TOXICOLOGICAL SCIENCES **62,** 339–352 (2001)

The Effects of Perinatal Tebuconazole Exposure on Adult Neurological, Immunological, and Reproductive Function in Rats

V. C. Moser,*[,1] S. Barone, Jr.,* R. J. Smialowicz,† M. W. Harris,‡ B. J. Davis,‡ D. Overstreet,‡ M. Mauney,§ and R. E. Chapin‡[,2]

**Neurotoxicology Division and †Environmental Toxicology Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711; ‡Reproductive Toxicology Group, National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina; and §ASI, Inc., Durham, North Carolina*

Received February 15, 2001; accepted April 24, 2001

Studies are under way to address concerns of potential persistent immunotoxic, reproductive, and neurotoxic effects of perinatal exposure to several pesticides. Tebuconazole, a triazole fungicide, was evaluated as part of this project. Sprague-Dawley dams were administered tebuconazole (0, 6, 20, or 60 mg/kg) by oral gavage daily from gestational day 14 to postnatal day (PND)7; the pups were then dosed daily at the same levels from PND7–42. Separate groups of rats were used for testing of immunological parameters, neurobehavioral testing using a screening battery of functional tests, and cognitive evaluations. Other groups of rats were evaluated for reproductive development and function, while yet others were sacrificed at the end of the dosing period for histological analyses of major organs systems, including neuropathological assessments. Pup viability and body weight were decreased in the highest dose group. There were no differences in the fertility indices in the exposed rats mated as adults. In the sheep RBC-immunized high-dose rats, spleen weights and cellularity were increased, and the ratio of cell types was altered compared to controls. There were, however, no biologically significant changes in the immune function of these rats. At necropsy on PND46 or 152, kidney, liver, and spleen weights were altered by tebuconazole treatment, but a dose-response relationship was not clear for most organs; only decreased kidney and increased liver weights were consistent in both sexes. Histological analyses were generally unremarkable outside of the brain. One month after the end of dosing, acquisition of learning the platform location in a water tank (i.e., Morris water maze) was impaired in the high-dose group; there were no differences in neuromuscular ability, motor activity, or swim speed to account for this finding. Furthermore, there was no effect on recall of the position during a free-swim trial. Neuropathological evaluations revealed pyknotic cells across hippocampal cell fields in animals of all tebuconazole treatment groups, with the highest incidence in the 20 and 60 mg/kg/day dose groups, coincident with cell loss within pyramidal cell layer of CA3-4 cell fields of the hippocampus and layer V of the neocortex. Thus, perinatal exposure to tebuconazole produced neurobehavioral deficits and neuropathology in rats, but did not alter immunological or reproductive function.

Key Words: **tebuconazole; developmental neurotoxicity; immunotoxicity; reproductive toxicity; rats.**

The nervous, immune, and reproductive systems were identified as potential targets of pesticide exposure in the 1993 National Research Council report entitled *Pesticides in the Diets of Infants and Children* (NRC, 1993). These three systems of interest undergo significant development well after birth, making these organ systems potential targets of toxicity due to pesticide exposure through food consumption. Perturbations in the development of these systems could result in long-term functional deficits. To address this scientific and regulatory concern, the National Institute of Environmental Health Sciences (NIEHS) and the U.S. Environmental Protection Agency (U.S. EPA) entered into a collaborative research project to focus on the long-term effects of developmental pesticide exposure on these three organ systems (Chapin *et al.*, 1996).

The design of this study was to mimic, in the rat, the exposure period of concern in humans, which ranges from late gestation to approximately age 18 years (NRC, 1993). To ensure exposure to pesticides during development, pregnant rats were dosed during gestation and for the first week postpartum. The rat pups were then directly dosed from 1 week of age until the approximate end of puberty, 42 days of age. This study design was conceived to ensure pesticide exposure during the critical windows of development for all three systems of interest (immune, nervous, and reproductive). The effects of methoxychlor, an endocrine-disrupting pesticide, on these systems have been reported previously (Chapin *et al.*, 1997a).

In the rat, different areas of the brain undergo synaptogen-

Portions of this research were presented at the 1999 annual Society of Toxicology meeting, *The Toxicologist* 48:16, 1999.

The information in this document has been funded in part by the U.S. Environmental Protection Agency. It has been reviewed by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

[1] To whom correspondence should be addressed at NTD (MD-74B), U.S. EPA., RTP, NC 27711. Fax (919) 541-4849. E-mail: moser.ginger@epa.gov.

