Disruption of Testosterone Homeostasis as a Mode of Action for the Reproductive Toxicity of Triazole Fungicides in the Male Rat

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Abstract

Triazole fungicides associated with a range of reported male reproductive effects in experimental animals were selected to assess potential toxic modes of action. Wistar Han rats were fed myclobutanil (100, 500, or 2000 ppm), propiconazole (100, 500, or 2500 ppm), or triadimefon (T: 100, 500, or 1800 ppm) from gestation day 6 to postnatal day (PND) 120. One male per litter was necropsied on PND1, 22, 50, or 92. Measurements included anogenital distance (AGD) at PND0, body and organ weights, serum hormone levels, age at preputial separation (PPS), sperm morphology and motility, and fertility and fecundity. AGD was increased by the high dose of all three triazoles, indicating hypervirilization. Triadimefon delayed PPS, consistent with delayed puberty, at 1800 ppm. Relative liver weights were increased at PND1, 50, and 92 by all three triazoles. Hepatocellular hypertrophy was present at PND50 from propiconazole and triadimefon and at PND92 from all three high dose triazole treatments. Relative pituitary weights were decreased at PND92 by middle and high dose myclobutanil treatment. Absolute testis weights were increased at PND1 by myclobutanil, at PND22 by myclobutanil and triadimefon, and at PND50 by propiconazole and triadimefon treatment. Relative ventral prostate weights were increased at PND92 by myclobutanil and triadimefon treatment. Serum testosterone was increased at PND50 by triadimefon and at PND92/99 by all three triazole treatments. Insemination and fertility were impaired by myclobutanil and triadimefon treatment. In addition to the reproductive system effects, total serum thyroxine levels were decreased at PND92 by high dose triadimefon. These reproductive effects are consistent with the disruption of testosterone homeostasis as a key event in the mode of action for triazole-induced reproductive toxicity.

Key Words: myclobutanil, propiconazole, triadimefon, development, steroidogenesis.

Introduction

Triazole fungicides are used agriculturally to control rust and mildew on fruit, vegetables, cereals and seeds, residential and commercial turf; and in pharmaceutical applications for the treatment of local and systemic fungal infections. The three agrichemical fungicides selected for this study (myclobutanil, propiconazole and triadimefon) were 1,2,4-triazole compounds, structurally distinct from the 1,3-imidazole fungicides such as ketoconazole and prochloraz.

The fungicidal mode of action of triazoles involves disruption of fungal cell membranes and walls, by the mechanism of inhibiting fungal lanosterol-14α-demethylase (cyp51; Ghannoum and Rice, 1999). Cyp51 is evolutionarily conserved between plants, fungi and animals, and in animals is critical for cholesterol synthesis and therefore steroid biosynthesis (Zarn et al., 2003). Besides Cyp51, triazoles also modulate the gene expression and enzyme activity of multiple CYPs and other metabolic enzymes in mammalian liver and other tissues (Barton et al., 2006; Goetz et al., 2006; Ronis et al., 1994; Sun et al., 2005, 2006; Tully et al., 2006). CYPs are phase I metabolizing enzymes that increase hydrophilicity and facilitate subsequent elimination of xenobiotics. CYP enzymes are also necessary for biosynthesis and catabolism of sterols, steroids, vitamin D, and other endogenous biochemicals (Nelson et al., 2004; Zarn et al., 2003). For example, the steroidogenic enzymes Cyp17α hydroxylase/ 17, 20 lyase and Cyp19 aromatase are critical to the biosynthesis of testosterone and estradiol. Propiconazole and triadimefon have been shown to inhibit Cyp19 aromatase *in vitro* (Vinggaard et al., 2000). These data suggest that CYP modulation by agrichemical triazoles has the potential to affect steroid homeostasis.

Male sexual development depends on appropriate levels of androgens during fetal and neonatal growth. The effects from androgen exposure in the developing male include masculinization of the brain, testicular development, and adult sexual behavior (Cummings and

Kavlock, 2004). Effects reported from triazole exposures of rodents include reproductive toxicity for myclobutanil and triadimefon and carcinogenicity for propiconazole and triadimefon. Two-generation reproduction studies in rats given myclobutanil resulted in testicular and prostatic atrophy, reduced litter size, and decreased pup weight gain (U.S. EPA, 2005a). Although propiconazole has been shown to inhibit aromatase *in vitro* it has been negative for reproductive effects in similar reproduction studies (U.S. EPA, 1999; U.S. EPA, 2005b). Two-generation reproduction studies in rats treated with triadimefon resulted in decreased fertility, litter size, and neonate viability, and increased serum testosterone in the F_1 generation (IPCS INCHEM, 1985; U.S. EPA, 2006). These data prompted our interest in gaining a better understanding of the modes and mechanisms of action for triazole reproductive toxicity, and led to the hypothesis of a common mode of action for this class of fungicides. The present study was designed to characterize the reproductive toxicity of myclobutanil, propiconazole and triadimefon following exposures from gestation through to adulthood. Endpoints examined were selected to test whether these triazoles shared a common mode of action for these reproductive effects.

Materials and Methods

Animals and animal husbandry

Animal care, handling, and treatment were conducted in an AAALAC-International accredited facility and all procedures were approved by the U.S. EPA NHEERL Institutional Animal Care and Use Committee. Rats were housed in polypropylene boxes containing Alpha-Dri® bedding (Shepherd Specialty Papers, Watertown, TN), with a 12 hour light: dark cycle under controlled temperature (72°F) and humidity (45%) with unlimited access to feed and water. Timed pregnant Wistar Han IGS rats were received from Charles River Laboratories (Raleigh, NC) on gestation day (GD) 1-3 and assigned to treatment groups so as to achieve equivalent weight means across treatment groups. Dams were single-housed and allowed to acclimate for 3 days prior to the start of treatment. Dams from each treatment group were divided evenly into daily blocks (7 total) to allow work to be done in manageable numbers. All dams within each block were of the same GD. Dams were allowed to deliver naturally, with day of delivery designated as PND0 for the F_1 offspring. On PND8 litters were weighed and then culled to 8 pups per dam, retaining males preferentially, to maximize uniformity in growth rates. Survival rates of offspring were based on percentage of animals remaining past PND8. The ratio of alive to dead male and female pups per treatment group was analyzed using Fisher's Exact test, measures with $p<0.05$ were considered significant. F_1 offspring were housed with their respective mothers until weaning at PND23. Males and females were then removed from the dams and housed by treatment in same-sex pairs until PND50. Males were single-housed after PND50, females remained housed in pairs.

Dosing regimen

Feed was prepared by Bayer CropScience (Kansas City, MO) as part of a materials Cooperative Research and Development Agreement between the U.S. EPA and the U.S. Triazole Task Force. Control animals were fed 5002 Certified Rodent Diet with acetone vehicle added. Treatment groups received feed containing either myclobutanil (M: 100, 500, or 2000 ppm), propiconazole (P: 100, 500, or 2500 ppm), or triadimefon (T: 100, 500, or 1800 ppm). Dams began treated feed diets on GD6, continuing through gestation, parturition and lactation. Dam feed intake and body weights were measured weekly during gestation and lactation periods and at necropsy. The F_1 generation continued on the same treated feed diets upon weaning at PND23. F_1 offspring feed intake and body weights were measured weekly until necropsy. One male from each litter was taken to necropsy at PND1, 22, 50, or 92 to assess affects on select organ weights, histology and hormone measures.

Anogenital Distance and Preputial Separation

On PND0, pup body weight and AGD were measured and footpads were tattooed for identification. AGD was measured under a dissecting microscope fitted with a calibrated ocular micrometer reticle, 15 units equaling 1 millimeter. AGD was defined as the distance from the anterior portion of the anus to the caudal portion of the genital tubercle. AGD was analyzed using analysis of covariance (ANCOVA) with a mixed effects model adjusting for body weight at PND0 and pup examiner (fixed effects) and dam block and individual dams (as random effects). Measures with $p \leq 0.05$ were considered significant.

F_1 males were examined for PPS beginning on PND38, continuing daily until complete cleavage of the epithelium lining the prepuce of the penis was observed indicating onset of puberty was achieved. Body weights were measured on the day of PPS. Delay of PPS was analyzed using analysis of variance (ANOVA) with Dunnett's post test and ANCOVA with

fixed predictors for body weight at PND43 and treatment, with addition of a random effect for block. Measures with $p \leq 0.05$ were considered significant.

Necropsy

At each selected time point (PND1, 22, 50, or 92) animals were anesthetized by CO_2 asphyxiation followed by exsanguination. Whole body weights was measured then brain, hypothalamus, hippocampus, pituitary, thyroid, liver, testis, epididymis, ventral prostate, and seminal vesicles were removed, weighed, and either fixed in 10% neutral buffered formalin for histology or snap frozen in liquid nitrogen for molecular work. Males within each treatment group were randomly assigned for histological or molecular biology tissue collection. Molecular tissues included brain, pituitary, thyroid, liver, testis, and ventral prostate. Blood was collected at PND22, 50, 92, and 99. Serum samples were collected and used for hormone analysis (see below). One testis and epididymis from each male at PND22, 50, and 92 necropsy were used for morphology analysis (see below). Statistical analysis of body weight was conducted using a one-way ANOVA, including a random effect for block. Absolute organ weights were analyzed using ANOVA with Dunnett's post test analysis. Relative organ weights were analyzed using a set of mixed effects linear models using Statistical Analysis System Proc Mixed (SAS Institute, Cary, NC), organ weights were adjusted for body weight and analyzed by ANCOVA for each triazole, by each necropsy time point, by each organ. Student's *t*-test was used for further comparisons between control and treatment groups. Measures with $p \leq 0.05$ were considered significant.

