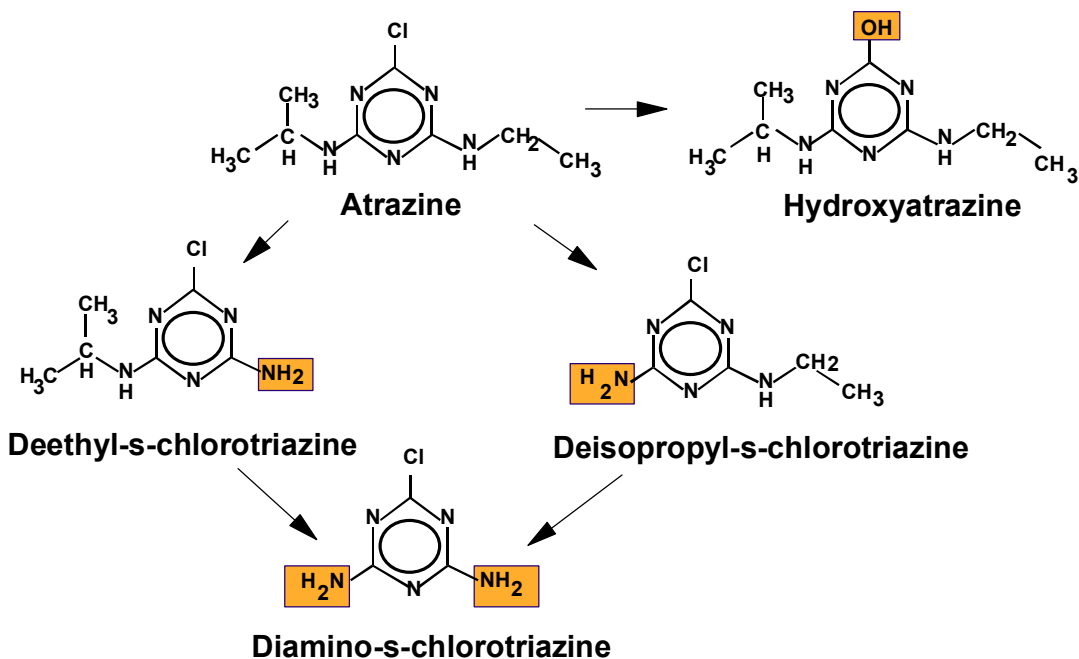


Figure 1 - Chemical structures of atrazine, its metabolites hydroxyatrazine and diamino-s-chlorotriazine, and its metabolic intermediates deethyl-s-chlorotriazine and deisopropyl-s-chlorotriazine

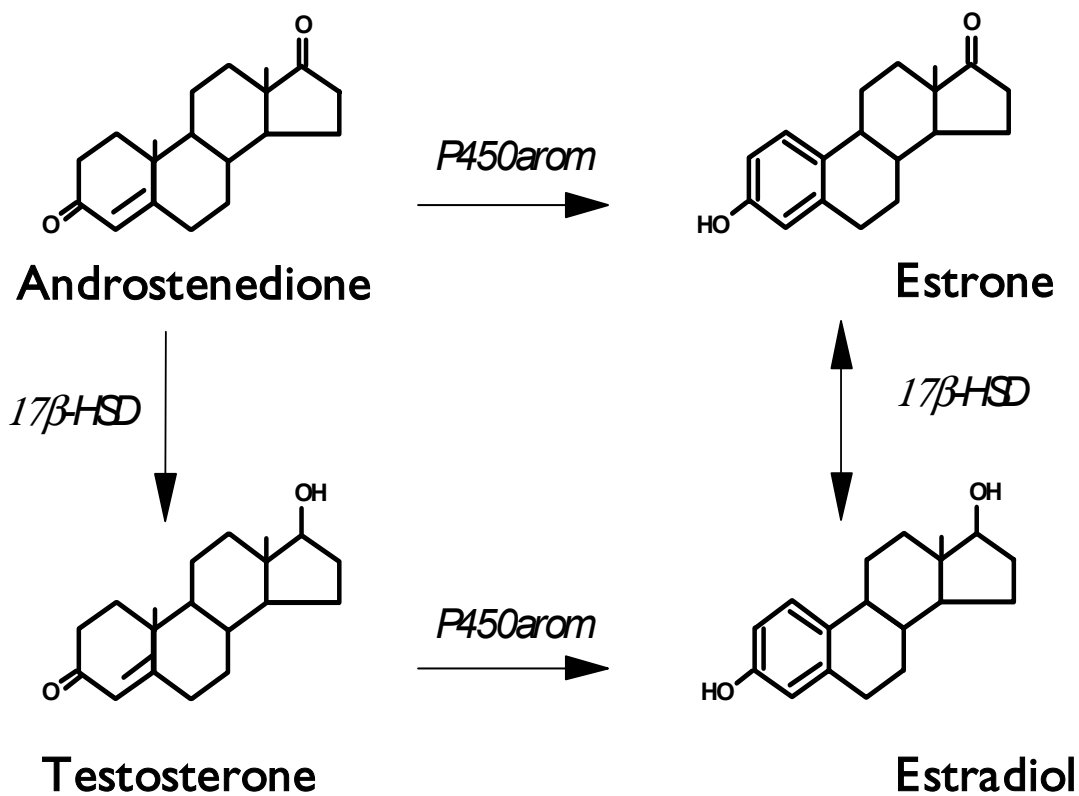


Figure 2 - Aromatization of androstenedione and testosterone to estrone and estradiol. P450arom and the ubiquitous NADPH cytochrome P450 reductase catalyze the aromatization of the A-ring of each androgen to produce each estrogenic equivalent.

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Experimental design

Animals

Male Wistar rats (50-53 days of age) were obtained from Charles River Laboratories, Raleigh, NC, and were housed 2 per cage under controlled temperature (20-24 °C), humidity (40-50%) and light conditions (12h light/ 12h dark), with Purina Laboratory Rat Chow (5001) and water available *ad libitum*. The lights were turned on and off at 0600 and 1800 hours, respectively. All references to time of day reported herein are given as military time and reported as the time perceived by the animal based on the 12h light/ 12h dark light cycle. Animals were allowed to acclimate for at least one week prior to the initiation of treatment. At 56 days of age animals were ranked by body weight (bw) and placed into treatment groups such that the mean body weight \pm SEM for all groups were similar. Unless noted otherwise, animal dosing began at 60 days of age.

Dosing solutions and procedures

Atrazine (97.1% purity; a gift from Syngenta Crop Protection, Inc., Greensboro, NC) was prepared as a suspension in 1.0% methyl cellulose (Sigma-Aldrich, St. Louis, Missouri, USA) in distilled water. Dose groups included 0 (vehicle), 50, and 200 mg atrazine/ kg body weight (bw), which were delivered in a volume of 5.0 ml dosing solution/ kg body weight. All doses were administered by oral gavage.

Time Course Studies

To characterize the temporal effects of exposure to atrazine on steroidogenesis, three separate studies were conducted (Table I). The first study evaluated changes in serum steroid

and CYP19 mRNA levels following a single dose of atrazine over a 24-hour time period. Male Wistar rats (60 days of age) received a single dose of 0 (vehicle), 50, or 200 mg atrazine/kg body weight (n = 10/treatment group at each time point) and were killed by decapitation 3, 6, or 24 hours after dosing. Animals killed at 3, 6, and 24 hours after treatment were dosed at 1000, 0900, and 1100 hours, respectively. The second study was designed to determine the effect of multiple doses of atrazine. Animals in the second study received one dose per day (0, 50 or 200 mg/kg; n=10/treatment group) at 1000 hours for 2, 3, or 4 days and were killed by decapitation 3 hours after the last dose, (1300). A third study was conducted to examine the effect of longer term (21-days) exposure to atrazine. In this study all animals (66 days of age) received a single dose of atrazine (0, 50, or 200 mg/kg) for 21 days and were killed by decapitation 3 hours after the final dose. Again, animals were dosed at 1000 and killed at 1300 hours. For all above studies, after decapitation the testes were removed and no more than 200 mg of testicular tissue was homogenized in TRI reagent (Sigma-Aldrich, St. Louis, Missouri, USA) for preparation of total RNA. The remaining testicular tissue was stored in cold TRIS/KCl buffer on ice until microsomal isolation could be preformed later that day. The hypothalamus was dissected from the whole brain using the optic chiasm, hypothalamic sulci, mammillary bodies, and tuber cinereum as the anterior, lateral, posterior, and basal boundaries respectively (McCann and Ojeda, 1996). After dissection the hypothalamus was homogenized in TRI reagent for preparation of total RNA. Serum was frozen for assay of serum steroids by radioimmunoassay.

Castrated Animal Study

To determine the role of the testes in the altered serum estrogen levels, a study was conducted using castrated animals. At 59 days of age, animals were sedated with 5% halothane (2-bromo-2chloro-1,1,1-trifluoroethane; Halocarbon Laboratories, River Edge, NJ) and 95% air administered with a Vapomatic (A.M. Bickford, Wales Center, NY) and surgically castrated. Beginning on postnatal day 66, groups of intact and castrated males were dosed once daily with atrazine (0, 50, or 200 mg/kg at 1000). All animals were killed by decapitation 3 hours after the final dose (1300). Serum steroid concentrations were measured for all animals by radioimmunoassay.

Radioimmunoassays

Serum levels of testosterone and corticosterone were measured using coat-a-count radioimmunoassay kits obtained from Diagnostic Products Corporation (Los Angeles, CA). The detection limits for testosterone and corticosterone were 0.04 ng/ml and 5.7 ng/ml, respectively. Serum levels of estrone and estradiol (3rd Generation) were measured using coat-a-count radioimmunoassay kits obtained from Diagnostic Systems Laboratories (Webster, TX). The detection limits for estrone and estradiol were 1.2 pg/ml and 0.6 pg/ml, respectively. Serum levels of androstenedione were measured using coat-a-count radioimmunoassay kits obtained from ICN Pharmaceuticals (Costa Mesa, CA). The detection limit for androstenedione was 0.03 ng/ml.

Serum luteinizing hormone (LH) was assayed by Dr. Tammy Stoker, Ashley Murr, and Keith McElroy, U.S.EPA, using materials obtained from the National Hormone and Pituitary Agency (iodination preparation I-9, reference preparation RP-3, and antisera S-11).

Iodination material was labeled with ^{125}I (DuPont/New England Nuclear) by a modification of the chloramines-T method of Greenwood *et al.* (1963). Labeled hormone was separated from unreacted iodine by gel filtration chromatography, as described previously by Goldman *et al.* (1986). Sample serum was pipetted with appropriate dilutions to a final volume of 500 μl with 100 mM phosphate buffer containing 1% bovine serum albumin (BSA). Standard reference solutions were serially diluted for the standard curves. 200 μl of primary antisera in 100 mM potassium phosphate, 76.8 mM EDTA, 1% BSA, and 3% normal rabbit serum were pipetted into each assay tube, vortexed, and incubated for 24 hours. Second antibody (goat anti-rabbit gamma globulin, Cal-Biochem, at a dilution of 1 unit/100 μl) was then added, vortexed, and incubated for 24 hours. The samples were then centrifuged at 1260 x g for 30 minutes, the supernatant aspirated, and the sample tube with pellet was counted on a gamma counter. The historical intra-assay coefficient of variation was 2.4% and the inter-assay coefficient of variation 9.1%.

$^3\text{H}_2\text{O}$ Aromatase Assay

Principals of the $^3\text{H}_2\text{O}$ -aromatase assay

The aromatase assay used herein utilizes the production of H_2O during the penultimate step of the aromatization reaction to quantify the level of aromatase activity. The assay utilizes 1-beta - [^3H] androst-4-ene-3,17-dione as the substrate. By positioning the ^3H at the C-1 beta position, $^3\text{H}_2\text{O}$ is only produced at the completion of the aromatase reaction. Thus, each mole of $^3\text{H}_2\text{O}$ produced represents the completion of a single aromatase reaction and the production of one mole of estrone. It is, for obvious reasons, critical that the

aromatase assay only quantify aromatase reactions that reach fruition if we are to examine the effect of varying conditions on the synthesis of estrone. The following discussion of the mechanism by which aromatase converts C-19 androgens into C-18 estrogens is included to illustrate the specificity of the ^3H -aromatase assay.

The stoichiometric requirements of the aromatase reaction are well understood; 3 moles of oxygen and 3 moles of NADPH are consumed per C-19 steroid aromatized (Thompson and Siiteri 1978), but the exact mechanism by which aromatase catalyzes the conversion of androgens to estrogens remains unknown. Mammalian P450s are invariably membrane bound. Thus, solubilization requires treatment with detergents and until very recently mammalian P450s have resisted characterization by X-ray crystallography. As a result, structure-function studies of aromatase have been limited to site-directed mutagenesis, directed at regions identified by comparative analysis of the amino acid sequence for aromatase from different species. The functionally significant regions identified by site-directed mutagenesis were then compared with cytochrome P450s for which the X-ray structure is available, primarily bacterial P450cam (Poulos *et al.*, 1985) and more recently the first mammalian cytochrome P450 to be analyzed, rabbit P450 2C5 (Williams *et al.*, 2000). This approach has allowed a hypothetical reaction mechanism for the aromatization of C-19 androgens to emerge.