[2] Present address: Dupont Pharmaceuticals, Haskell Laboratory, Newark, DE.

esis, proliferation, and differentiation at specific times, beginning during gestation and continuing about 3 weeks after birth. Myelination in both the central and peripheral nervous systems continues for another 3–4 weeks after weaning (Benjamins and McKhann, 1981; Woodbury, 1974). Establishment of the immune system requires a series of carefully timed and coordinated developmental events that begin early in embryonic/fetal life and continue through the early postnatal period. In rodents, the epithelial thymic rudiment forms during midgestation, followed by colonization of the thymus by precursor T cells from the fetal liver (Owens and Raff, 1970). B-cell lymphopoiesis also begins in the rodent liver during gestation (Hayakawa *et al*., 1994). Antibody responses to T-cell–dependent and –independent antigens occur soon after birth (Tyan, 1981), whereas natural killer (NK) cell activity does not appear until about 3 weeks of age (Santoni *et al*., 1982). The reproductive systems begin cellular population expansion in the middle of the second week of gestation. Spermatogenesis commences shortly after birth, whereas oocytes in the newborn are arrested in the first meiotic prophase. Development of the excurrent ducts continues after birth (Byskov and Høyer, 1994).

In order to characterize the impact of pesticide exposure, broad batteries of tests were used that evaluate many different aspects of the nervous, immune, and reproductive systems. Neurological and behavioral alterations were evaluated using a functional observational battery, which is a series of observational and manipulative tests designed to assess nervous system integrity, in conjunction with an automated measure of motor activity. Cognitive function was measured using passive avoidance training, which measures associative learning, and training in a Morris water maze, a task that requires learning and memory of a spatial location. This series of tests has been shown to be sensitive to a variety of toxicants, including pesticides (see, for example, Bammer, 1982; Brandeis *et al.*, 1989; Moser, 1989; Moser *et al.*, 1995) These tests began shortly after dosing ended, and testing continued for months in order to detect potential long-term or persistent effects of the perinatal exposures. The rats were also pharmacologically challenged to detect alterations in the biological response to triadimefon (another triazole fungicide). At 8–9 weeks of age (i.e., 2–3 weeks after cessation of dosing), offspring were evaluated for a variety of innate and specific immune function end points, including the evaluation of splenic lymphocyte subpopulations using flow cytometry. The end points employed have been identified as sensitive and predictive for the identification of immunotoxicants (Luster *et al.*, 1992). Developmental end points included parameters of maternal health, and numbers and viability of the offspring. Effects on the reproductive system were evaluated by monitoring development in males and females (anogenital distance at birth, day of vaginal opening, day of preputial separation), two mating trials with untreated mates, two weeks of monitoring vaginal cytology to assess cyclicity, and a necropsy including measures of sperm motility and count, as well as testicular and epididymal sperm counts, and organ weights and histology. In addition to specific focus on these systems, the study design included necropsy at the end of dosing and again in the adults to assess organ weight and histology. Levels of the test chemical in plasma and liver of dams and pups, as well as in milk, were measured at specific times to verify the actual exposure conditions.

Selected pesticides are being evaluated using this study design (see Chapin *et al.*, 1996). One of these test chemicals was tebuconazole, a triazole fungicide. Tebuconazole (Elite®, Folicur®, Raxil®, Lynx®) is a systemic fungicide used on crops such as barley, wheat, peanuts, and orchard fruits. Its mechanism of fungicidal activity is inhibition of α-lanosterol demethylase, which decreases ergosterol biosynthesis (Kwok and Loeffler, 1993; Lamb *et al.*, 1998). The reported LD_{50} in rats is quite high ($\sim$4 g/kg), but systemic toxicity, teratogenicity, and developmental toxicity have been reported at doses as low as 50 mg/kg/day (review of data submissions, U.S. EPA, 1999). However, there are almost no published studies on the biological effects of tebuconazole. In a study that compared acute effects of tebuconazole with other triazole fungicides on motor activity in adult rats, tebuconazole was ineffective (Crofton, 1996). Although another triazole, triadimefon, has been shown to cause neurotoxic and developmental effects (Moser *et al.*, 1995; Narotsky and Kavlock, 1995), no information was available concerning tebuconazole's effects on the developing nervous and immune systems. This study was therefore undertaken to determine whether perinatal exposure to tebuconazole would alter function of the nervous, reproductive, or immune systems in adult rats.