Histology

Brain, pituitary, thyroid, liver, testis, epididymis, and ventral prostate were collected for histological evaluation from each necropsy time point. Testis and epididymis were fixed in

Bouin's and embedded in paraffin. Brain, pituitary, thyroid, liver, and ventral prostate were fixed in 10% neutral buffered formalin at 4℃ and embedded in paraffin. Testis samples were sectioned transversely incorporating the rete testis. Five micron sections were cut from the paraffin-embedded tissues, stained with hematoxylin and eosin and prepared for light microscope examination. All slides were read without knowledge of treatment or time. Liver lesions were graded based on severity of hepatocyte hypertrophy. Lesion grading: 0 = no lesions present; 1 = centrilobular hypertrophy; 2 = centrilobular and midzonal hypertrophy; 3 = pan lobular hypertrophy; 4 = pan lobular hypertrophy with cytoplasmic vacuolization.

Serum Hormone Levels

Blood samples were collected in 6ml Vacutainer tubes containing SST Gel and Clot Activator (Becton-Dickinson, Franklin Lakes, NJ) and set on ice. Serum was prepared from the blood on the same day of necropsy and stored in siliconized microcentrifuge tubes at -80℃. Estradiol, Testosterone, Total Triiodothyronine (T_3), and Total Thyroxine (T_4) levels were assayed in duplicate using their appropriate Coat-A-Count radioimmunoassay (RIA) kits (Diagnostic Products Co., Los Angeles, CA) according to manufacturer's instructions. Luteinizing Hormone (LH) levels were quantified using the rat LH dissociation enhanced lanthanide fluorometric immunoassay (DELFIA®) as described by Bielmeier et al. (2004). Rat LH, for standards, was provided by the National Hormone and Pituitary Program. The capture antibody, 518B7, was provided by Dr. Jan Roser, UC-Davis, CA. The tracer antibody, clone 5303, was provided by Medix Biochemica, Kauniainen, Finland. Perkin Elmer Life Sciences (Gaithersburg, MD) performed antibody biotinyllation and europium labeling, and provided all other reagents. Thyroid Stimulating Hormone (TSH) levels were quantified using specific RIA adapted from Thibodeaux et al. (2003). Serum hormone levels were analyzed using ANOVA,

measures with $p \leq 0.05$ were considered significant. Student's *t*-test was used for further comparisons between control and treatment groups.

Sperm preparation for morphology and motility

One cauda epididymis was placed in a 35-mm culture dish containing 2 ml of 37°C modified-Hank's Balanced Salt Solution (mHBSS) supplemented with 0.035% sodium bicarbonate, 0.42% HEPES, 0.09% D-glucose, 1% sodium pyruvate, 0.0025% soybean trypsin inhibitor and 0.2% bovine serum albumin. Tubules of the cauda epididymis were pierced several times to release the epididymal fluid into the medium. The dish was maintained at 37°C for 5 min to allow dispersion.

Sperm morphology

Fifty microliters of sperm suspension was diluted 1:10 with Dulbecco's Phosphate Buffered Saline (Invitrogen Corp., Carlsbad, CA) containing 10% formaldehyde and 0.5% sucrose and stored in 4ºC. Sperm were examined with 40x magnification and phase-contrast illumination. Sperm were assigned categories based upon morphological examination: normal sperm, abnormal sperm, abnormal head and tail, or abnormal tail. A sperm head was deemed abnormal if it was misshapen and a sperm tail was considered abnormal if it was sharply bent, had thinning, exhibited splayed fibers, or missing completely. Five hundred sperm per animal were categorized. General Linear Modeling analysis was used (SAS Institute, Cary, NC). Analysis for each triazole was run separately, measures with $p \leq 0.05$ were considered significant.

Sperm image collection and motion analysis

Sperm suspensions were diluted in mHBSS (1:50) and loaded by capillary action into a 100 µm deep microslide (VitroCom, Inc., Mountain Lakes, NJ) and loaded into a HTM TOX-IVOS (Hamilton Thorne Biosciences, Inc., Beverly, MA), using version 12 software, set to

collect images at 60 Hz for 1 s at 37ºC. Motile sperm were previewed, and sperm concentration was adjusted as needed to yield 25-30 motile sperm per field of view. Eight fields for each biological sample were analyzed for percent motile, percent progressive sperm, and percent progressive tracks. Progressive sperm were user defined as those having an average path velocity greater than 100 μm/s and a ratio of straight line velocity to smoothed path velocity (STR) greater than 50%. At least 200 sperm per animal were tracked for determination of mean velocity and other motion parameters (Perreault and Cancel, 2001).

Insemination and Fertility Indices

Mating assays were conducted on PND78 or older treated males with untreated virgin Wistar Han females PND56 or older. Wistar Han female rats were housed by threes under the same conditions described above. After 10 days of acclimation, daily vaginal smears were collected, examined, and cycles were recorded. Females with 4 day cyclicity were used for mating assays. Upon second proestrus, one female was placed per treated male just before lights were turned off for the duration of one dark cycle. Mating behavior was observed at 5 and 20 minutes after introduction, and active mounting by the male and responsiveness of the female was noted. Females were removed the following morning, vaginal smears were examined for presence of sperm and females were placed back in their original housing. Insemination index was assessed by the ratio of vaginal smears positive for sperm to all smears and analyzed using Fisher's Exact test.

If spermatozoa were found in the vaginal smear the following morning, the female was designated as being at GD0. Females were euthanized and necropsied with removal of the intact uterus and ovaries on GD13-16 to evaluate fertility outcome. Ovaries were removed and observed for gross abnormalities and corpora lutea (CL) were counted. Uteri were excised,

numbers of live fetuses and resorptions were recorded, and fetuses were examined for abnormalities. Absence of fetuses or resorption sites was followed up with uterine staining in 5 ml of 2% ammonium sulfide solution for 5 minutes to distinguish implantation sites if any. Fertility index was assessed by the ratio of successful pregnancies to mated females and analyzed using Fisher's Exact test. Fecundity of the treated males was analyzed by the number of live fetuses per litter, the ratio of live fetuses to the number of CL, and the number of implants relative to the number of live fetuses. Results were analyzed using ANOVA with Dunnett's post test, measures with $p \leq 0.05$ were considered significant.

Ejaculated sperm counts after natural breeding

Procedures for housing, acclimation and determination of cyclicity in females were identical to the first mating assay. Control, M2000 and T1800 treated males PND118-120 were used in this assay. Propiconazole treated males were unavailable at this time point due to inadequate numbers. Shortly after lights were turned off, each treated male was cohabited for 4 hours in a wire-bottom cage with an untreated proestral virgin female. Mating behavior was observed and recorded at 5 and 20 minutes after introduction. After 4 hours the females were separated from the males and the number of copulatory plugs recorded. Females were euthanized with CO_2 asphyxiation for uterine sperm enumeration. The cervix of each female was ligated, uterine horns were removed and placed into 35-mm Petri dishes containing 2 ml of 35℃ Medium 199 (Sigma-Aldrich Corp., St. Louis, MO), and opened using small curved scissors. Dishes were shaken gently for 5–10 minutes at 35℃ for dispersion of sperm. Sperm suspensions were transferred to 15 ml conical tubes and total volumes were recorded. One hundred microliters of each sample was diluted 1:10 with distilled water and spermatozoa were counted using a hemacytometer.

Results

F_0 Dam & F_1 male feed consumption, estimated dose levels, and body weight gain

The daily feed consumption and calculated dose levels for F_0 dams during the last two weeks of gestation and three weeks of lactation prior to weaning are available in Rockett et al. (2006). Exposure to the triazole fungicides at the lower doses (100 and 500 ppm) did not have an effect on feed consumption during gestation or lactation. Dams exposed to M2000 had a reduced feed consumption rate during the first and second weeks of lactation but not the third. This may have been due to the F_1 pups beginning to transfer over to solid food. Dams exposed to P2500 had a reduced feed consumption rate during the last two weeks of gestation, and recovered close to control levels during lactation. Dams exposed to T1800 had a reduced feed consumption rate beginning the first week of the study and were still lagging behind controls at the time of weaning. In relation to the control animals, all dams in all treatment groups had increased feed consumption by 2 fold or greater during lactation. Dam body weights during gestation and lactation showed the historical decrease at parturition with normal weight gain during the lactation period (Rockett et al., 2006).

Table 1 presents the F_1 male food consumption and dose levels calculated during gestation, lactation, juvenile, and adult exposure periods. The dams consumed more feed and hence received increased doses during gestation and lactation, most likely due to the increased energy demand during this period. Dose levels, dependent on feed consumption and body weight gain, decreased in a similar pattern among the treatment groups compared to controls as the animals aged. Similar to the response by F_0 dams, exposure to the three different triazole fungicides at the lower doses (100 and 500 ppm) did not have a robust effect on F_1 male feed consumption. T1800 treated F_1 males had a consistent reduction in feed consumption throughout

the study. Feed efficiency (the percent of consumption to body weight ratio) increased at later time points in a consistent fashion by M2000 and T1800. M2000 and T1800 F_1 male feed consumption did decrease, however the significant loss in body weight resulted in a difference in dose levels of these high dose groups.

Body weights of F_1 males were measured weekly (Table 2). There were no consistent treatment effects after exposure to 100 or 500 ppm of any treatment. Males in all three high treatment groups had significant decreases in body weight relative to controls. Significant loss in body weight was observed for M2000 from weaning and onward throughout the study, P2500 began to show a similar reduction in body weight shortly after weaning. T1800 F_1 males had reduced body weight from birth and remained less that control weight throughout the study.