Most recently Chen *et al.* (2003) have used comparative analysis of the rabbit P450 2C5 to refine the mechanism proposed by Koa *et al.* (2001) which was based on the mechanism originally proposed by Graham-Lorence *et al.* (1995). In this model, (Figure 3) the D-ring of the androgen is anchored via the interaction of the O-17 keto group with Lys-119 and Arg-435. This positions the A-ring near the hypothetical catalytic triad of Asp-309,

Ser-478, and His-480 and places the C-19 methyl group near the heme bound iron atom. Once docked in the active site, the steroid is aromatized by a series of hydroxylation reactions at the C-19 methyl group and C-3 keto group eventually resulting in the decarboxylation of the C-19 carbon.

The first two hydroxylation events occur at the C-19 methyl group. Asp-309 is believed to activate oxygen by breaking the oxygen-oxygen bond. This allows for the formation of an iron-oxo intermediate that is responsible for extracting hydrogen from the C-19 methyl group. The iron-hydroxy radical is then recombined with the C-19 carbon radical, resulting in the first hydroxylation at the C-19 carbon. The second C-19 hydroxylation is believed to occur via the same reaction, though some evidence points to the involvement of Ser-478 (Koa *et al* 2001). The gem-diol structure generated by the second hydroxylation is unstable and is converted to a 19-aldehyde androgen by the removal of a molecule of water.

After the two C-19 hydroxylations, the aromatization of the A-ring is initiated by the Asp-309 mediated extraction of a C-2 hydrogen. This commences a series of rearrangements resulting in the formation of a C-2, C-3 double bond and the conversion of the C-3 keto group to a hydroxyl group. His-480 is believed to be the hydrogen donor at the C-3 keto group. His-480 is then restored to its initial conformation by the transfer of hydrogen from Asp-309. Finally, the third molar equivalent of oxygen, bound to the heme iron, is thought to form an enzyme bound peroxyhemiacetal-like intermediate with the C-19 carbon. Rearrangement of this complex results in the release of water, formic acid, and the formation of a C-1, C-10 double bond creating the aromatic A-ring characteristic of estrogens. During the formation of the C-1, C-10 double bond, the C-1 carbon donates the hydrogen nearest the heme iron, the beta-hydrogen, to the formation of water. It is only during this penultimate

step that the ^3H is extracted from the substrate, 1beta - [^3H] androst-4-ene-3,17-dione. This ensures that only the aromatase catalyzed reactions that reach completion are measured by the isolation and quantification of $^3\text{H}_2\text{O}$.

Optimized Protocol: $^3\text{H}_2\text{O}$ Aromatase Assay

Our general assay conditions followed the protocol and guidance provided by Dr. Thomas Sanderson, University of Utrecht, the Netherlands. In order to establish that the assay was appropriately standardized in our laboratory and to ensure that assay conditions were conducive for use in tissue with fairly low levels of aromatase activity, a series of experiments were conducted (See *Standardization: $^3\text{H}_2\text{O}$ Aromatase Assay*). These tests were designed to determine the optimal microsomal concentration and optimal length of incubation to ensure that the availability of the enzyme was the rate limiting variable. After optimizing the reaction conditions, the specificity of the assay system was tested using a known inhibitor, 4-OH-androstenedione, and the K_m and V_{max} were experimentally determined.

The final optimized reaction conditions for measuring aromatase activity using testicular microsomes were as follows: 11.6 μg of microsomal protein (final protein concentration in reaction = 0.029 mg/ml) were incubated for 75 minutes at 37 $^{\circ}\text{C}$ with 136.42 nM ^3H -androstenedione (Perkin Elmer Life Sciences, NET-926, SA = 25.3 Ci/mmol) and 0.31 mM NADPH+ prepared in 50 mM HEPES, 5 mM MgCl buffer, pH 7.8, in a total reaction volume of 400 μl . After incubation, $^3\text{H}_2\text{O}$, the byproduct of the aromatization of ^3H -androstenedione to estrone, was isolated from the reaction products and quantified by liquid scintillation counting to serve as a measure of microsomal aromatase activity.

To isolate the $^3\text{H}_2\text{O}$ produced during the conversion of ^3H -androstenedione to estrone, the reaction solution was placed on ice and 200 μl of each reaction mixture was vortexed with 500 μl of chloroform. The chloroform solution was then centrifuged at 11,000 x g for 2 to 3 minutes. 100 μl of the supernatant was transferred to 100 μl of a charcoal-dextran solution and vortexed vigorously. The charcoal dextran solution was centrifuged (11,000 x g) for 15 minutes at 4 $^{\circ}\text{C}$. The supernatant was then added to a scintillation vial with 400 μl of Ultima Gold scintillate and counted for one minute. The final concentration of $^3\text{H}_2\text{O}$ was determined by conversion of the reported DPMs to pmols. The production of $^3\text{H}_2\text{O}$ was adjusted to account for background activity. Background activity was determined by the production of $^3\text{H}_2\text{O}$ by testicular microsomes from ^3H -androstenedione in the absence of NADPH^+ .

Microsomal Preparation

Immediately after dissection, the testes from a single animal were placed in 6 mls of cold 50 mM TRIS, 1.15% KCL, pH 7.5 buffer and stored on ice. The tissue was then homogenized with a Con-Torque (Eberbach) homogenizer, and an additional 2 mls of the TRIS buffer were added after homogenization. The homogenate was then centrifuged at 10,000 x g at 4 $^{\circ}\text{C}$ for 20 minutes. The supernatant was removed and centrifuged at 100,000 x g for one hour, 4 $^{\circ}\text{C}$. Following the ultra-centrifugation, the supernatant was discarded, and the pellet was gently resuspended in 500 μl of a 0.25 M sucrose buffer by drawing the buffer and microsomes up and down through a 25 gauge needle with a 1ml syringe. After resuspension microsomes were flash frozen in liquid nitrogen and stored at -80 $^{\circ}\text{C}$.

Microsomes intended to be used in the standardization of the $^3\text{H}_2\text{O}$ -assay were prepared from 12 untreated animals, pooled, and gently vortexed before being flash frozen in liquid nitrogen and stored at $-80\text{ }^\circ\text{C}$. To evaluate aromatase activity in animals following exposure to atrazine, testicular microsomes were prepared for each individual animal and frozen in two separate aliquots. The concentration of protein in each microsomal preparation was determined using the Pierce Bicinchonic Acid Assay (Rockford, IL) with bovine serum albumin as the standard. Protein concentrations (mg of protein/ml) were used to standardize the quantity of microsomal protein used in each reaction.

Standardization: $^3\text{H}_2\text{O}$ Aromatase Assay

A series of experiments were conducted to demonstrate that the $^3\text{H}_2\text{O}$ aromatase assay was optimized for use with testicular microsomes. The initial experiments were designed to determine the optimal microsomal protein concentration, considered to be the concentration of microsomes which yielded the greatest concentration of product, at a constant rate over time, when using the optimized concentrations of substrate and cofactor. The assay was conducted using varying amounts of microsomes in each reaction with a final protein concentration ranging from 0.088 to 0.029 mg/ml (35.2 to 11.6 μg protein in total reaction volume of 400 μl). All other components of the reaction were as described for the optimized assay (see *Optimization: $^3\text{H}_2\text{O}$ Aromatase Assay*). Each microsomal protein concentration was tested at 3 time points, ranging from 5 to 120 minutes, to determine the length of time over which each protein concentration continued to produce $^3\text{H}_2\text{O}$ in a linear fashion. Results were plotted as pmol product formed/mg of microsomal protein versus time of incubation and as the reaction velocity (pmol product formed/minute) versus time of

incubation. These data are shown in Figure 4. Both graphs demonstrate that at 0.029 mg microsomal protein/ml, estrone was produced at a constant rate over the duration of incubation. The linear progression of reactions using 0.029 mg/ml of microsomal protein indicated that at no point was the availability of reactants the limiting factor. Therefore, despite producing lower quantities of $^3\text{H}_2\text{O}$, the optimal final assay concentration of microsomal protein was determined to be 0.029 mg/ml. Based on these results, all subsequent reactions were performed at a final assay concentration of 0.029 mg of protein/ml.

A second series of experiments were conducted to determine the period of time over which the reaction progressed in a linear fashion. The time point at which the greatest concentration of product formed while the reaction still progressed in a linear fashion was considered the optimal length of incubation. Using the established optimal microsomal protein concentration, testicular microsomes were incubated under the conditions described for the optimized protocol (see *Optimization: $^3\text{H}_2\text{O}$ Aromatase Assay*) for varied lengths of time ranging from 15 to 150 minutes. At each time point, the quantity of product formed (pmol) was assayed in quadruplicate and plotted versus the length of incubation (min). These results are shown in Figure 5. The reaction was linear between 30 and 90 minutes of incubation. After 90 minutes of incubation, $3.3 \times 10^{-3} \pm 4.5 \times 10^{-4}$ pmol of trace had been aromatized. An additional hour of incubation resulted in only a 12.4% increase in aromatized substrate ($4.1 \times 10^{-4} \pm 2.4 \times 10^{-4}$ pmol) from that observed at the 90 minute time point. The R^2 of the linear portion of the line was 0.99. Based on these results, subsequent assays were incubated for 75 minutes to ensure the reaction was proceeding in a linear fashion at the time of measurement.

Finally, the K_m and V_{max} of the *in vitro* aromatase reaction were determined experimentally using testicular microsomes. To determine the K_m and V_{max} , the experimental velocity was determined for each of fourteen different substrate concentrations ranging from 24.48 to 388.20 nM. With the exception of the concentration of 3H -androstenedione, the reaction conditions were identical to those described for the optimized protocol. A Lineweaver Burke plot of these data (Figure 6) yielded an experimental K_m of 130 ± 14.61 nM and a V_{max} equal to $5.0 \times 10^{-3} \pm 1.17 \times 10^{-3}$ pmol/min/mg. The experimental K_m is comparable to the Michaelis constant for microsomes prepared from human placental tissue, reported by Reed and Ohno, (1976) of 100 nM. Kellis and Vickery (1987) using antibody purified aromatase from human placental tissue reported a K_m of 60 nM. While the values reported by Kellis and Vickery are notably lower, this was not unexpected. In the absence of other microsomal proteins a purified aromatase preparation would be expected to form the enzyme substrate complex with greater frequency, resulting in a lower K_m . Furthermore, Kellis and Vickery were able to calculate the velocity as a measure of pmol/min/mg of aromatase, while our measurements are reflective of pmol/min/mg of microsomal protein. Assay sensitivity was demonstrated using 4-hydroxy-androstenedione, and under the assay conditions described for the optimized protocol 4-hydroxy-androstenedione demonstrated greater than 90% inhibition over two log units with an $IC_{50} = 0.74 \mu M$ (Figure 7A). These results were used as confirmation that the assay conditions described above did in fact quantify, in a fashion sensitive to change, the aromatization of the C-19 steroid androstenedione to the C-18 steroid estrone.

Real Time RT – PCR for Testicular and Hypothalamic CYP19 mRNA

RNA Isolation

RNA was isolated using TRI reagent (Sigma-Aldrich, St. Louis, Missouri, USA). Tissue samples were homogenized in TRI reagent with a Pellet pestle motor (Kontes, Dusseldorf, Germany) immediately following dissection at a ratio of roughly, but not exceeding, 200 mg of tissue per 1.0 ml of TRI reagent. Chloroform was then added in a chloroform to TRI reagent ratio of 1:5 and centrifuged at 12,000 x g for 15 minutes at 4 °C. The upper layer was then removed, and the RNA precipitated with a volume of isopropanol equal to half the initial TRI reagent volume. After ten minutes at room temperature, samples were centrifuged at 12,000 x g for 15 minutes at 4 °C, and the pellet was then twice washed with 1 ml of 75% ethanol, and stored at negative 80 °C under 1 ml of 75% ethanol.

CYP19 mRNA Quantification

Prior to CYP19 mRNA quantification, RNA samples were digested with DNase I (Promega Corp., Madison, WI) and the quantity of total RNA was determined using RiboGreen quantification reagent (Molecular Probes R11490), according to manufacturer's instructions. CYP19 mRNA was quantified by real time RT-PCR with a dual-labeled (Flourescein, Black Hole-1) hydrolysis probe. The CYP19 forward primer TCATTAACGAGAGCCTGCGG, reverse primer, TTAACCGGGTAGCCGTCAATC, and the dual labeled Taq man probe, TGTCGTGGACTTGGTCATGCGCA were designed from the rat CYP19 gene sequence (NM_017085.1) with assistance from Dr. Gary Held, U.S. EPA. The primers and probe were selected so that they would overlap the conserved helical

region of aromatase, as described by Hickey *et al.* (1990). The primers and probe were synthesized by IDT (Coralville, IA).