Litters exposed to M2000 or T1800 treatment had decreased survival rates of 82.6% and 47.37% respectively (Table 3). Eighteen of the 30 litters exposed to M2000 had deaths within, one whole litter was lost due to treatment. Eighteen of the 21 litters exposed to T1800 had deaths within, nine whole litters were lost due to treatment. There were no treatment related deaths in animals that survived past PND8.

Anogenital Distance and Preputial Separation

AGD was significantly increased following exposure to M2000, P2500, and T1800 (Figure 1). Individual triazole trend analysis using a regression model to compare the slope across dose, showed a significant increasing trend for myclobutanil ($p<0.001$), propiconazole ($p=0.013$), and triadimefon ($p=0.024$). Table 4 presents the day PPS was achieved, the mean body weight on the day of PPS and on PND43 across treatment groups. PPS was delayed following T1800 exposure based on mixed effects ANCOVA and ANOVA models. The body weight at the time of PPS was significantly reduced in males following M2000 (7.8%), P2500

(4.6%), T500 (3.9%), and T1800 (13.2%) treatment based on ANOVA analysis. F_1 males had decreased body weights at PND43 following M2000 (10.3%), P2500 (8.7%), and T1800 (24.6%) treatment. Due to reduced body weights by some treatments, ANCOVA analysis using body weight as a covariate could not be used to examine effects of treatment on endocrine development.

Organ Weights

Relative liver weights were increased at PND1 following exposure to M2000 (13%), P2500 (15.5%), and all three doses of triadimefon (4.25%, 15.5%, and 17.5%; Table 5). There was no effect on relative liver weight at PND22. The relative liver weight of peripubertal (PND50) males was increased following exposure to M2000 (11.5%), P2500 (25.8%), and T500 (5.8%). Due to limited numbers of T1800 animals surviving to weaning, insufficient animals were available at this dose to include a PND50 necropsy. Adult (PND92) relative liver weights were increased following exposure to M2000 (9.0%), P2500 (23.8%), and T1800 (26.3%).

Absolute testis weight was increased at PND1 following M100 and M2000 treatments, at PND22 following M500 and T500 treatment, and at PND50 following P500, T100, and T500 treatment. By PND92, there were no effects observed on absolute testis weight (Table 6). Changes in accessory sex gland weights were observed at PND92 (Table 6). Relative ventral prostate weight was increased following M500, T100, and T500 treatment. Absolute ventral prostate weight was increased following M500 treatment. Absolute epididymal and seminal vesicle weights decreased following exposure to T1800.

Relative pituitary weights were decreased at PND92 following M500 and M2000 exposure (Table 7). Relative brain weight was decreased by propiconazole at PND22 by P100, at PND50 by P100 and P500, and at PND92 by P500. High dose triadimefon also decreased

relative brain weight at PND92. Absolute pituitary to brain weight ratios were significantly reduced at PND92 following M500 (16.8%) and M2000 (16.5%) exposure.

Histology

Liver histology of the PND1 and 22 animals were not different from controls. At PND50, P2500 males had centrilobular and slight midzonal hypertrophy; T500 males had centrilobular hepatocyte hypertrophy (Table 8). At PND92, M2000 and P2500 males had mild centrilobular hepatocyte hypertrophy, and T1800 males had strong pan lobular hypertrophy. Pituitary, thyroid, testis, epididymis, and ventral prostate histology results did not show any consistent histologic alterations across the treatment groups (data not shown).

Serum Hormone Levels

Serum testosterone levels were measured at PND50 and PND92. At PND50, serum testosterone was increased following exposure to T500 and T1800, and at PND92 following exposure to M2000, P500, P2500, T500, and T1800 (Figure 2). Serum levels of estradiol and LH were unaffected by treatment. Total T_4 levels were not changed at PND50 but decreased at PND92 from T1800 treatment (control = 5.61 $\pm$ 1.38 μg/dl; T1800 = 4.22 $\pm$ 0.95 μg/dl). Total T_3 and Thyroid Stimulating Hormone levels measured at PND50 and PND92 did not show a significant change across any treatment group. Effects of the triazoles on hormones of the hypothalamo-pituitary-gonadal (HPG) and thyroid (HPT) axes are depicted in Figure 4, and put into context relative to effects on the liver.

Sperm morphology and motility

There were no significant differences in sperm head or tail morphology or sperm motility across treatment groups (Table 9).

Fertility Outcomes

Fertility parameters included the insemination index, fertility index, total number of implantation sites, number of early and late dead embryos, and number of live and dead fetuses. Insemination index was reduced in mating pairs of untreated females with M2000 or T1800 males to 31.3% or 7.7% respectively (Table 9, Figure 3A). Fertility index was reduced in mating pairs of untreated females with M500, M2000, or T1800 males to 54.5%, 25.0%, and 0.0% respectively (Table 9, Figure 3B). Of those females that checked positive for sperm in their vaginal smears, not all of them became pregnant (false positive). There were also four false negative females, one from M500, M2000, T100, and T500 treatment groups (Table 9). There were no significant effects on the total number of implantation sites, live or dead fetuses, live or dead embryos or the number of resorptions by any of the triazoles tested (Table 9). All successful pregnancies, independent of the treatment group, produced normal healthy litters with little to no post implantation loss.

In the 4 hour cohabitation natural breeding assay, numbers of copulatory plugs and ejaculated sperm counts from ligated uteri were measured. Four of the five control animal mating pairs produced multiple copulatory plugs during the four hour cohabitation breeding period. One of the five M2000 mating pairs was found with one copulatory plug under its cage. There were no copulatory plugs found under any of the five T1800 mating pairs (data not shown). Ejaculated sperm counts were only available for the control animals, there was complete lack of sperm in the uterine horns of females from the M2000 and T1800 mating pairs.

The observed modes of action for the reproductive toxicity of the three triazoles are tabulated with accompanying observed effects and potential mechanisms of action (Table 10). Doses representing the no observed effect levels (NOEL) and lowest observed effects levels (LOEL) from this current study are also included.

Discussion

This study was designed to identify potential modes of action for the reproductive toxicity of three triazole fungicides: myclobutanil, propiconazole, and triadimefon. Mode of action is the sequence of key cellular and biochemical events which result in a toxic effect, and when multiple chemicals cause a common toxic effect(s) by the same, or essentially the same sequence of biochemical events this constitutes a common mode of action (Seed et al., 2005). A mechanism of action is a more detailed understanding of the molecular basis of the toxic effect. It is significant when a group of chemicals share a common toxic effect and mode of action, and oftentimes this requires a cumulative assessment of human health risk. Based on earlier studies with these triazoles, it was hypothesized that disruption in steroid homeostasis is a common mode of action leading to abnormal reproductive development and diminished reproductive function. To establish this mode of action we examined the dose-response and onset of a series of neonatal, peripubertal and adult endpoints.

Serum testosterone levels were elevated in peripubertal males following triadimefon exposure, and in adult males following exposure to all three triazoles. These serum testosterone results are consistent with previous studies in rats exposed to myclobutanil (14 day exposure to 150 mg/kg/day; Tully et al., 2006) or triadimefon (IPCS INCHEM, 1985). Typically, increased testosterone will result in reduced gonadotropin-releasing hormone (GnRH) synthesis and release from the hypothalamus, and reduced synthesis and release of LH and FSH from the anterior pituitary. In the present study, LH was not decreased and the LH to testosterone ratio was altered, suggesting disruption of the HPG axis. Differences in the onset and dose-response of altered serum testosterone between the three triazoles may have been due, in part, to differences in pharmacokinetics (Barton et al., 2006).

Increased AGD at PND0 was observed in F_1 males following gestational exposure to the high dose of all three triazoles. The AGD of F_1 females was also increased following myclobutanil exposure (Rockett et al., 2006). Increased F_0 maternal serum testosterone at GD20 (unpublished data) could account for the increased AGD of male and female pups following myclobutanil treatment. Androgens are responsible for normal elongation of the AGD in neonatal males (Clemens et al., 1978). The fetal rat testis begins synthesizing androgens after GD14, during the period of sexually dimorphic development (Clemens et al., 1978). Disruption of maternal and fetal steroid biosynthesis by myclobutanil and subsequent elevation in circulating and tissue levels of androgens in the male and female fetuses likely resulted in the increased AGD. This effect was unrelated to body weight, as has been seen in other studies, because AGD increased even when body weight was controlled as a constant (Gallavan et al., 1999).

Several factors are involved in regulating testosterone levels and controlling steroid homeostasis. By GD19, the fetal hypothalamic pituitary gonadal axis provides both positive and negative feedback to facilitate regulation of synthesis and release of gonadotropins and stimulating Leydig cell steroidogenesis. Naturally occurring androgen metabolism in the liver is catalyzed by a variety of endogenous enzymes, including hydroxysteroid dehydrogenases and Cyp3a and Cyp2b isoforms which are known to hydroxylate testosterone (Goodwin et al., 2002). Exposure to myclobutanil, propiconazole, and triadimefon induced expression of CAR and PXR stimulated pathways which are involved in xenobiotic metabolism and clearance in the liver (Goetz et al., 2006; Plant and Gibson, 2003; Sun et al., 2005, 2006; Tully et al., 2006). Since both CAR and PXR regulate the expression of CYP genes and the associated enzyme activity of

oxidative metabolism of steroid hormones as well as xenobiotics, it may be that CAR and PXR are also components of a regulatory network that regulates circulating hormone levels.

Assessment of liver CYP enzyme activities through alkoxyresorufin O-dealkylation assays demonstrated that myclobutanil, propiconazole, and triadimefon induced Cyp2c and Cyp3a enzyme isoforms in the rat liver (Barton et al., 2006; Sun et al., 2005, 2006). It has been reported that a likely mode of action for liver enzyme induction and weight increase is through CAR-mediated gene expression, based on the lack of hepatic response to cyproconazole in CAR knock out mice (Peffer et al., 2006). The observed increase in relative liver weight and evidence of hepatocyte hypertrophy in the current study is consistent with previous studies (Tully et al., 2006; U.S. EPA, 2005b, 2006). There was not a robust increase in absolute liver weights; however the significantly increased relative weights suggest the increase was at least in part, due to a treatment effect (Uemitsu et al., 1986). These combined results suggest that triazole fungicides disrupt testosterone homeostasis, at least in part, by induction of metabolic enzymes in the liver.

High dose triadimefon delayed the onset of puberty as marked by the delay in complete balano-preputial separation. Preputial separation is an androgen-dependent process that occurs just prior to puberty, before a significant rise in circulating androgens, and prior to the appearance of mature sperm in the caput epididymis in rats (Korenbrot et al., 1977). Preputial separation is not synonymous with puberty, however it is a necessary event for normal copulatory behavior, and a useful index of male pubertal development. Prenatal androgens influence the timing of puberty by controlling neural GnRH secretion in the fetal and neonatal brain. In addition to circulating androgens, fetal and neonatal development of GnRH secretion is also important in testicular development, masculinization of the brain, and subsequent sexual

maturation and behavior (Cummings and Kavlock, 2004). Triadimefon may affect the onset of puberty by inhibiting glutamatergic transmission and the function of N-methyl-D-aspartate and gamma-amino butyric acid receptors on the GnRH neurons within the developing central nervous system (Ebling, 2005; Reeves et al., 2004; Stoker et al., 2000). Elevated levels of testosterone can also directly inhibit pituitary sensitivity and response to GnRH (Kalra and Kalra, 1982). Thus high dose triadimefon may have delayed puberty by disrupting GnRH activation. However, the significant effects of triadimefon on body weight may have also contributed to the delayed onset of puberty (Carney et al., 2004).

Although body weights were reduced by the high dose treatment of these triazoles, this was not likely the cause of increased reproductive tissue weights. Studies on the effects of feed restriction alone, during pre- and post-natal development, demonstrate that a reduction in body weight will effect puberty and decrease the absolute weight of the adult ventral prostate, however produces no effect on adult reproductive function or on the absolute weight of the epididymis, seminal vesicles or the testis (Carney et al., 2004). At PND50, increase in absolute testis weight from propiconazole and triadimefon treatment was not accompanied by a significant decrease in feed consumption nor did body weight reduction exceed 4.8% from controls. Adult exposure to triadimefon increased intratesticular concentrations of testosterone, possibly explaining this increase in weight (unpublished data). This increase in testis weight was not statistically identified at PND92 suggesting acclimation over time. At PND92, increased absolute ventral prostate from myclobutanil treatment was not accompanied by significant weight loss and feed consumption was greater than controls. Increased epididymis and seminal vesicle weight from triadimefon treatment at PND92 was accompanied by a 24.9% body weight reduction and 7.5% decrease in feed consumption, however weight reduction to this degree has not been reported to

affect the weights of these tissues (Carney et al., 2004), indicating these effects were elicited in part by treatment. Propiconazole reduced body weights similar in magnitude to myclobutanil; however, had no effect on the testicular or accessory sex gland weights supporting the interpretation that myclobutanil and triadimefon treatment had an effect on these tissues. In addition to reproductive tissue effects, myclobutanil decreased adult pituitary weight. The pituitary to brain weight ratios were significantly reduced following exposure to myclobutanil, further indicating the effect on pituitary weight was not related to changes in body weight (House et al., 1985). This decreased pituitary weight may have been due to disruption of GnRH neuron development and sensitivity (Kalra and Kalra, 1982; Grober et al., 1998).

Mating pairs with myclobutanil or triadimefon treated males had a significantly reduced insemination index and associated reduced fertility. Complete lack of sperm in the uterine horns indicated a failure to inseminate and may suggest a possible impact on the male reproductive development or behavioral response (Burke et al., 1999). Masculinization of the developing brain is due in part to the metabolism of testosterone into estradiol (aromatization) and dihydrotestosterone (5α-reductase). Estradiol increases the number of GnRH cells in male rodents, causing a sexual dimorphic difference between males and females. However, too much estradiol early in development reduces the number of GnRH cells (Elkind-Hirsch et al., 1981). Reduction in GnRH cells during this critical stage in development could explain the reduced fertility in the adult males. Alternatively, disruption in the number or size of the spinal nucleus of the bulbocavernosus (SNB) motoneurons and associated dendrites can have a negative impact on reproductive performance in the male rat (Burke et al., 1999). Sexually dimorphic development of SNB motoneuron size and number is androgen and estrogen dependent and occurs during early development (Breedlove and Arnold, 1983; Burke et al., 1999), alteration in

testosterone and estrogen levels during this developmental stage could potentially explain the reproductive effects by myclobutanil and triadimefon. Triadimefon, but not myclobutanil, has been reported to do reduce insemination (IPCS INCHEM, 1985). Reduced fertility by myclobutanil and triadimefon suggested a possible reduced fertilizing ability, yet sperm morphology and motility were normal. The reduced fertility index for myclobutanil and triadimefon is consistent with registrations studies submitted to the U.S. EPA (U.S. EPA, 1999, 2005a, 2005b). Differences in reproductive performance may be a reflection of the differences in metabolism and clearance of the triazoles (Barton et al., 2006).

The doses used for this study were selected to match doses used in regulatory studies for registering these triazoles with the U.S. EPA. From two generation reproductive toxicity studies, the current lowest observed adverse effect level (LOAEL) and no observed adverse effect level (NOAEL) are 60.1 and 12.1 mg/kg/day for myclobutanil (U.S. EPA 2005a). Propiconazole was negative for any reproductive effects in registration studies (U.S. EPA, 2005b). The LOAEL for triadimefon is 90.0 and the NOAEL is 15.0 mg/kg/day (U.S. EPA, 2006). However, the NOAEL values used to determine the reference dose (RfD) for each of these triazoles are based on two year chronic studies; 2.5 mg/kg/day for myclobutanil (RfD 0.025 mg/kg/day; U.S. EPA, 2005a), 10.0 mg/kg/day for propiconazole (RfD 0.10 mg/kg/day; U.S. EPA, 2005b) and 3.4 mg/kg/day for triadimefon (RfD 0.0034 mg/kg/day; U.S. EPA, 2006).

Based on elevated serum testosterone, reduced reproductive function, or decreased pituitary weights in this study, the NOEL for observed effects in the present study are higher than the NOAEL from registration studies for all three triazoles. Unlike the higher NOEL, the LOEL in the present study was lower than the LOAEL for propiconazole and triadimefon. The

decrease in LOEL was likely due to differing endpoints measured, and it appears that changes in serum testosterone is a more sensitive endpoint than fertility and fecundity outcomes.

A common mode of action for the reproductive toxicity of the triazole fungicides appears to be disruption of testosterone homeostasis. Elevated serum testosterone, increased testis weights and AGD, and hepatomegaly indicative of altered liver metabolism of steroids are the key events consistent with this common mode of action. In addition, effects on reproductive function, pituitary weight, and the onset of puberty suggest possible neuroendocrine effects for myclobutanil and triadimefon. To further characterize mechanisms of action we are profiling gene expression in the livers and testes of rats from this study, in order to monitor effects of triazoles on steroidogenic, P450 and xenobiotic metabolizing enzyme genes in these tissues. Similar genomic and proteomic analyses are also underway with thyroids from this study. Results from these ongoing studies should help us better define the mechanisms underlying the mode of action defined by the current dataset (Table 10) and expand our understanding of triazole reproductive toxicity and potential impact on human health.

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Figure Legend

1. Increase in AGD at PND0 following gestational exposure to myclobutanil, propiconazole, or triadimefon. AGD is given in millimeters $\pm$ SEM. *p<0.05; **p<0.01; ***p<0.001.

2. Serum testosterone at PND50 and PND92/99 following exposure to myclobutanil, propiconazole, or triadimefon. Serum testosterone values in ng/ml + SEM, weights in grams + SEM. *p<0.05; **p<0.01; ***p<0.001.

3. Insemination (A) and Fertility (B) index for male rats exposed to myclobutanil, propiconazole, or triadimefon. *p<0.05; **p<0.01; ***p<0.001.

4. Effects of triazoles on hormones and enzymes of the hypothalamo-pituitary-gonadal (HPG) and thyroid (HPT) axes and the liver. Arrows between tissues indicate directional influence of hormones within the axes. Effects on gonadotropin releasing hormone (GnRH), thyrotropin releasing hormone (TRH), thyroid stimulating hormone (TSH), luteinizing hormone (LH), follicle stimulating hormone (FSH), triiodothyronine (T_3), thyroxine (T_4), cytochrome P450 enzymes (P450), and xenobiotic metabolizing enzymes (XME) are indicated by ↑ (increased), ↓ (decreased), NC (no change) or ND (not determined). Testosterone and thyroid hormones act back on the hypothalamus and pituitary creating a negative feedback loop to regulate hormone release (not shown).