RT-PCR reactions were conducted using 100 ng of each RNA sample per reaction. RNA was added to a reaction cocktail containing 0.4 mM dNTP, 8 mM MgCl₂, 1X Improm buffer (Promega A3803), 20 units Rnasin (Promega N2515), 0.5 µl Improm enzyme, 0.24 µM forward primer, 0.24 µM reverse primer, 0.12 µM dual-labeled fluorescent probe, and 2.5 units of Taq polymerase antibody (Life Technologies 10965-028) bound to 2.5 units of Taq polymerase (Promega M1688). RT-PCR cycling conditions began with a 15 minute incubation at 42 °C, followed by 3 minutes at 95 °C and forty cycles of 95 °C for fifteen seconds, 56 °C for twenty seconds, and ten seconds at 72 °C. Quantitative real-time RT-PCR was performed in a BioRad iCycler. Multi-well plates were organized so that an equal number of samples from each treatment group at each time point were assayed on every plate. This was done to minimize the bias of any intra-assay variation.

A CYP19 standard curve was also run on each plate with experimental samples. The standard curve was used to quantify the total number of molecules of CYP19 mRNA per 100 ng of RNA. CYP19 cDNA, used in the generation of the standard curve, was synthesized by reverse transcription of RNA from ovarian tissue and amplification of the CYP19 fragment with the reverse and forward primers described above. The cDNA was extracted with phenol and then precipitated with ethanol. The cDNA was resuspended and reprecipitated to further purify the cDNA. The concentration of cDNA was then determined with Pico-Green (Molecular Probes), and the total ng of cDNA were calculated. Because the cDNA fragment is of a known size, 93 base pairs, Avogadro's number was used to calculate the total number of CYP19 cDNA molecules. The standard CYP19 cDNA solution was then diluted to known

concentrations that were then used to create a standard curve ranging from 100 molecules of CYP19 mRNA to 31,600 molecules of CYP19 mRNA.

Statistical Analyses

Data were tested for treatment effects by analysis of variance (ANOVA) using the General Linear Model (GLM) procedure (Statistical Analysis Systems (SAS), SAS Institute, Inc. Cary, NC). When a significant treatment event was observed ($p < 0.05$), Dunnett's test (control vs each treatment group) was used to compare treatment groups. Bartlett's test (Graph Pad InStat, Graph Pad Software, San Diego, CA) was used to test for homogeneity of variance. When heterogeneity of variance was observed, the Kruskal-Wallis Nonparametric Test and Dunn's Multiple Comparison Test were used ($p < 0.05$).

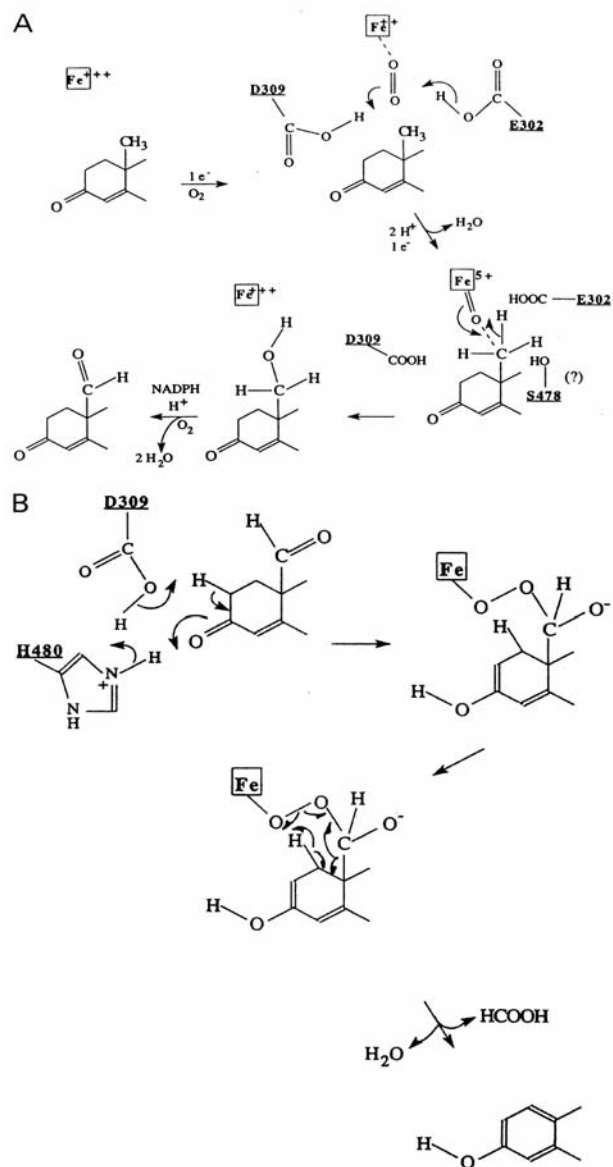


Figure 3 - Proposed reaction mechanism of aromatase (A) The first and second hydroxylations resulting in the formation of a 19-aldehyde androgen. (B) Aromatization of the A-ring. (Replicated from Koa *et al.*, 2001)

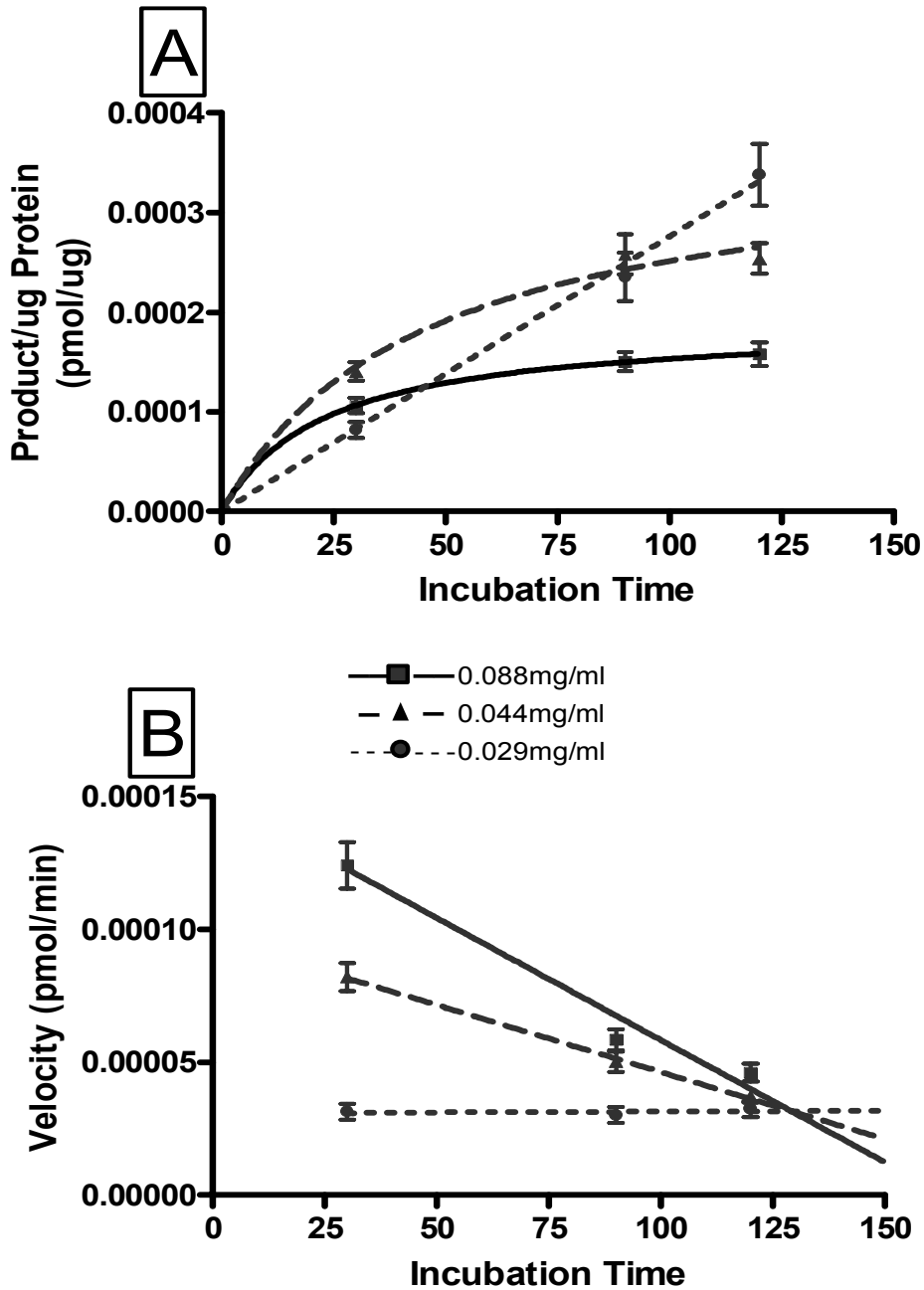


Figure 4 – The effect of testicular microsomal protein concentration on the rate of ^3H -androstenedione aromatization over time. Varied protein concentrations of testicular microsomes, were incubated at 37°C with 136.42 nM trace and 0.31mM NADPH^+ in a total reaction volume of $400\ \mu\text{l}$. The pmols of $^3\text{H}_2\text{O}$ produced [Mean \pm SEM ($n = 4$)] were determined. The production of $^3\text{H}_2\text{O}$ was adjusted to account for background activity as determined by the production of $^3\text{H}_2\text{O}$ by testicular microsomes from ^3H -androstenedione in the absence of NADPH^+ at each time point for each microsomal protein concentration. **A** – Product formed/ μg of microsomal protein over time. **B** – The reaction velocity at each microsomal concentration (pmol/min) versus the time of incubation (minutes)

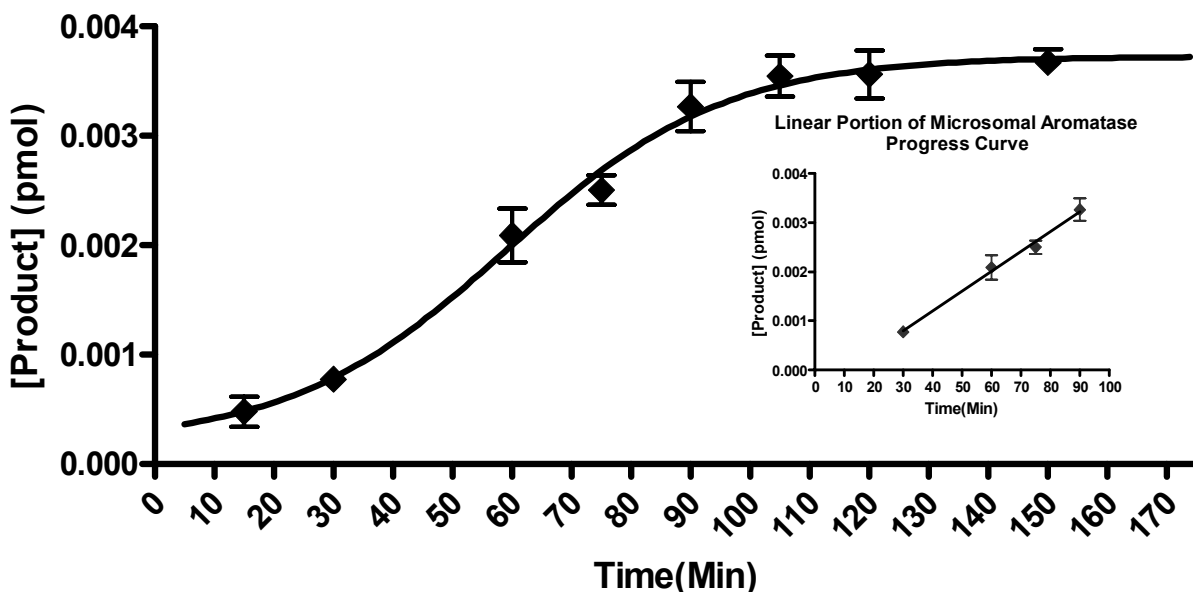


Figure 5 – $^3\text{H}_2\text{O}$ produced by the aromatization of ^3H -androstenedione by testicular microsomes over time. Testicular microsomes (0.029 mg protein/ml), were incubated at 37°C with 136.42 nM trace and 0.31 mM NADPH^+ in total reaction volume of 400 μl for varied lengths of time. $^3\text{H}_2\text{O}$, the byproduct of the aromatization of ^3H -androstenedione to estrone, was isolated from the reaction mixture and expressed in pmols [Mean \pm SEM (n = 4)]. The production of $^3\text{H}_2\text{O}$ was adjusted to account for background activity. Background activity was determined by the production of $^3\text{H}_2\text{O}$ by testicular microsomes from ^3H -androstenedione in the absence of NADPH^+ at each time point. The reaction proceeds in a linear fashion between 30 and 90 minutes of incubation. The slope of the linear portion of the curve (inset) was 4.0×10^{-5} with an R^2 value of 0.99.

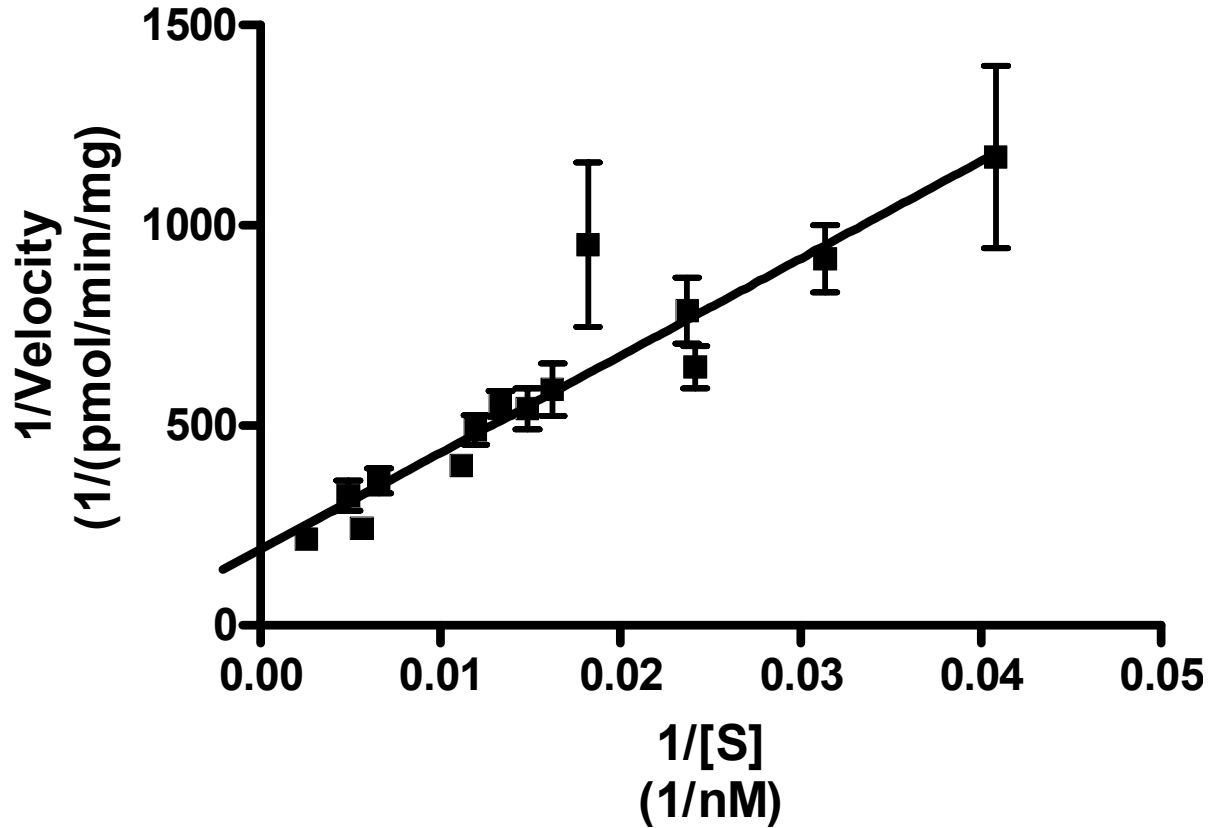


Figure 6 – Experimental determination of the K_m and V_{max} of testicular microsomes. Testicular microsomes (0.029 mg protein/ml) were incubated at 37 °C with varied concentrations of ^3H -androstenedione and 0.31 mM NADPH^+ for 75 minutes in a final assay volume of 400 μl . After incubation the pmols of $^3\text{H}_2\text{O}$ produced [Mean \pm SEM (n = 4)] were measured. The production of $^3\text{H}_2\text{O}$ was adjusted to account for background activity as determined by the production of $^3\text{H}_2\text{O}$ by testicular microsomes from ^3H -androstenedione in the absence of NADPH^+ at each concentration of ^3H -androstenedione. A double reciprocal Lineweaver Burke plot of the results yielded an experimental K_m of 130 ± 14.6 nM and a V_{max} equal to $5.0 \times 10^{-3} \pm 1.17 \times 10^{-3}$ pmol/min/mg.

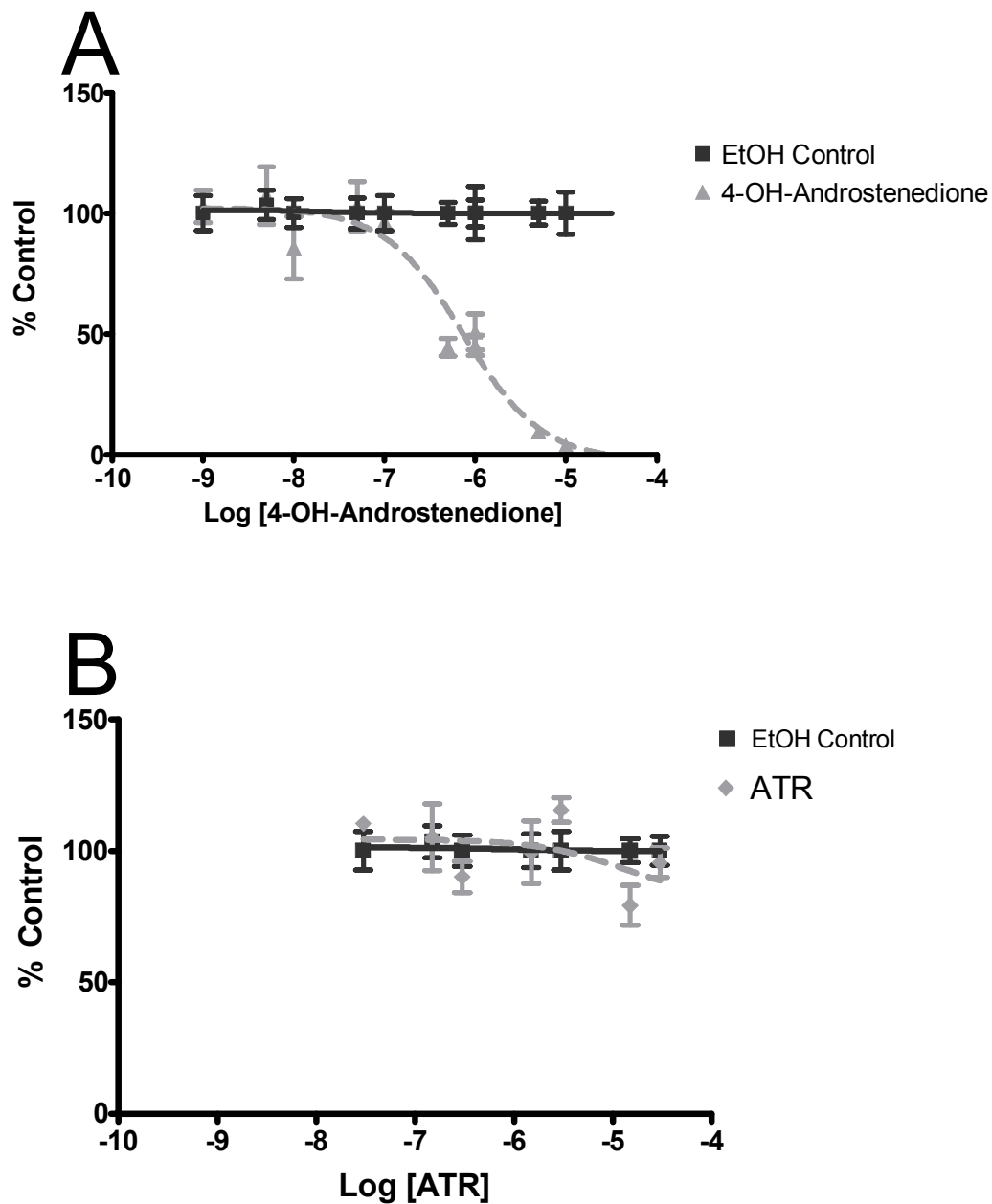


Figure 7 – The effect of *in vitro* exposure to 4-hydroxy-androstenedione (**A**) and atrazine (ATR) (**B**) on the aromatization of ^3H -androstenedione by testicular microsomes. Testicular microsomes (0.029 mg protein/ml), were incubated at 37 °C with 136.42 nM ^3H -androstenedione and 0.31 mM NADPH⁺ with log dilutions of either 4-hydroxy-androstenedione or atrazine dissolved in ethanol for 75 minutes (Total reaction volume = 400 μl). Production of $^3\text{H}_2\text{O}$ is displayed as % control. Controls were prepared with concentrations of ethanol equal to the concentration of ethanol in the groups incubated with either atrazine (diamonds) or 4-hydroxy-androstenedione (triangles). The production of $^3\text{H}_2\text{O}$ was adjusted to account for background activity. Background activity was determined by the production of $^3\text{H}_2\text{O}$ by testicular microsomes from ^3H -androstenedione in the absence of NADPH⁺ at each concentration of ethanol.

Table I

The experimental design of the time course studies

	Time Point	Number of Times Dosed	Days of Treatment	Time of Dosing^{a,b}	Time of Death^{a,b}	Endpoints
Single Dose Study	3 Hrs	1	1	1000	1300	Steroids ^c , CYP19 mRNA
	6 Hrs	1	1	0900	1500	Steroids ^c , CYP19 mRNA
	24 Hrs	1	1	1100	1100	Steroids ^c , CYP19 mRNA
Multiple Dose Study	2 Days	2	2	1000	1300	Steroids ^c , CYP19 mRNA
	3 Days	3	3	1000	1300	Steroids ^c , Aromatase Activity CYP19 mRNA
	4 Days	4	4	1000	1300	Steroids ^c , CYP19 mRNA
	21 Days	21	21	1000	1300	Steroids ^c CYP19 mRNA
Castrated Male	3 Days	3	3	1000	1300	Steroids ^d , LH

^a All times are represented as the time as perceived by the animal. Lights were maintained on a 12:12 on/off light schedule. Lights on at 0600 (6AM) and off at 1800 (6PM)

^b All animals were killed by decapitation on the same day of the last dose except for the single dose, 24hr time point.

^c Testosterone, Androstenedione, Estrone, Estradiol, Corticosterone, and Progesterone

^d Testosterone, Androstenedione, Estrone, Estradiol

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Serum Steroid Levels Following a Single Dose of Atrazine

The effects of a single dose of atrazine on the concentration of androstenedione, testosterone, estrone, estradiol, corticosterone and progesterone 3, 6, and 24 hours after treatment are reported in Figure 8 and Table II. As early as 6 hours after a single dose of 50 and 200 mg atrazine/kg of body weight, the androgenic steroids, testosterone and androstenedione, were significantly increased as compared with the controls. A dose of 200 mg/kg significantly elevated not only the androgenic steroids but also estradiol. These changes did not persist beyond the 6 hour time point. By 24 hours, testosterone, androstenedione and estradiol had returned to control levels. However, estrone was significantly increased at the 24 hour time point (113% of the control). A dose dependent increase was also observed with corticosterone (Table II, significantly different from control for 200 mg/kg at 3 and 24 hrs post dosing) and progesterone (significantly different from control for 200 mg/kg at 3 and 6 hours post dosing).

Serum Steroid Levels Following Multiple Doses of Atrazine

Table III and Figure 9 show the changes in serum androstenedione, testosterone, estrone, estradiol, corticosterone and progesterone following exposure to atrazine for 2, 3, 4, and 21 days. A significant increase was observed in androstenedione and testosterone for the 50 mg/kg group following 2 days of exposure, but neither steroid was significantly altered following 3 or 4 days of exposure. While the elevated levels of testosterone and androstenedione appear transient, the changes in the serum concentrations of estrone and estradiol were sustained for the duration of treatment. At the highest dose estrone levels were significantly increased after 2 and 3 days of exposure. The greatest estrone

concentration was observed after 4 days of treatment and was 163% of control. Estradiol levels in the 200 mg/kg group were significantly greater than control levels by 3 days of dosing. This change was conserved at the 4 and 21 day time points as well. In addition, corticosterone was increased for the 200 mg/kg group at 2, 4, and 21 days. While a dose dependent increase was noted for progesterone at all time points, the 200 mg/kg group was significantly different from the respective controls at 2 and 21 days.

Serum Steroid and LH Levels of Castrated Males Following 3 Daily Doses of Atrazine

The serum steroid and LH levels of castrated males following 3 daily doses of atrazine are shown in Table IV and Figure 10. As expected, castration drastically reduced the concentrations of both androstenedione and testosterone. The LH levels in the castrated animals were as great as 8.89 ± 0.682 ng/ml. Treatment with 50mg/kg caused a significant reduction in LH levels in castrated males compared to the castrated control.

Estrone and estradiol were significantly increased in the castrated males receiving 200mg/kg of atrazine. Despite the greatly reduced availability of their respective metabolic precursors, both steroids were available in the castrated animals (estrone = 112.15 ± 9.25 pg/ml, estradiol = 48.52 ± 3.19 pg/ml at 200mg/kg dose) at concentrations similar to those observed in the intact animal (estrone = 131.94 ± 8.01 pg/ml, estradiol = 43.87 ± 1.62 pg/ml at 200mg/kg dose).

Testicular Microsomal Aromatase Activity

In Vitro Experiments. Figure 7 compares aromatase activity in testicular microsomes following the *in vitro* additions of increasing concentrations of 4-hydroxy-androstenedione

(7A) and atrazine (7B). While 4-hydroxy-androstenedione demonstrated a greater than 90% inhibition over two log units, atrazine did not significantly alter the rate of enzyme activity over concentrations ranging from 30nM to 30uM.