Figure 1

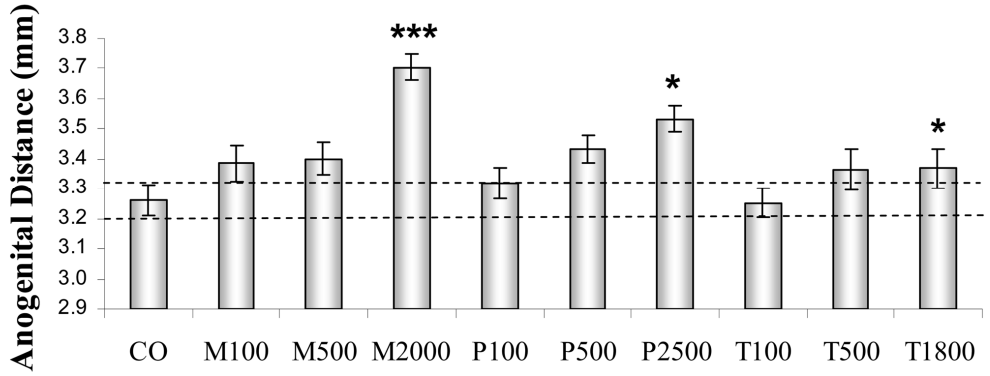

Figure 2

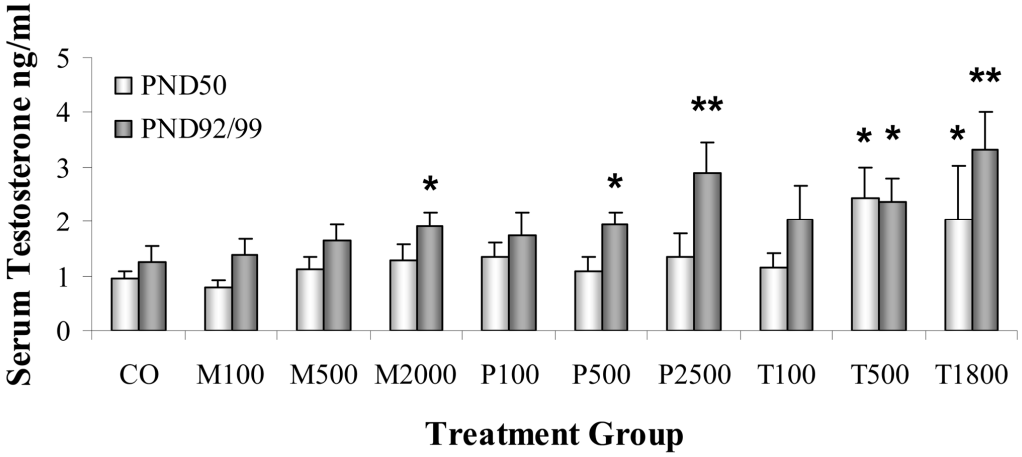

Figure 3

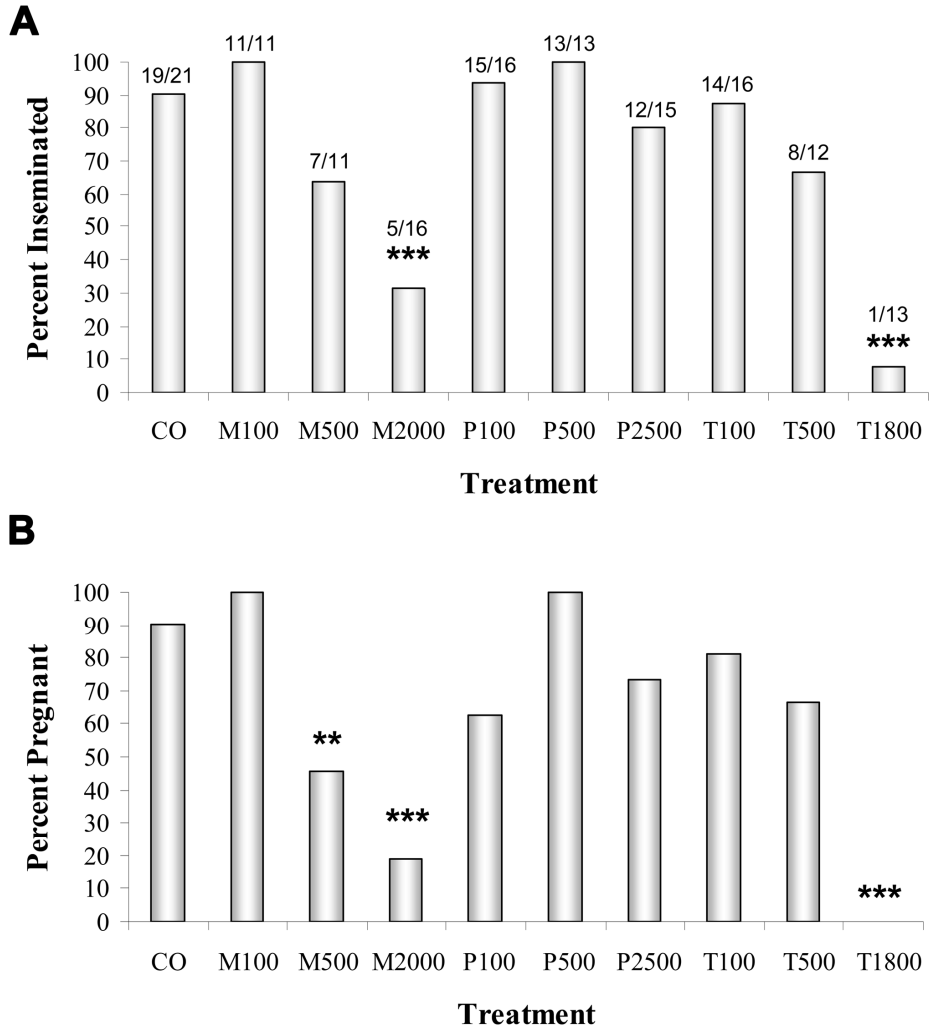
A
Percent Inseminated
100
90
80
70
60
50
40
30
20
10
0
19/21
11/11
7/11
5/16
***
15/16
13/13
12/15
14/16
8/12
1/13
***
CO
M100
M500
M2000
P100
P500
P2500
T100
T500
T1800
Treatment
B
Percent Pregnant
100
90
80
70
60
50
40
30
20
10
0
**
***
***
CO
M100
M500
M2000
P100
P500
P2500
T100
T500
T1800
Treatment

Figure 4

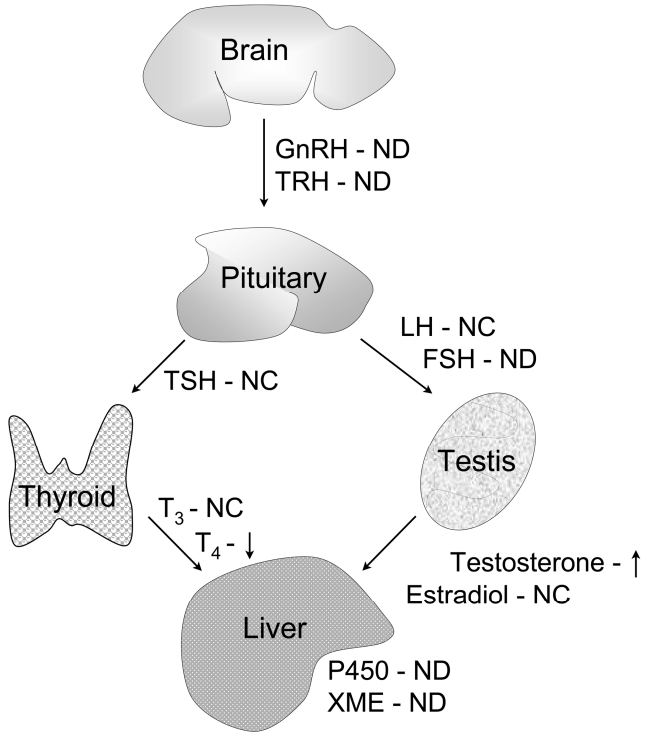
Brain
GnRH - ND
TRH - ND
Pituitary
LH - NC
FSH - ND
TSH - NC
Testis
Thyroid
T3 - NC
T4 - ↓
Testosterone - ↑
Estradiol - NC
Liver
P450 - ND
XME - ND

Table 1. Daily food consumption and calculated dose levels.