In Vivo Experiments: The $^3\text{H}_2\text{O}$ aromatase assay was also used to examine the effect of atrazine on aromatase activity in microsomes prepared from the testes of animals treated for three days with 0 (vehicle), 50, or 200 mg/kg of atrazine. The three day time point was selected because it was at this time point that lasting changes in the levels of estrone and estradiol were observed. No significant change in activity was observed for either of the treatments compared to the control (Figure 11).

Hypothalamic and Testicular CYP19 mRNA

Mean levels of CYP19 mRNA in the hypothalamus and testis are shown in Figures 12 and 13, respectively. Eight animals were evaluated for each time and treatment group. Each sample was assayed in duplicate. The mean value of the duplicates was used as a single point in the determination of the mean \pm SEM of a treatment group. No treatment effect was observed at any time point.

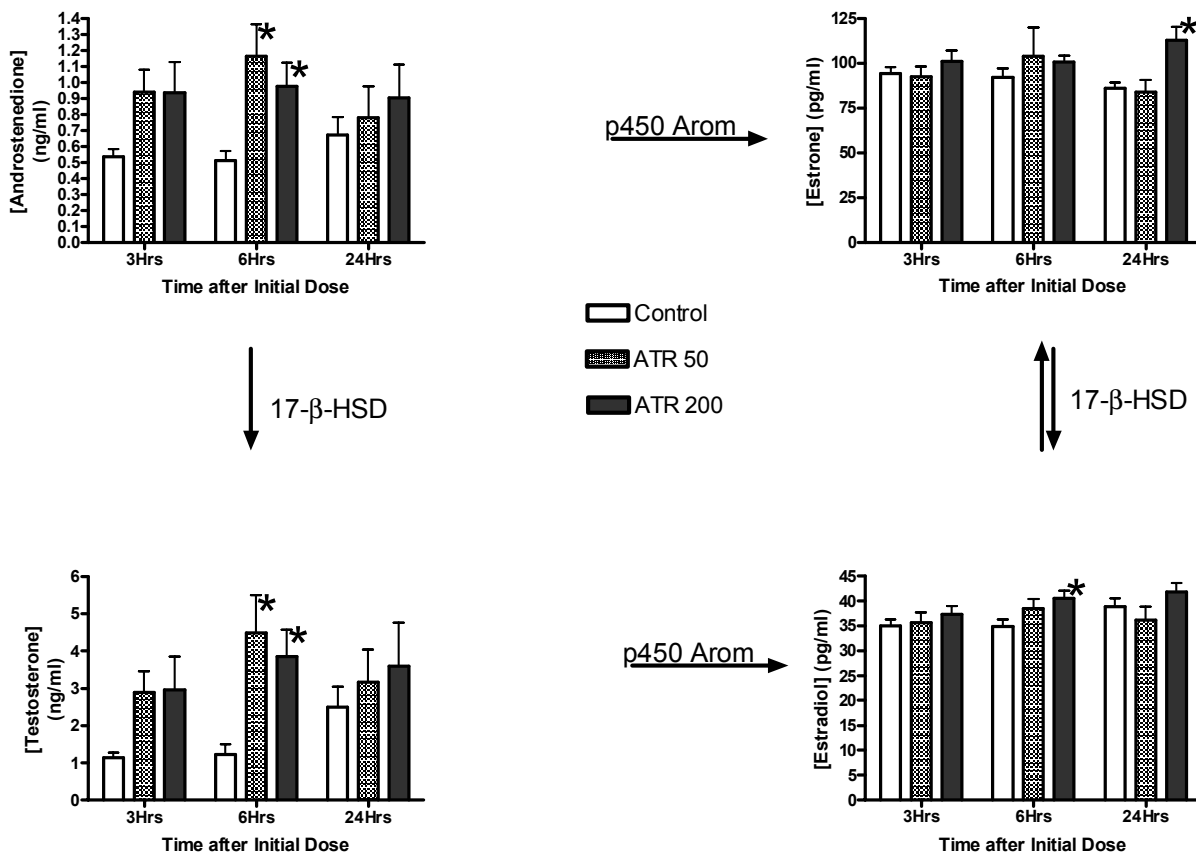


Figure 8 – Effect of a single dose of atrazine on the serum steroid levels of 60 day old male wistar rats. Animals received a single dose of atrazine with methyl cellulose vehicle, by oral gavage and were killed 3, 6, or 24 hours after dosing. Steroid concentrations of testosterone and androstenedione are reported in ng/ml while estrone and estradiol are reported as pg/ml. All values are represented as Mean \pm SEM with an n = 10 except for estradiol (3 hours, 50 mg/kg of atrazine (n = 8)) and estrone (6 hours, 50 mg/kg of atrazine (n = 9)). For statistical analysis the animals were grouped by treatment (e.g., concentration of atrazine) and hours after dosing. *Significant effect by ANOVA or Kruskal-Wallis nonparametric Anova test ($p = 0.05$) with comparison to control by the Dunnett's multiple comparison test or Dunn's multiple comparison test ($p < 0.05$).

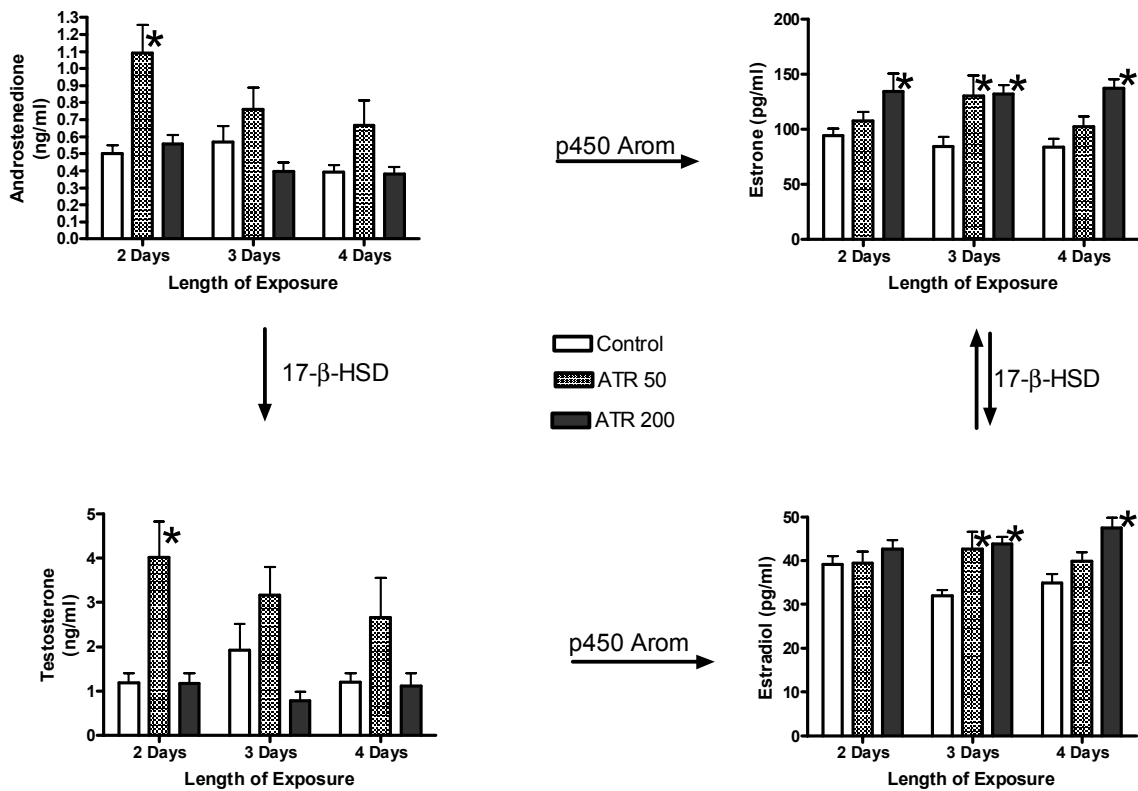


Figure 9 – The effect of multiple doses of atrazine on the serum steroid levels of 60 day old male wistar rats. The animals received a single dose of atrazine with methyl cellulose vehicle by oral gavage, each day for 2, 3, or 4 days. Animals were killed 3 hours after the final dose. Testosterone and androstenedione are reported as ng/ml while estrone and estradiol are reported as pg/ml. All values are represented as Mean \pm SEM with an n = 10 except after 2 days of treatment with 200 mg/kg (n = 9). For statistical analysis the animals were grouped by treatment (e.g., concentration of atrazine) and the number of days treated with atrazine. * Significant effect by ANOVA or Kruskal-Wallis nonparametric Anova test (p = 0.05) with comparison to control by the Dunnett's multiple comparison test or Dunn's multiple comparison test (p < 0.05).

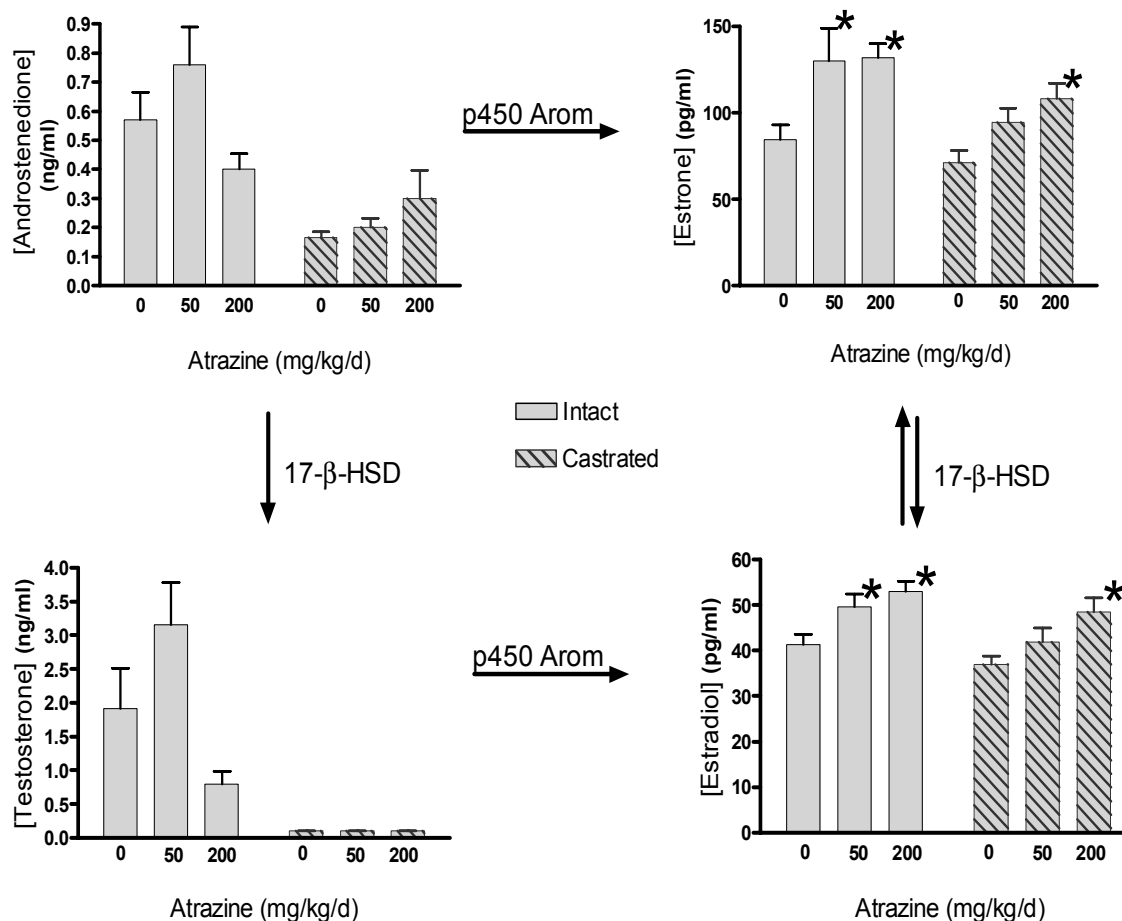


Figure 10 – The effect of treatment with atrazine on serum steroid levels of castrated and intact 60 day old male wistar rats. The animals received a daily dose of atrazine with methyl cellulose by oral gavage for three days. Animals were killed 3 hours after the final dose. Testosterone and androstenedione concentrations are reported as ng/ml while estrone and estradiol are shown as pg/ml. All values are represented as Mean \pm SEM (n=10) except for atrazine 200 mg/kg, castrated (n=9). Because all values for testosterone were below the lowest detectable limit of the assay for the castrated animals, values are reported as 0.10 ng/ml, the lowest standard curve used in the assay. Data for the intact animals was taken for the multiple dose study reported in Figure 9 and Table III. For statistical analysis the animals were grouped by treatment (e.g., concentration of atrazine) and separated into groups of intact and castrated animals. * Significant effect by ANOVA or Kruskal-Wallis nonparametric Anova test ($p = 0.05$) with comparison to control by the Dunnett's multiple comparison test or Dunn's multiple comparison test ($p < 0.05$).