		Feed Consumption (g/rat/day)											
		GD6 - PND0	PND1 – 22	PND 23-29	PND 30-36	PND 37-43	PND 44-50	PND 51-57	PND 58-64	PND 65-71	PND 72-78	PND 79-85	PND 86-92
Treatment	ppm			Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12	Week 13
Control	0	17.0-19.7	23.1-45.9	9.3 ± 1.9	15.1 ± 1.6	17.8 ± 3.9	21.0 ± 4.2	21.7 ± 2.8	24.8 ± 3.0	22.7 ± 2.9	25.3 ± 2.9	23.9 ± 2.5	22.0 ± 4.4
Myclobutanil	100	18.2-20.5	21.0-48.9	9.2 ± 1.2	15.1 ± 1.2	18.1 ± 1.9	20.3 ± 2.6	21.1 ± 3.4	22.9 ± 2.9 [a]	23.3 ± 3.2	24.1 ± 2.6	24.6 ± 2.8	23.7 ± 2.3
	500	17.4-19.4	20.1-48.6	9.9 ± 1.4	15.2 ± 1.4	18.0 ± 2.4	20.6 ± 2.7	22.0 ± 2.7	23.1 ± 3.1	22.7 ± 4.1	22.9 ± 6.2	24.7 ± 2.3	23.8 ± 2.9
	2000	15.7-19.0	19.9-44.3	8.6 ± 1.9	13.1 ± 1.7 [b]	16.6 ± 1.6	18.9 ± 1.6 [b]	20.6 ± 4.9	21.2 ± 2.3 [b]	20.3 ± 3.6 [a]	23.2 ± 3.7	23.7 ± 4.0	21.4 ± 2.4
Propiconazole	100	16.9-20.8	22.2-49.1	9.3 ± 1.0	15.3 ± 1.1	18.5 ± 2.2	21.2 ± 1.2	22.6 ± 2.2	24.4 ± 1.8	23.7 ± 3.5	25.2 ± 2.4	24.7 ± 2.4	24.0 ± 2.4
	500	16.0-18.8	20.6-48.1	8.7 ± 2.8	14.7 ± 2.1	17.4 ± 1.9	20.3 ± 1.8	20.8 ± 2.5	22.6 ± 2.4 [a]	23.6 ± 1.7	23.6 ± 2.5	23.5 ± 2.0	23.0 ± 1.7
	2500	12.9-17.6	21.3-42.6	8.3 ± 1.3	13.7 ± 2.2 [b]	17.7 ± 3.3	19.1 ± 1.9 [a]	21.4 ± 3.1	22.9 ± 1.9 [a]	22.3 ± 2.4	24.1 ± 2.6	23.5 ± 2.3	22.5 ± 1.8
Triadimefon	100	17.4-20.0	23.4-46.0	10.4 ± 1.4 [a]	14.8 ± 1.7	17.5 ± 1.7	20.1 ± 3.5	23.9 ± 3.5	23.8 ± 2.7	22.2 ± 2.2	25.3 ± 3.0	24.7 ± 2.2	23.7 ± 2.8
	500	15.8-19.3	19.4-47.6	9.5 ± 1.1	14.7 ± 1.8	17.6 ± 1.9	20.2 ± 1.7	19.7 ± 3.8	22.3 ± 1.5 [b]	22.7 ± 1.6	22.9 ± 1.8	23.3 ± 1.9	23.4 ± 2.7
	1800	11.0-17.5	16.6-40.6	7.1 ± 0.9 [b]	11.1 ± 2.7 [b]	14.7 ± 2.0 [b]	16.1 ± 2.3 [b]	18.4 ± 5.1 [b]	18.4 ± 2.6 [b]	18.0 ± 2.6 [b]	20.6 ± 5.2 [b]	21.7 ± 3.1 [b]	20.4 ± 4.0
		Dose level (mg/kg/day)											
Control	0	0.0 - 0.0	0.0 - 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Myclobutanil	100	8.0 - 8.1	8.1 - 19.1	15.8 ± 2.0	13.7 ± 1.5	12.4 ± 1.1	10.6 ± 1.1	9.7 ± 0.6	8.8 ± 0.4	7.9 ± 0.4	7.5 ± 0.4	7.3 ± 0.5	6.1 ± 2.0
	500	38.8 - 38.7	39.4 - 93.8	77.2 ± 8.4	69.4 ± 5.1	60.8 ± 4.1	54.8 ± 3.5	48.8 ± 3.4	44.2 ± 4.3	40.3 ± 3.5	38.2 ± 3.7	36.6 ± 3.5	32.9 ± 2.7
	2000	141.3 - 149.9	155.0 - 347.3	279.1 ± 22.5	280.2 ± 19.8	245.4 ± 16.0	219.0 ± 13.8	197.5 ± 23.0	180.2 ± 17.3	163.3 ± 11.1	153.5 ± 8.6	153.6 ± 20.5	133.9 ± 13.8
Propiconazole	100	7.6 - 8.3	8.6 - 19.0	15.3 ± 2.0	13.9 ± 1.6	12.6 ± 1.3	10.8 ± 0.7	9.7 ± 0.6	9.0 ± 0.8	7.8 ± 0.3	7.6 ± 0.7	7.2 ± 0.5	6.7 ± 0.3
	500	36.0 - 37.5	39.0 - 92.4	73.3 ± 7.6	70.1 ± 6.3	61.2 ± 6.1	53.3 ± 6.1	46.7 ± 3.5	42.9 ± 2.4	39.3 ± 2.4	36.1 ± 1.8	34.6 ± 2.6	31.9 ± 1.9
	2500	144.9 - 173.6	204.9 - 412.8	334.2 ± 39.9	326.5 ± 42.6	307.2 ± 22.2	251.7 ± 27.7	251.7 ± 31.1	228.3 ± 22.0	202.2 ± 16.3	190.0 ± 10.9	175.1 ± 10.5	169.7 ± 7.0
Triadimefon	100	7.9 - 8.1	9.4 - 17.8	14.8 ± 0.6	14.3 ± 1.4	12.6 ± 1.0	11.0 ± 1.1	9.6 ± 0.7	8.6 ± 0.5	7.8 ± 0.3	7.3 ± 0.3	7.1 ± 0.5	6.5 ± 0.3
	500	35.9 - 38.5	37.3 - 94.1	74.6 ± 4.7	68.5 ± 10.6	62.2 ± 7.8	53.1 ± 3.2	47.3 ± 3.0	43.3 ± 2.2	39.8 ± 3.7	36.4 ± 1.4	35.4 ± 1.8	33.1 ± 1.8
	1800	94.3 - 135.7	124.8 - 300.1	235.0 ± 0.0	261.6 ± 0.0	230.6 ± 0.0	230.6 ± 0.0	185.7 ± 34.0	169.7 ± 12.2	156.9 ± 11.3	144.4 ± 18.6	160.1 ± 27.3	139.1 ± 17.0

Gestation and lactation feed consumption was based on F_0 dam feed intake (Rockett et al., 2006). Feed consumption from PND22 onward was based on F_1 animal feed intake. Feed consumption presented as grams/rat/day ± SD. Dose levels calculated as mg/kg/day ± SD. ppm, parts per million; PND, postnatal day.

[a] $p<0.05$; [b] $p<0.01$.

Table 2. Weekly body weights.

Treatment	ppm	Lactation PND 0	PND 1	PND 8	PND 22	Week 4 PND 23-29	Week 5 PND 30-36	Week 6 PND 37-43
Control	0	5.8 ± 0.7	6.5 ± 0.9	16.7 ± 1.9	50.7 ± 8.1	84.0 ± 16.0	129.3 ± 15.4	170.1 ± 16.8
Myclobutanil	100	6.0 ± 0.7	7.1 ± 1.0	17.4 ± 3.0	48.9 ± 4.9	82.3 ± 7.6	126.2 ± 8.4	169.4 ± 15.7
	500	6.0 ± 1.1	6.3 ± 1.4	18.1 ± 3.2 [a]	52.4 ± 7.1 [a]	86.5 ± 11.5 [a]	127.7 ± 12.3	168.8 ± 17.1
	2000	6.5 ± 0.7[a]	7.2 ± 0.9	17.3 ± 2.5	48.5 ± 6.7 [b]	78.4 ± 13.4 [b]	119.2 ± 15.5 [c]	156.7 ± 16.6 [c]
Propiconazole	100	5.7 ± 0.5	6.3 ± 0.5	17.6 ± 2.2	49.8 ± 6.1	83.2 ± 8.2	130.6 ± 9.7	176.6 ± 14.5
	500	6.1 ± 0.6	7.0 ± 0.7	18.3 ± 2.2	50.1 ± 5.4	81.2 ± 13.7	127.2 ± 17.9	168.8 ± 21.3
	2500	6.1 ± 0.8	6.5 ± 1.2	16.1 ± 2.4	47.5 ± 5.6 [b]	80.1 ± 8.5	119.6 ± 12.3 [b]	160.4 ± 12.0 [c]
Triadimefon	100	5.6 ± 1.0	6.0 ± 1.3	17.1 ± 2.7	53.1 ± 6.5	89.4 ± 11.0 [a]	128.7 ± 12.6	164.6 ± 16.3
	500	6.1 ± 0.5	6.5 ± 0.9	17.5 ± 1.9	48.4 ± 3.4	82.4 ± 5.7	126.8 ± 9.0	166.2 ± 10.5
	1800	5.4 ± 0.5	5.3 ± 0.5 [b]	12.2 ± 2.3 [b]	42.1 ± 5.1 [c]	64.0 ± 8.1 [c]	99.7 ± 9.8 [c]	132.7 ± 12.5 [c]

Treatment	ppm	Week 7 PND 44-50	Week 8 PND 51-57	Week 9 PND 58-64	Week 10 PND 65-71	Week 11 PND 72-78	Week 12 PND 79-85	Week 13 PND 86-92
Control	0	212.5 ± 21.0	253.5 ± 27.7	292.5 ± 26.1	318.4 ± 28.5	340.4 ± 28.7	351.7 ± 33.3	364.5 ± 37.1
Myclobutanil	100	205.8 ± 17.9	245.1 ± 28.1	270.8 ± 44.0 [a]	300.0 ± 51.2	327.0 ± 37.7	343.7 ± 33.9	356.6 ± 33.7
	500	208.2 ± 21.4	252.8 ± 25.1	282.1 ± 36.5	306.0 ± 34.1	326.0 ± 47.9	349.0 ± 31.4	362.6 ± 29.7
	2000	191.7 ± 18.1 [c]	223.2 ± 30.8 [c]	252.4 ± 28.8 [c]	272.7 ± 35.8 [c]	289.0 ± 39.9 [c]	309.4 ± 33.0 [c]	321.0 ± 35.6 [c]
Propiconazole	100	217.9 ± 18.3	261.8 ± 23.6	296.3 ± 27.7	320.7 ± 25.1	345.7 ± 29.0	359.1 ± 34.0	374.6 ± 34.7
	500	211.0 ± 22.4	254.3 ± 26.4	289.1 ± 29.1	317.1 ± 31.2	340.3 ± 34.2	355.0 ± 34.4	369.0 ± 35.7
	2500	196.9 ± 16.1 [c]	232.1 ± 25.4 [c]	270.4 ± 18.5 [b]	295.8 ± 20.9 [b]	317.3 ± 22.4 [b]	331.5 ± 22.9 [b]	345.3 ± 24.8 [a]
Triadimefon	100	208.9 ± 24.6 [a]	251.2 ± 24.3	280.4 ± 35.5	306.5 ± 30.6	326.7 ± 32.4	342.4 ± 32.2	359.5 ± 34.1
	500	202.3 ± 12.3 [b]	240.5 ± 14.2	271.1 ± 18.0	300.2 ± 19.5	322.0 ± 22.9	336.6 ± 24.5	350.4 ± 26.3
	1800	158.0 ± 16.5 [c]	182.3 ± 30.2 [c]	208.5 ± 28.1 [c]	225.8 ± 29.4 [c]	240.1 ± 41.4 [c]	258.1 ± 34.8 [c]	273.8 ± 30.9 [c]

Body weights in grams presented as mean ± SD. ppm, parts per million; PND, postnatal day.