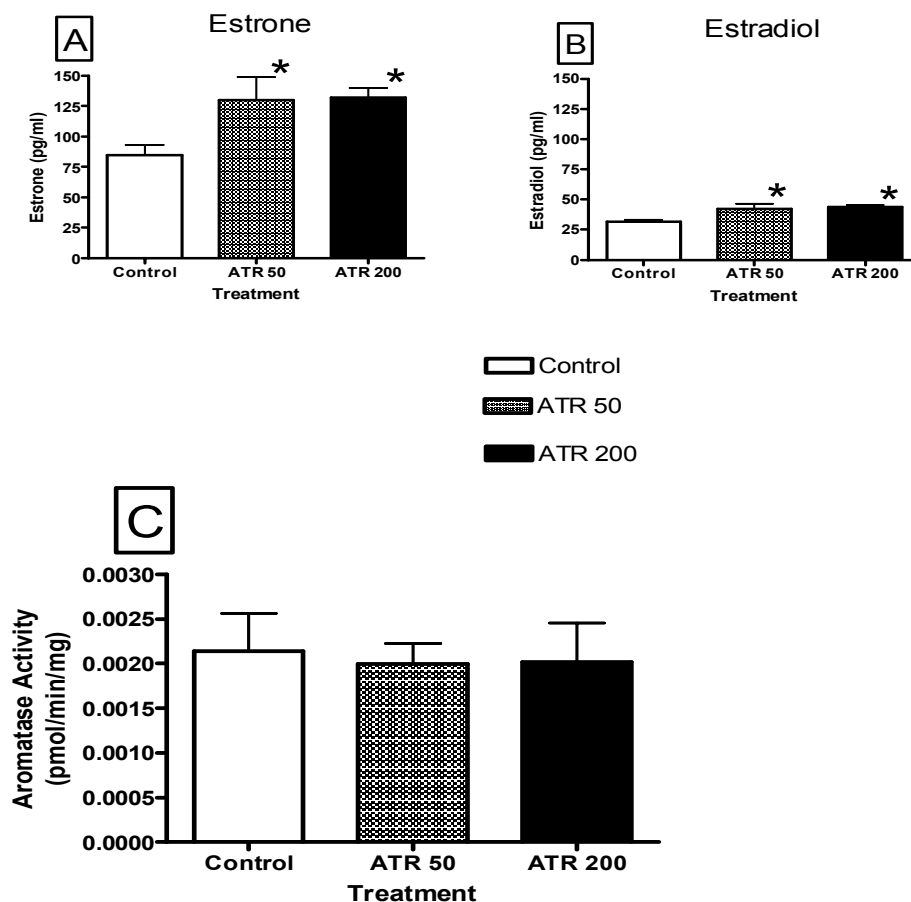


Figure 11 – The effect of atrazine on aromatase activity (C), serum estrone (A), and serum estradiol (B) after three days of treatment. **(A and B)** Animals were treated with atrazine for 3 days and killed 3 hours after the final dose. Steroid levels of estrone and estradiol are shown as pg/ml. All values are represented as Mean \pm SEM with an n = 10. For statistical analysis the animals were grouped by treatment (e.g., concentration of atrazine) and the number of days treated with atrazine. * Significant effect by ANOVA or Kruskal-Wallis nonparametric Anova test ($p = 0.05$) with comparison to control by the Dunnett’s multiple comparison test or Dunn’s multiple comparison test ($p < 0.05$). **(C)** $^3\text{H}_2\text{O}$ produced by the aromatization of ^3H -androstenedione by testicular microsomes exposed to atrazine *in vivo*. Microsomes were prepared from the testicular tissue of animals treated with atrazine once a day for three days. 0.029mg/ml of testicular microsomes, as determined by the bicinchonic acid protein assay, were incubated at 37°C with 136.42nM trace and 0.31mM NADPH⁺ for 75 min. $^3\text{H}_2\text{O}$, the byproduct of the aromatization of ^3H -androstenedione to estrone, was isolated from the reaction mixture and is expressed in pmols/hr/mg of microsomal protein. The production of $^3\text{H}_2\text{O}$ was adjusted to account for background activity. Background activity was determined by the production of $^3\text{H}_2\text{O}$ by testicular microsomes from ^3H -androstenedione in the absence of NADPH⁺ at each time point. Each sample was run in quadruplicate. The mean of the quadruplicates was then used as a single point in the calculation of the mean values for each treatment group. Data are reported as the Mean \pm SEM (n = 10) and was analyzed by ANOVA. No significant treatment effect was observed.

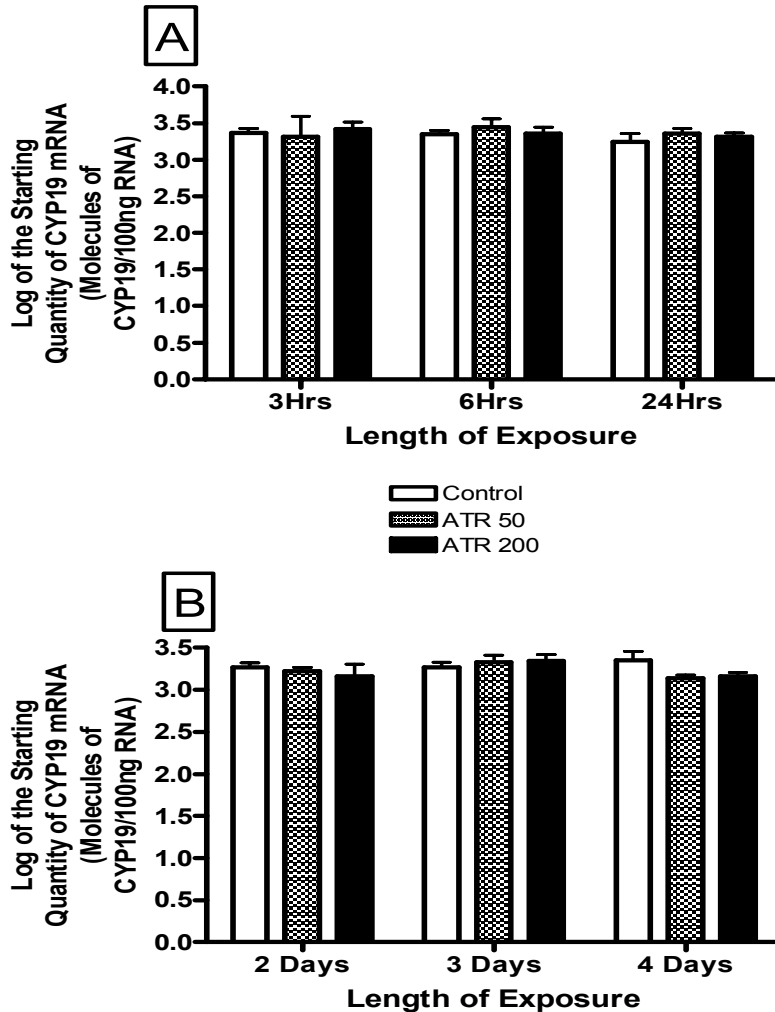


Figure 12 - CYP19 mRNA in the hypothalamus after treatment with either a single dose (A) or multiple doses (B) of atrazine. Quantification of CYP19 mRNA was performed as described in the methods. All samples were run in duplicate. The mean of the duplicates was then used as a single point in the calculation of the mean values for each treatment group at a specific time point. (A) Hypothalamic CYP19 levels after a single dose of atrazine. The standard curves from each of the two plates needed to assay these samples were $Y = -3.45x + 37.63$ and $Y = -3.370x + 38.139$ where x equals the log of the starting quantity of CYP19 mRNA. Data are presented as Mean \pm SEM ($n=8$) except at 3 hours, 50 mg/kg ($n = 7$) and 200 mg/kg ($n = 7$) and 24 hours in the control ($n = 7$) and 200 mg/kg ($n = 7$) groups. For statistical analysis the animals were grouped by treatment (e.g., concentration of atrazine) and time of death, as expressed by hours after dosing and analyzed by ANOVA. No significant treatment effect was observed at any time point. (B) Hypothalamic CYP19 levels after multiple doses of atrazine. The standard curves from the two plates needed to assay these samples were $Y = -3.686x + 38.43$ and $Y = -3.510x + 38.7$ where x equals the log of the starting quantity of CYP19 mRNA. Data are presented as Mean \pm SEM with an $n = 8$, except in the 200 mg/kg treatment group after 2 days ($n = 7$) and 3 days ($n = 7$) of treatment. For statistical analysis the animals were grouped by treatment (e.g., concentration of atrazine) and time of death, as expressed by hours after dosing and analyzed by ANOVA. No significant treatment effect was observed at any time point.

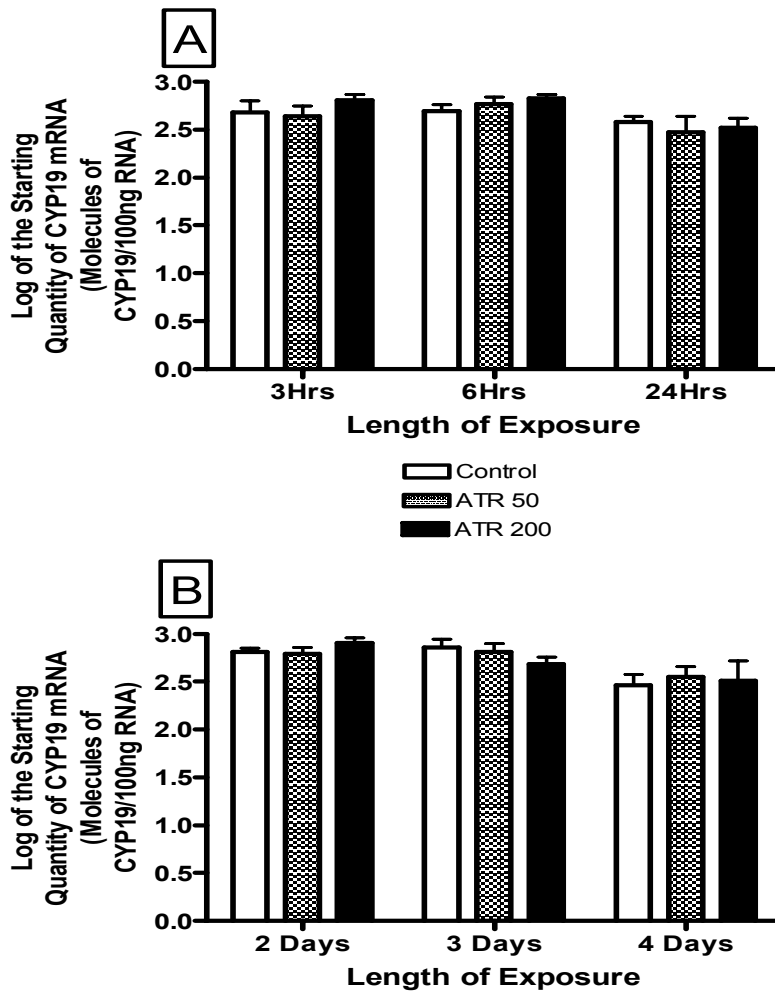


Figure 13 - CYP19 mRNA in the testis after treatment with either a single dose (A) or after multiple doses (B) of atrazine. Quantification of CYP19 mRNA was performed as described in the methods. All samples were run in duplicate. The mean of the duplicates was then used as a single point in the calculation of the mean values for each treatment group at a specific time point. (A) Testicular CYP19 levels after a single dose of atrazine. The standard curves from the two plates needed to assay these samples were $Y = -3.330x + 37.00$ and $Y = -3.337x + 36.420$ where x equals the log of the starting quantity of CYP19 mRNA. Data are presented as Mean \pm SEM with an $n = 8$, except in the 50 mg/kg treatment group at 3 hours ($n = 7$) and 24 hours ($n = 7$) after the first dose. For statistical analysis the animals were grouped by treatment (e.g., concentration of atrazine) and time of death, as expressed by hours after dosing and analyzed by ANOVA. No significant treatment effect was observed at any time point. (B) Testicular CYP19 levels after multiple doses of atrazine. The standard curves from the two plates needed to assay these samples were $Y = -3.340x + 35.41$ and $Y = -3.336x + 37.22$ where x equals the log of the starting quantity of CYP19 mRNA. Data are presented as Mean \pm SEM with an $n = 8$, except at 4 days of treatment in the control ($n = 6$), 50 mg/kg ($n = 7$), and 200 mg/kg ($n = 7$) treatment groups. For statistical analysis the animals were grouped by treatment (e.g., concentration of atrazine) and time of death, as expressed by hours after dosing and analyzed by ANOVA. No significant treatment effect was observed at any time point.