[a] $p<0.05$; [b] $p<0.01$; [c] $p<0.001$.

Table 3. Survival rates.

Treatment	ppm	Litter size	Total litters	Total litters with deaths[a]	Litters lost entirely[b]	Offspring total	Offspring deaths	Percent survival
Control	0	9.8 ± 1.34	27	3	1	276	12	95.65
Myclobutanil	100	10.1 ± 2.18	17	2	1	172	13	92.44
	500	9.0 ± 2.52	19	4	1	171	11	93.57
	2000	7.5 ± 3.13	30	18	1	230	40	82.61[c]
Propiconazole	100	9.3 ± 2.19	18	1	0	167	3	98.20
	500	8.6 ± 2.56	15	1	0	129	1	99.22
	2500	9.2 ± 2.06	20	4	1	184	12	93.48
Triadimefon	100	8.5 ± 2.38	21	6	0	180	7	96.11
	500	8.9 ± 2.00	15	1	0	133	1	99.25
	1800	7.8 ± 2.49	21	18	9	165	87	47.27[c]

Litter size presented as mean ± SD. ppm, parts per million; PND, postnatal day.

[a] Litters with one or more deaths within the litter.

[b] Entire litter lost, subset of total litters with deaths.

[c] $p<0.001$.

Table 4. Triazole dose, age and body weight at preputial separation.

Treatment	ppm	Dose at PPS (mg/kg/day)	PND of PPS (PND ± SEM)	Body weight on PND of PPS (g ± SD)	Body weight on PND43 (g ± SD)
Control	0	0.0 ± 0.0	42.5 ± 0.27	168.01 ± 2.14	174.37 ± 2.38
Myclobutanil	100	12.2 ± 1.0	42.5 ± 0.30	166.05 ± 2.30	170.54 ± 2.89
	500	57.7 ± 7.7	41.9 ± 0.31	163.32 ± 3.01	170.77 ± 2.98
	2000	244.1 ± 18.8	42.7 ± 0.34	154.83 ± 2.15[c]	156.34 ± 2.65[c]
Propiconazole	100	11.9 ± 2.5	42.5 ± 0.27	171.84 ± 2.50	177.61 ± 2.68
	500	61.5 ± 5.9	42.9 ± 0.39	169.35 ± 2.64	170.49 ± 2.87
	2500	311.2 ± 27.2	43.2 ± 0.30	160.21 ± 2.32[a]	159.26 ± 2.84[c]
Triadimefon	100	12.6 ± 1.8	41.9 ± 0.22	161.80 ± 2.35	166.29 ± 2.69
	500	61.3 ± 5.7	42.2 ± 0.28	161.54 ± 1.71[a]	167.12 ± 2.89
	1800	207.6 ± 32.8	47.4 ± 0.44[c]	145.77 ± 3.20[c]	131.41 ± 3.56[c]

Body weights presented as mean ± SD. ppm, parts per million; PND, postnatal day.

[a] $p<0.05$; [b] $p<0.01$; [c] $p<0.001$.

Table 5. Liver weights.

Diet	ppm	PND 1		PND 22		PND 50		PND 92	
		Absolute (g)	Relative (%)	Absolute (g)	Relative (%)	Absolute (g)	Relative (%)	Absolute (g)	Relative (%)
Control	0	0.26 ± 0.01	4.00 ± 0.09	1.89 ± 0.12	4.14 ± 0.15	10.84 ± 0.30	5.04 ± 0.07	14.59 ± 0.52	3.91 ± 0.11
Myclobutanil	100	0.29 ± 0.01	4.00 ± 0.10	2.01 ± 0.10	3.97 ± 0.10	10.43 ± 0.33	5.01 ± 0.06	13.57 ± 0.56	3.85 ± 0.05
	500	0.27 ± 0.02	4.21 ± 0.08	2.28 ± 0.15	4.19 ± 0.18	10.13 ± 0.27	5.03 ± 0.05	14.59 ± 0.85	4.09 ± 0.09
	2000	0.33 ± 0.01[b]	4.52 ± 0.10[b]	2.04 ± 0.14	4.30 ± 0.17	11.04 ± 0.32	5.62 ± 0.07[c]	14.50 ± 0.83	4.26 ± 0.09[a]
Propiconazole	100	0.26 ± 0.01	4.21 ± 0.06	2.13 ± 0.09	4.03 ± 0.08	10.96 ± 0.39	5.01 ± 0.10	14.90 ± 1.01	4.05 ± 0.16
	500	0.29 ± 0.01	4.14 ± 0.06	2.01 ± 0.13	3.90 ± 0.12	10.74 ± 0.47	5.15 ± 0.11	15.43 ± 0.81	4.17 ± 0.15
	2500	0.30 ± 0.02	4.62 ± 0.07[c]	1.92 ± 0.12	4.20 ± 0.13	12.67 ± 0.36[a]	6.34 ± 0.09[c]	16.59 ± 0.50	4.84 ± 0.06[c]
Triadimefon	100	0.25 ± 0.02	4.17 ± 0.13[b]	2.01 ± 0.08	4.10 ± 0.13	10.17 ± 0.64	5.04 ± 0.10	14.12 ± 0.70	3.89 ± 0.09
	500	0.30 ± 0.01	4.62 ± 0.09[c]	2.04 ± 0.06	4.17 ± 0.08	10.74 ± 0.26	5.33 ± 0.06[b]	13.78 ± 0.42	4.00 ± 0.13
	1800	0.25 ± 0.02	4.70 ± 0.42[c]	n/a	n/a	n/a	n/a	13.60 ± 0.70	4.94 ± 0.09[c]

Absolute weight in grams, relative weight adjusted for body weight. Weights presented as mean ± SEM. T1800 animals were not available (n/a) for PND 22 or PND 50 assessments. ppm, parts per million; PND, postnatal day.

[a] $p<0.05$; [b] $p<0.01$; [c] $p<0.001$

Table 6. Testis and accessory sex gland weights.

Treatment	ppm	PND1 Testis		PND22 Testis		PND50 Testis		PND92 Testis		PND92 Ventral Prostate		PND92 Epididymis		PND92 Seminal Vesicle	
		Absolute (mg ± SEM)	Relative (%)	Absolute (mg ± SEM)	Relative (%)	Absolute (g + SEM)	Relative (%)	Absolute (g + SEM)	Relative (%)	Absolute (g + SEM)	Relative (%)	Absolute (g + SEM)	Relative (%)	Absolute (g + SEM)	Relative (%)
Control	0	2.83 ± 0.59	0.049 ± 0.003	127.24 ± 8.59	0.28 ± 0.01	1.11 ± 0.06	0.54 ± 0.01	1.77 ± 0.04	0.48 ± 0.01	0.40 ± 0.01	0.11 ± 0.00	0.51 ± 0.01	0.14 ± 0.00	1.30 ± 0.04	0.36 ± 0.01
Myclobutanil	100	4.10 ± 0.24[b]	0.053 ± 0.002	149.93 ± 4.21	0.30 ± 0.01	1.22 ± 0.03	0.60 ± 0.02	1.61 ± 0.12	0.46 ± 0.03	0.42 ± 0.02	0.12 ± 0.01	0.49 ± 0.01	0.14 ± 0.00	1.31 ± 0.06	0.36 ± 0.02
	500	3.66 ± 0.25	0.063 ± 0.006[a]	162.26 ± 8.61[b]	0.30 ± 0.01	1.06 ± 0.18	0.61 ± 0.02	1.66 ± 0.07	0.47 ± 0.01	0.48 ± 0.02 [a]	0.13 ± 0.01[a]	0.48 ± 0.01	0.14 ± 0.00	1.33 ± 0.06	0.37 ± 0.01
	2000	4.16 ± 0.24[b]	0.059 ± 0.004	155.38 ± 10.14	0.33 ± 0.01[b]	1.19 ± 0.07	0.65 ± 0.03[a]	1.70 ± 0.08	0.51 ± 0.02	0.44 ± 0.02	0.14 ± 0.01	0.48 ± 0.02	0.15 ± 0.01	1.28 ± 0.08	0.40 ± 0.03
Propiconazole	100	3.02 ± 0.10	0.048 ± 0.001	145.16 ± 7.38	0.28 ± 0.01	1.28 ± 0.06	0.56 ± 0.02	1.70 ± 0.06	0.47 ± 0.01	0.39 ± 0.01	0.11 ± 0.00	0.49 ± 0.01	0.13 ± 0.00	1.20 ± 0.04	0.33 ± 0.01
	500	3.73 ± 0.15	0.052 ± 0.001	150.40 ± 7.70	0.29 ± 0.01	1.32 ± 0.07[a]	0.61 ± 0.02[a]	1.88 ± 0.08	0.51 ± 0.01	0.40 ± 0.02	0.11 ± 0.00	0.52 ± 0.01	0.14 ± 0.00	1.24 ± 0.07	0.34 ± 0.02
	2500	3.59 ± 0.21	0.056 ± 0.004	138.96 ± 8.04	0.30 ± 0.01[a]	1.22 ± 0.02	0.61 ± 0.01[a]	1.74 ± 0.06	0.51 ± 0.02	0.40 ± 0.01	0.12 ± 0.00	0.50 ± 0.01	0.15 ± 0.00	1.32 ± 0.07	0.39 ± 0.02
Triadimefon	100	3.11 ± 0.21	0.054 ± 0.003	139.18 ± 5.84	0.28 ± 0.02	1.30 ± 0.04[a]	0.62 ± 0.02[b]	1.65 ± 0.03	0.46 ± 0.02	0.47 ± 0.02	0.13 ± 0.01[a]	0.51 ± 0.01	0.14 ± 0.00	1.39 ± 0.07	0.38 ± 0.02
	500	3.67 ± 0.23	0.053 ± 0.003	154.34 ± 5.76[a]	0.32 ± 0.01[b]	1.27 ± 0.03[a]	0.63 ± 0.01[b]	1.77 ± 0.06	0.52 ± 0.02	0.49 ± 0.02	0.14 ± 0.00[b]	0.52 ± 0.01	0.15 ± 0.00	1.42 ± 0.05	0.41 ± 0.01[a]
	1800	2.70 ± 0.10	0.050 ± 0.003	n/a	n/a	n/a	n/a	1.68 ± 0.05	0.61 ± 0.02[b]	0.35 ± 0.02	0.13 ± 0.00	0.47 ± 0.01[a]	0.17 ± 0.01	1.05 ± 0.05[c]	0.39 ± 0.01