Table II

Serum steroid concentrations following a single dose of atrazine

	Dose (mg/kg/d)	Androstenedione [#] (ng/ml)	Testosterone [#] (ng/ml)	Estrone [#] (pg/ml)	Estradiol [#] (pg/ml)	Corticosterone [#] (ng/ml)	Progesterone [#] (ng/ml)
3 Hours after First Dose	0	0.54 ± 0.049	1.15 ± 0.14	94.26 ± 3.38	34.92 ± 1.30	115.53 ± 20.61	0.805 ± 0.15
	50	0.94 ± 0.14	2.89 ± 0.55	92.36 ± 5.61	35.62 ± 2.09 ^b	167.90 ± 28.49	1.35 ± 0.27
	200	0.94 ± 0.19	2.96 ± 0.91	101.14 ± 5.83	37.33 ± 1.68	199.048 ± 27.22*	1.60 ± 0.30*
6 Hours after First Dose	0	0.51 ± 0.063	1.23 ± 0.28	92.15 ± 5.12	34.81 ± 1.52	213.98 ± 23.69 ^c	1.32 ± 0.25
	50	1.17 ± 0.20*	4.49 ± 1.01*	103.83 ± 16.14 ^a	38.40 ± 1.90	210.75 ± 23.75 ^c	1.49 ± 0.17
	200	0.98 ± 0.15*	3.85 ± 0.73*	100.58 ± 3.78	40.53 ± 1.49*	229.47 ± 36.87 ^c	2.05 ± 0.39*
24 Hours after First Dose	0	0.67 ± 0.12	2.49 ± 0.54	83.88 ± 6.79	38.77 ± 1.74	57.24 ± 11.16	0.607 ± 0.086 ^a
	50	0.78 ± 0.20	3.17 ± 0.87	85.99 ± 3.14	36.06 ± 2.68	84.04 ± 21.11	0.700 ± 0.12
	200	0.91 ± 0.21	3.60 ± 1.16	112.65 ± 7.59*	41.78 ± 1.75	171.34 ± 27.78*	1.23 ± 0.24

[#]Mean ± SEM (*n* = 10 unless indicated otherwise)^a*n* = 9^b*n* = 8^cAnimals killed at near the peak of circadian rhythm controlled steroid levels* Significant effect by ANOVA followed by Kruskal-Wallis nonparametric test (*p* = 0.05) with comparison to control by the Dunn's multiple comparison test (*p* < 0.05).

Table III

Serum steroid concentrations following multiple doses of atrazine

	Dose (mg/kg/d)	Androstenedione [#] (ng/ml)	Testosterone [#] (ng/ml)	Estrone [#] (pg/ml)	Estradiol [#] (pg/ml)	Corticosterone [#] (ng/ml)	Progesterone [#] (ng/ml)
2 Days of Treatment	0	0.50 ± 0.051	1.19 +/- 0.21	94.35 ± 6.37	39.25 ± 1.79	137.87 ± 26.23	1.02 ± 0.18
	50	1.090 ± 0.17*	4.017 +/- 0.81*	107.74 ± 8.10	39.42 ± 2.59	141.82 ± 26.43 ^a	1.30 ± 0.22 ^a
	200	0.55 ± 0.060 ^a	1.20 +/- 0.26 ^a	139.27 ± 17.98* ^a	43.37 ± 2.15 ^a	223.73 ± 33.96* ^c	1.91 ± 0.40* ^c
3 Days of Treatment	0	0.57 ± 0.094	1.92 +/- 0.59	84.39 ± 8.50	31.97 ± 1.27	172.95 ± 20.93	1.17 ± 0.12
	50	0.76 ± 0.13	3.16 +/- 0.63	130.10 ± 18.81*	42.61 ± 3.93*	140.86 ± 22.13	1.27 ± 0.20
	200	0.40 ± 0.055	0.79 +/- 0.19	131.94 ± 8.01*	43.87 ± 1.62*	211.86 ± 38.27	1.54 ± 0.36
4 Days of Treatment	0	0.40 ± 0.040	1.20 +/- 0.20	84.07 ± 7.47	34.94 ± 2.03	108.45 ± 20.73	0.78 ± 0.21
	50	0.66 ± 0.15	2.65 +/- 0.90	102.48 ± 8.90	39.90 ± 2.07	123.08 ± 18.56	1.07 ± 0.26
	200	0.38 ± 0.042	1.12 +/- 0.28	137.03 ± 8.00*	47.54 ± 2.23*	231.10 ± 51.84*	1.44 ± 0.34
21 Days of Treatment	0	0.93 ± 0.25	2.73 +/- 0.62	94.37 ± 7.28	46.24 ± 2.21	47.80 ± 7.73	0.44 ± 0.058
	50	1.60 ± 0.24*	4.54 +/- 0.92	96.56 ± 7.90	46.72 ± 1.62	90.64 ± 13.04	0.80 ± 0.11
	200	1.00 ± 0.23	2.81 +/- 0.73	136.80 ± 8.86*	61.18 ± 2.92*	158.75 ± 29.54*	1.23 ± 0.20*

[#]Mean ± SEM (*n* = 10 unless indicated otherwise)^a*n* = 9^c*n* = 8* Significant effect by ANOVA followed by Kruskal-Wallis nonparametric test (*p* = 0.05) with comparison to control by the Dunn's multiple comparison test (*p* < 0.05).

Table IV

Serum steroid and LH concentrations in castrated male Wistar rats after 3 days of treatment with atrazine

	Treatment (mg/kg/d)	Androstenedione (ng/ml)	Testosterone (ng/ml)	Estrone (pg/ml)	Estradiol (pg/ml)	LH (ng/ml)	Body weight ^b (g) at 66 days	Body weight ^c (g) at 68 days
Castrated^a	0	0.16 ± 0.019	0.10 ± 0.0 ^f	74.06 ± 6.86	37.00 ± 1.89	8.89 ± 0.68	299.4 ± 4.9	299.4 ± 4.9
	50	0.20 ± 0.031	0.10 ± 0.0 ^f	94.46 ± 8.51	41.87 ± 3.11	4.94 ± 1.16 ^{g*}	291.5 ± 2.8	290.0 ± 2.5
	200	0.20 ± 0.027 ^g	0.10 ± 0.0 ^{f,g}	112.15 ± 9.25 ^{g*}	48.52 ± 3.19 ^{g*}	6.86 ± 1.054 ^g	295.1 ± 2.5	282.8 ± 3.0

^aMean ± SEM (*n* = 10 unless indicated otherwise)^bBody weight on first day of treatment^cBody weight on final day of treatment^d*n* = 8^fAll values were below the lowest detectable level of the assay. Therefore values are expressed as the lowest detectable value 0.10ng/ml^g*n* = 9* Significant effect by ANOVA followed by Kruskal-Wallis nonparametric test (*p* = 0.05) with comparison to control by the Dunn's multiple comparison test (*p* < 0.05).

Discussion

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The impetus for this study were previous reports that exposure to atrazine alters serum concentrations of estrone and estradiol in the male rat, as well as *in vitro* studies showing increased aromatase activity and CYP19 mRNA in the H295R cell line. Herein, we report the consistent duplication of increased serum estrone and estradiol in the male Wistar rat following 3-4 days of exposure to atrazine. Additionally, our studies show that atrazine not only altered serum estrogens, but also caused significant increases in serum androgens, progesterone, and corticosterone. Thus it appears that atrazine has a broader effect on steroidogenesis. Our work also demonstrates that changes in estrogen concentrations are not the result of increased availability of substrate. Nor are the changes in estrone and estradiol likely the result of increased testicular aromatase activity or increased transcription of CYP19 mRNA in the testes. Rather a different atrazine-mediated event appears to be responsible for the observed changes in serum estrone and estradiol levels.

Prior to beginning this work, little to no information was available regarding the effect of atrazine on the steroids from which estrogens are synthesized. We report that significant changes in the estrogen precursors, androstenedione and testosterone, occur as early as 6 hours after an initial dose of atrazine. Within 24 hours, following a single dose, these changes in androgen concentrations have dissipated. Yet, it is not unreasonable to assume that during the eighteen hour window, between the six and twenty-four hour observation times, the excess androgens could have been metabolized. The modest increase in estrone levels observed at the 24 hour time point lends some credence to such a hypothesis.

The effect of multiple doses of atrazine on serum androgens was not conserved for both treatment groups. The serum concentration of androstenedione and testosterone

remained significantly elevated on the second day of treatment with the lower dose of atrazine (50mg/kg). However, the androgens in the highest treatment group were similar to control. Although not statistically significant from the control, this U-shaped response curve for both androgens prevailed following 3, 4, or 21 days of exposure. The U-shaped androgen response curve may be caused by altered LH levels in animals treated with atrazine. Atrazine has been reported to inhibit the LH surge in female rats and to reduce basal serum LH levels in male rats (Stoker *et al.*, 2000, Cooper *et al.*, 1996). Under normal physiological conditions, gonadotropin releasing hormone (GnRH) controls the pulsatile release of LH from the pituitary gland. Release of LH initiates a series of events in the male that result in production of testosterone in the testes. Testosterone then participates in a negative feedback mechanism to regulate the release of LH (Griffin 1996) (Figure 14). Thus, the loss of testosterone, and in turn the negative feedback mechanism, will cause elevated levels of LH, as observed in the castrated males. Conversely, a reduction in the release of GnRH and/or LH will result in lower testosterone concentrations. Therefore, if, as previously reported, atrazine depresses the levels of LH in the intact male (Stoker *et al.*, 2000) one would expect a reduction in serum androgen levels to be observed as well. Further work will be necessary to definitively demonstrate the effects of atrazine on LH and androgens. Especially interesting will be the effect atrazine has on LH in males receiving only a single dose of atrazine, when androgens did not demonstrate a U-shaped response.

The dose dependent changes in corticosterone observed in these animals were also interesting. At all but two of the six time points examined, serum corticosterone was significantly greater in the 200 mg/kg group as compared with control. While it is known that corticosterone follows a daily circadian rhythm (Atkinson and Waddell, 1997), the

increases observed during the study can not be explained by circadian rhythms because, with exception of one time point, all animals were dosed and killed at the same time of day (Table I). It is interesting that the corticosterone levels in animals killed 6 hours after the single dose were not significantly elevated. These animals were killed later in the day when corticosterone levels were approaching their peak (Atkinson and Waddell, 1997). This would suggest that any atrazine-mediated increase in corticosterone 6 hours after treatment was perhaps masked by the circadian peak at that time point.

In addition to above changes in serum steroid concentrations, the studies reported here replicated previous reports of the effect of atrazine on estrone and estradiol. The fact that changes in both estrone and estradiol occur after only 3 days of treatment is notable as previous studies (Stoker *et al.*, 2000) have focused on considerably longer periods (30 days) of exposure. Here, atrazine altered estrone and estradiol levels after only 3 daily exposures and with continual treatment persisted at least through 21 days. These data should prove germane to the design of future investigations to identify the specific molecular event responsible for the elevation of serum estrogens following exposure to atrazine.

The changes in estrogen levels observed *in vivo* do not appear to be caused by a change in aromatase activity. No change in aromatase activity was observed in testicular microsomes prepared from animals exposed to atrazine for 3 days, although serum estrone and estrogen was significantly elevated at that time point (Figure 11). Nor was there a change in aromatase activity in testicular microsomes prepared from untreated males and exposed to atrazine *in vitro* (Figure 7B). While other explanations for the changes in estrone and estradiol levels exist, these studies indicate that the effect of atrazine on estrogen levels is most likely not the result of a direct interaction between atrazine and aromatase.

Although serum steroid concentrations are not totally reflective of tissue concentrations, the early increases in androstenedione and testosterone concentrations observed after a single dose of atrazine provided a potential explanation for the elevated levels of estrone and estradiol. A comparison of the substrate concentrations of androstenedione in the serum to the experimentally determined K_m for aromatase does not exclude a substrate driven model of elevated enzyme activity. That is, at steroid concentrations below the K_m , subtle changes in steroid concentrations could produce a substantial change in estrogen concentrations. Here, the experimental K_m for androstenedione was determined to be 130 ± 14.6 nM. Following 3 days of exposure to atrazine, the serum concentrations of androstenedione and testosterone in the control animals were 1.98 ± 0.33 nM and 6.66 ± 2.0 nM, respectively. While at concentrations so far below the K_m it may be theoretically feasible that changes in estrogen levels are the result of increased substrate levels closer examination of the steroid data refutes this hypothesis.

The steroid data from the time course and castrated male studies demonstrates that changes in the serum estrogen levels are not the result of increased availability of substrate. Were the changes in estrogen levels propagated by an increase in substrate availability, the higher levels of androstenedione and testosterone observed in the lower atrazine treatment group would yield the greatest concentration of estrone and estradiol. Rather the results indicate that in animals treated with atrazine, there is not a direct relationship between the serum concentration of androgens and estrogens. Despite the greater availability of androgens in the 50 mg/kg treatment group (i.e., 2-fold and 3-fold increases in serum androstenedione and testosterone, respectively, as compared with the highest atrazine treatment group), at no time point was the concentration of estrone or estradiol in animals

treated with the lower dose of atrazine greater than those observed in the higher atrazine group (Figure 9). Furthermore, the effect of atrazine on serum estrone and estradiol is conserved in the castrated male. If the changes in estrone and estradiol observed in the intact animal were the result of increased substrate, the considerable reduction in androgen levels in the castrated males (Table IV) would nullify the effect of atrazine on estrone and estradiol. Not only was a dose dependent increase in estrone and estradiol observed in the castrated animal, but the magnitude of the change was similar to that observed in the intact male. Treatment with 200 mg/kg of atrazine resulted in concentrations of estrone and estradiol in the intact animal of 131.1 ± 8.0 pg/ml and 42.8 ± 1.62 pg/ml, respectively. In the castrated animal, despite the lower concentrations of substrate (Table IV), the same treatment produced estrone and estradiol levels of 112.15 ± 9.25 pg/ml and 48.52 ± 3.19 pg/ml respectively. Clearly, exposure to atrazine results in elevated concentrations of estrone and estradiol independent of the concentration of substrate in the serum.