Absolute weight in milligrams or grams, relative weight adjusted for body weight. Data are presented as mean ± SEM. T1800 animals were not available (n/a) for PND 22 or PND 50 assessments. ppm, parts per million; PND postnatal day.

[a] $p<0.05$; [b] $p<0.01$; [c] $p<0.001$.

Table 7. Pituitary and brain weights.

		PND1	PND22			PND50			PND92		
Treatment	ppm	Brain (%)	Brain (%)	Pituitary (%) E-03	Ratio E-04	Brain (%)	Pituitary (%)E-03	Ratio E-04	Brain (%)	Pituitary (%)E-03	Ratio E-04
Control	0	4.78 + 0.72	3.18 + 0.13	5.80 + 0.40	18.36	0.86 + 0.02	3.30 + 0.16	36.82	0.53 + 0.01	2.71 + 0.08	51.53
Myclobutanil	100	4.61 + 0.19	2.90 + 0.12	5.33 + 0.58	18.92	0.88 + 0.02	3.15 + 0.18	36.11	0.54 + 0.02	2.61 + 0.10	48.11
	500	5.38 + 0.76	2.61 + 0.15	4.19 + 0.63	17.75	0.88 + 0.03	3.60 + 0.19	40.89	0.54 + 0.01	2.23 + 0.11[c]	42.86[b]
	2000	4.51 + 0.23	3.16 + 0.11	4.77 + 0.38	15.36	0.93 + 0.04	2.97 + 0.22	33.93	0.58 + 0.03	2.43 + 0.16[c]	43.05[b]
Propiconazole	100	4.91 + 0.30	2.67 + 0.08[b]	4.06 + 0.62	19.14	0.80 + 0.01[a]	3.65 + 0.30	45.63	0.52 + 0.01	2.61 + 0.14	49.64
	500	4.49 + 0.25	2.92 + 0.08	4.68 + 0.55	16.47	0.81 + 0.02[a]	3.31 + 0.28	40.73	0.51 + 0.02[a]	2.76 + 0.14	54.43
	2500	4.57 + 0.35	3.23 + 0.12	5.31 + 0.82	16.91	0.88 + 0.01	3.46 + 0.22	39.20	0.56 + 0.01	2.68 + 0.07	48.00
Triadimefon	100	4.91 + 0.28	3.07 + 0.10	5.07 + 0.61	16.76	0.89 + 0.04	3.26 + 0.37	36.27	0.54 + 0.02	2.48 + 0.13	46.52
	500	5.02 + 0.36	2.96 + 0.05	4.85 + 0.48	16.37	0.88 + 0.01	3.00 + 0.32	34.25	0.55 + 0.01	2.32 + 0.17	42.60
	1800	4.38 + 0.16	n/a	n/a	n/a	n/a	n/a	n/a	0.63 + 0.03[b]	2.69 + 0.22	43.82

Brain and pituitary weights presented as percentage of body weight, data presented as mean $\pm$ SEM. Pituitary to brain weight ratio is based on absolute tissue weights. Pituitary weights were unavailable at PND1; T1800 animals were not available (n/a) for PND 22 or PND 50 assessments. ppm, parts per million; PND, postnatal day.

[a] $p<0.05$; [b] $p<0.01$; [c] $p<0.001$.

Table 8. Liver histopathology.

Treatment	ppm	PND50 Incidence (mean grade ± SD)	PND92 Incidence (mean grade ± SD)
Control	0	0/5	0/5
Myclobutanil	100	0/5	0/5
	500	1/5 (1.0 ± 0.0)	0/5
	2000	1/5 (1.0 ± 0.0)	2/5 (0.4 ± 0.6)
Propiconazole	100	0/5	0/5
	500	0/5	0/5
	2500	4/5 (1.4 ± 1.5)	2/5 (0.8 ± 1.1)
Triadimefon	100	1/5 (1.0 ± 0.0)	0/5
	500	4/5 (0.8 ± 0.5)	0/4
	1800	n/a	4/4 (3.0 ± 1.2)

Lesions grades based on severity of hepatocyte hypertrophy (see Methods). Data presented as ratio of samples with lesions. Parenthetical values indicate the average lesion grade ± SD of the subset of animals with lesions. ppm, parts per million; PND, postnatal day.

Table 9. Breeding performance of male rats exposed to myclobutanil, propiconazole, or triadimefon.

Treatment	ppm	Sperm positive females	Insemination Index	Pregnant females[d]	Fertility Index	Percent Fertility[e]	Postimplantation loss (%)[f]	Sperm Morphology % normal ± SEM	Sperm Motility	
									VAP[g]	STR[h]
Control	0	19/21	90.5	19/19	100.0	84.97	6.61	88.0 ± 0.7	136.3	79
Myclobutanil	100	11/11	100.0	11/11	100.0	81.24	11.76	88.1 ± 0.8		
	500	7/11	63.6	5/7 (6/11)[b]	54.5[b]	85.86	8.41	87.8 ± 1.1		
	2000	5/16[c]	31.3[c]	3/5 (4/16)[c]	25.0[c]	82.06	9.42	87.3 ± 1.4	134.0	79
Propiconazole	100	15/16	93.8	14/15	93.3	90.03	6.92	87.3 ± 0.8		
	500	13/13	100.0	13/13	100.0	86.19	12.41	88.7 ± 0.6		
	2500	12/15	80.0	11/12	91.7	84.62	9.29	88.8 ± 1.1	132.7	78
Triadimefon	100	14/16	87.5	13/14 (14/16)	87.5	76.23	13.77	88.4 ± 1.2		
	500	8/12	66.7	8/8 (9/12)	75.0	83.97	10.91	87.3 ± 1.4		
	1800	1/13[c]	7.7[c]	0/0[c]	0.0[c]	-	-	86.8 ± 1.1	138.4	75

Insemination index: percent of inseminated females to number of females mated. Fertility index: percent of pregnant females to number of females mated. Parenthetical values: include litters born to false negative sperm checks. ppm, parts per million.
[a] $p<0.05$; [b] $p<0.01$; [c] $p<0.001$.
[d] number of sperm positive matings with litters.
[e] number of live fetuses/ number of corpora lutea.
[f] number of implants/ live fetuses.
[g] Smooth Path Velocity (μm/ sec)
[h] Straightness (%), ratio of Straight Line Velocity/ Smoothed Path Velocity

Table 10. Modes of action and potential mechanisms of triazole-induced reproductive toxicity.

Modes of Action	Potential Mechanisms	Observed Effects	Triazole	LOEL (mg/kg/day)	NOEL (mg/kg/day)
Altered steroid homeostasis	Increased testicular steroidogenesis (e.g., Cyp17). Decreased liver steroid metabolism.	Elevated serum testosterone Increased testis weight Increased AGD Hepatomegaly Delayed puberty	Myclobutanil [a] Propiconazole [a] Triadimefon [a]	133.9 31.9 33.1	32.9 6.7 6.5
Reduced insemination and fertility indices	Demasculinization of SNB.[d] Reduced copulatory behavior.	Failure to ejaculate Reduced pregnancy rate	Myclobutanil [b] Propiconazole Triadimefon [b]	32.9 - 139.1	6.1 - 33.1
Altered neuroendocrine function	Elevated steroid hormones during development; reduction in GnRH cell function.	Decreased pituitary weight	Myclobutanil [c] Propiconazole Triadimefon	32.9 - -	6.1 - -

[a] Lowest observed effect level (LOEL) and no observed effect level (NOEL) based on serum testosterone at PND92.

[b] LOEL and NOEL based on fertility index at PND92

[c] LOEL and NOEL based on pituitary weight at PND92

[d] Spinal nucleus of the bulbocavernosus