This project also examined the effect of atrazine on the concentration of CYP19 mRNA in the hypothalamus and testis. The results in both tissues and at all time points indicated that atrazine had no effect on CYP19 mRNA. Yet, the significance of these data may be limited due to a recent case study, wherein Bouraima *et al.* (2003) examine a feminizing adrenocortical adenoma which had caused major hyperestrogenism in the patient from which it was removed. Although this patient had extremely high serum estrogen concentrations, the RT-PCR analysis did not detect a significant change in aromatase CYP19 expression in the adrenocortical adenoma. Bouraima *et al.* (2003) interpret this to mean that it may be possible that a small change in CYP19 mRNA levels, undetectable by RT-PCR analysis, could yield altered estrogen concentrations. However, other explanations also exist,

for example Bouriamia *et al.* (2003) do not exclude the possibility that the elevated serum estrogens are the result of extra-adrenal aromatization, activated by aromatase inducing factors produced by the adrenal carcinoma. While the Bouriamia *et al.* (2003) report documents only a single incident, it does suggest that caution should be taken not to base definitive conclusions about aromatase activity solely on RT-PCR data.

Despite questions raised by Bouriamia *et al.* (2003) the castrated male study allows for a more definitive conclusion to be drawn about the role of testicular CYP19 mRNA in the elevated estrogen levels after exposure to atrazine. As previously discussed, the quantity of serum estrogens in animals receiving the same dose of atrazine was not affected by castration. Therefore, one can surmise that testicular aromatase is not a significant contributor to the altered estrogen levels. Furthermore, because castration does not diminish the effect of atrazine on serum estrogen levels, a biologically significant change in CYP19 mRNA levels, undetectable by RT-PCR, could not have occurred in the testes. This conclusion is in agreement with the data gathered by real time RT-PCR analysis of testicular tissue discussed above. Finally, this would suggest that *in vivo* exposure to atrazine most likely does not affect the transcription of the PII driven aromatase promoter.

The data reported here does not eliminate the possibility that exposure to atrazine alters the transcription or enzymatic activity of aromatase in a different tissue. The significant changes in serum corticosterone provides an intriguing mode of action by which atrazine could alter the expression of aromatase in the adipose tissue. In humans, the expression of aromatase in the adipose tissue is regulated thorough the I.4 promoter that is activated by TNF-alpha and class I cytokines via a JAK1/STAT3 pathway. This promoter also requires the presence of glucocorticoids and glucocorticoid receptors for transcriptional

activation (Zhao *et al.*, 1995). The biologically limiting factors of I.4 expression have not yet been established. Yet, if the availability of glucocorticoids were the limiting factor in I.4 driven transcription, changes in corticosterone could potentially alter CYP19 mRNA transcription resulting in elevated levels of estrone and estradiol. If the rat possesses a promoter analogous the human I.4 promoter, then aromatase in adipose tissue could provided a site for conversion of adrenal androgens to estrogens. Although it has not yet been demonstrated that rodent adipose tissue has a similar promoter, the possibility of an analogous promoter in the rat is notable in light of the data reported here. The elevation of serum corticosterone observed at almost all time points following exposure to atrazine could play a role in the activation of adipose aromatase and explain, in part, the altered levels of serum estrogens after exposure to atrazine.

The possibility that changes in aromatase activity are a secondary or tertiary event should not be overlooked. Treatment with atrazine may well induce discomfort, mild nausea, or illness, events that the studies herein and *in vitro* work are unable to recognize or quantify. The biological reaction to “stress” for lack of a better term, induced by these potential responses to atrazine could affect the pituitary-adrenal axis. It could be these stress related events that directly, or indirectly (i.e. via altered levels of serum corticosterone) alter aromatase activity and/or transcription.

While aromatase has been the focus of this work, it can not be ignored that changes in estrone and estradiol levels after exposure to atrazine could occur independently of aromatase. Changes in the degradation and clearance of estradiol could be responsible for elevated estrone and estradiol concentrations. The metabolism of estrogens is controlled by the activity of P450 enzymes, other then aromatase (for review of enzymes see Martucci and

Fishman, 1993). For the most part, estrogen metabolism begins with the hydroxylation of either the A-ring of the steroid at the 2 or 4 carbon positions or at carbon 16 of the D-ring. Hydroxylation at either A-ring position allows for the excretion of the metabolite in urine or conjugation by methylation, sulfation, or glucuronidation (Lippert *et al.*, 1999). The metabolic clearance rate of 2-hydroxyestrodial and 4-hydroxyestradiol are 11 and 4 times greater than estradiol, respectively (Ball *et al.*, 1983, Ball *et al.*, 1985). The metabolic clearance rate of 16- α hydroxyestrone remains undefined as it, until recently, was considered to only exist as an intermediate in the formation of estriol. Estriol, the product of reduction of the C17 ketone has a reported metabolic clearance rate similar to that of 4-hydroxyestradiol (Flood *et al.*, 1976, Ball *et al.*, 1983, Ball *et al.*, 1985). Bradlow *et al.*, (1995) demonstrated in the estrogen receptor positive breast cancer cell line MCF-7, that exposure to atrazine significantly increased the ratio of 16- α hydroxyestrone to 2-hydroxyestrone formation. Assuming the metabolic clearance rate of 16- α hydroxyestrone is similar to estriol a change in this ratio would reduce the rate at which estrogens are removed from the system. If such a change were to occur *in vivo* it seems likely that the reduced rate of estrogen clearance could cause the serum concentrations of estrone and estradiol to rise, as observed in this and previous studies. The observation that treatment with atrazine results in a constant change in estrone and estradiol levels, despite depleted substrate levels after castration, would support this theory.

It is also important to note that changes in the ratio of C16 and C2 hydroxylation result in physiological changes consistent with atrazine mediated effects, further supporting the *in vitro* reports on the effect of atrazine on C16 and C2 hydroxylation (Bradlow *et al.*, 1995). It has been shown that 16- α -hydroxyestrone promotes carcinogenesis while the

antiproliferative effects of 2-hydroxyestrone inhibit carcinogenesis (for review see Mueck *et al.*, 2002). In fact, the ratio of C16 to C2 hydroxylation is used as an indication of cancer risk (Bradlow *et al.*, 1995). Atrazine, once believed to be carcinogenic, is known to affect the hypothalamic-pituitary-gonadal axis (Copper *et al.*, 1998, 2000). Cooper *et al.*, demonstrated that changes in catecholamine levels altered the secretion of luteinizing hormone and prolactin. The A-ring hydroxylated estrogens are referred to as catechol estrogens because of their ability to bind both estrogen and catecholamine receptors (Ball and Knuppen 1980, Ball and Knuppen 1990, MacLusky *et al.*, 1981 and Paul *et al.*, 1980). It is possible that changes in the ratio of C16 to C2 hydroxylation in the brain, reduces the availability of catecholamine receptor agonists, altering catecholamine levels, and thus altering the secretion of luteinizing hormone and prolactin. The known effect of atrazine on the ratio of C16 to C2 hydroxylation *in vitro* as well as the similarities between the effects of *in vivo* exposure to atrazine and alterations in the ratio of C16 to C2 hydroxylation present an interesting hypothetical role for estrogen metabolites in the catecholamine levels of atrazine treated animals. A considerable amount of work is necessary to test such a hypothesis. Yet, the correlation between the effects of *in vivo* exposure to atrazine and changes in the ratio of C16 to C2 hydroxylation lend legitimacy to the assertion that atrazine may alter the rate of estrogen clearance, resulting in the altered estrogen serum levels described herein.

Finally, there are recently characterized mechanisms, and likely others that have not yet been established, involved in the regulation of aromatase activity that have not been examined with respect to atrazine. Balthazart *et al.* (2003) have shown that calcium dependant kinases are responsible for regulation of aromatase activity in the preoptic-hypothalamic area of the quail brain. It is possible not only that a similar pathway exists in

the rodent, but that atrazine somehow affects this kinase pathway and in doing so alters aromatase activity. This hypothesis further complicates the task of assessing the effect of atrazine on aromatase activity; as such native regulatory systems would be disturbed if not eliminated by the microsomal preparation used in the $^3\text{H}_2\text{O}$ assay.

In summary, this report demonstrates that atrazine alters the steroidogenic pathway, not only altering serum estrogen concentrations as previously reported, but also increasing androgens and corticosterone in a temporal fashion. In addition, we present evidence that changes in estrone and estradiol levels are not the result of increased availability of substrate. The results of the *in vitro* aromatase assay minimize the likelihood that atrazine is able to directly affect the kinetic properties or rate of aromatase activity. Data from the castrated male study and real time RT-PCR demonstrate that exposure to atrazine does not change testicular aromatase activity or CYP19 mRNA. Furthermore, we demonstrate that the testes are not involved in altered serum estrone and estradiol levels in rats treated with atrazine. While unable to identify the mode and site of action by which atrazine alters estrogen levels, this study does eliminate the testicular tissue as a possible site of atrazine mediated changes in serum estrogens.

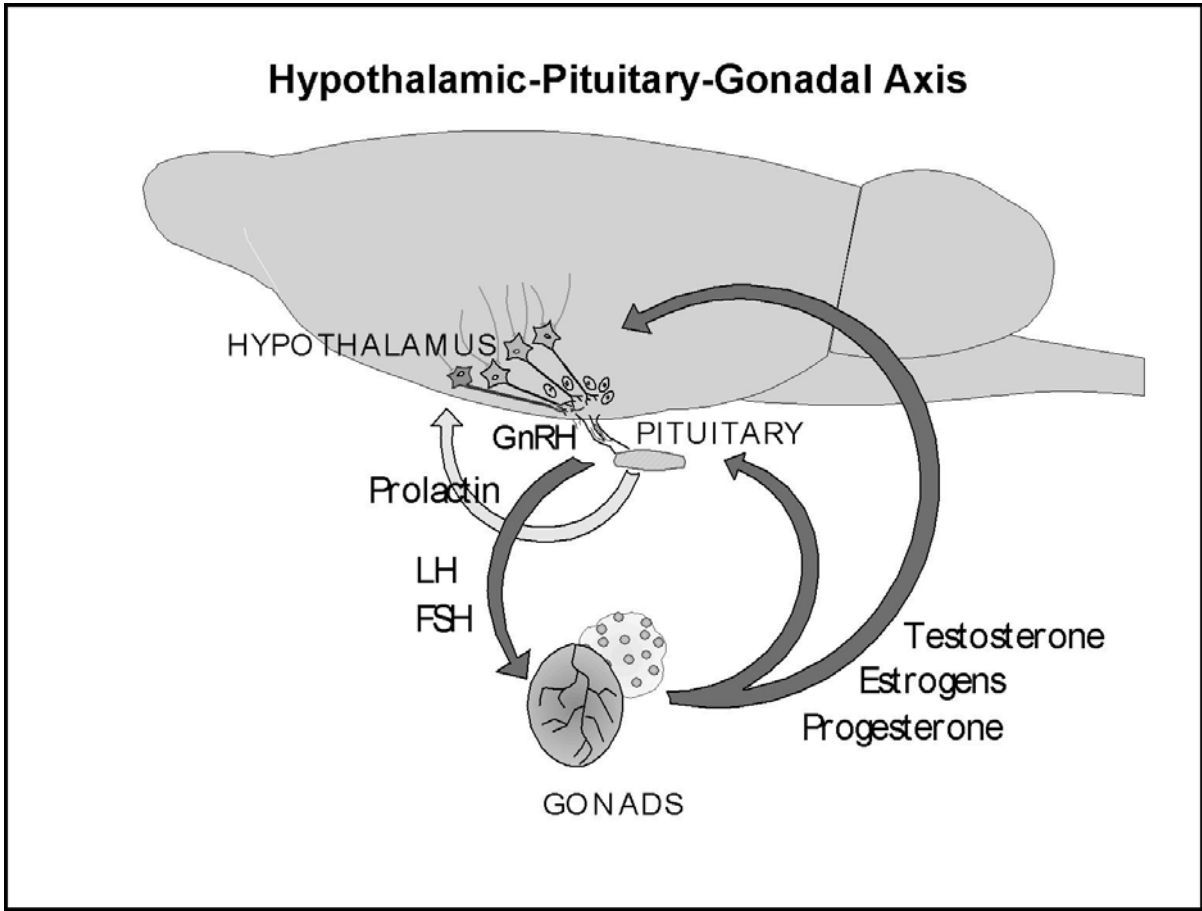


Figure 14 - Schematic representation of the hypothalamic-pituitary-gonadal axis. Hypothalamic signals induce pituitary secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH). These signals induce gonadal steroidogenesis. The products of gonadal steroidogenesis, testosterone, estradiol, and progesterone then participate in the regulation of pituitary secretion by acting at both the hypothalamus and the pituitary.

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