MATERIALS AND METHODS

Chemical. Tebuconazole was obtained from Bayer AG, Agricultural Division (Stillwell, KS), with a labeled purity of 97.4%. The chemical was verified by infrared and nuclear magnetic resonance (NMR) spectroscopies. A 1-h NMR spectrum was obtained from 0–15 ppm for a solution of the test article in deuterated chloroform. High performance liquid chromatography (HPLC) was used to detect impurities greater than or equal to 0.05%; none were detected. A C18 column was used with an acetonitrile:water eluent and ultraviolet detection at 222 nm. Neat compound was homogenized and sonicated into suspension in 0.7% methylcellulose. Dosing solutions were prepared every 30 days and analyzed for tebuconazole using a Waters 510 HPLC with UV detection; all dosing solutions were within 10% of nominal value. Stability studies indicated that the formulations were stable for 3 h at simulated dosing conditions and for at least 35 days at ambient storage conditions. Suspensions were tested and shown to be homogeneous.

Animals. Pregnant Sprague-Dawley rats (Tac:N(SD)fBR) were received from Taconic Farms (Germantown, NY) on gestational day (GD)4–5. All procedures were approved by the NIEHS and U.S. EPA Institutional Animal Care and Use Committees. Due to the size of this study, the experiment was run in two cohorts (neuro- and immunotoxicity studies were conducted with one cohort, reproductive toxicity the other; PND46 necropsy was conducted with rats from both cohorts and the data indicated no cohort differences). Pregnant F_0 dams subject to prenatal dosing at the beginning of the study were acclimated for $\sim$7 days before dosing; naive adults for mating with adult tebuconazole-treated rats were allowed an acclimation period of 7–10 days prior to mating. Dams ($n \geq 15$/dose/cohort) were assigned to treatment groups

by stratified randomization to assure equivalent body weight means across groups prior to dosing.

All rats during dosing as well as rats destined for the reproductive studies, were singly housed in polycarbonate cages in the animal facility with a 12:12-h light:dark cycle and maintained at 20 ± 1°C and 50 ± 10% humidity. They were allowed *ad lib* access to NIH-07 certified feed (Zeigler Bros, Inc., Gardners, PA) and deionized water. After dosing ended, two sets of rats were transferred to another facility with similar environmental conditions, the only difference being that they were switched to Purina Rat Chow #5001 (Ralston Purina Co., St. Louis, MO). These rats, used for subsequent immunological and neurological testing, were allowed at least 1 week to acclimate after moving.

Experimental design. The general design of this collaborative study is described in Chapin *et al.* (1997a). Pregnant F_0 dams were orally dosed at 2 ml/kg with either 0, 6, 20, or 60 mg/kg/day from GD14 to postnatal day (PND) 7. The F_1 pups were then directly dosed using the same dose levels until PND42. Litters were standardized to four males and four females on PND7 and weaned on PND21. The day after birth (PND1), each pup was marked with a paw tattoo, and an individual identification number was made by tail tattoo at weaning on PND21. Subsets of rats (one male and one female from each litter) were used for the reproductive, immunological, and neurological evaluations. Additional pups and litters were used for lactational assessment and the pubertal necropsy. All procedures (necropsy, milk expression, maternal observations, etc.) in rats from different treatment groups occurred in a random order.

Maternal and pup assessment. F_0 dams were weighed daily from the day dosing began, and any obvious toxicity was noted by personnel unaware of each rat's treatment level. Number of newborns, newborn weight, individual anogenital distance, and any external abnormalities were recorded for each litter. Pups were weighed daily during dosing (PND7–42).

Tissue levels of tebuconazole. On PND7, a subset of dams and litters was used to determine the amount of tebuconazole in milk and in plasma and liver of dams and pups; milk quality was also assessed. Five to seven hours after the daily tebuconazole dose, dams (n = 7–8/dose) were anesthetized in a random order with ketamine/xylazine, followed by 1 IU oxytocin dissolved in water and injected intraperitoneally. Milk was manually expressed. The quantity, proportion of lipids, and total protein, triglycerides, and lactose content were analyzed (Dostal *et al.*, 1990).

Dams' milk and plasma, as well as plasma from the pups, were analyzed for tebuconazole content by extracting tissues with acetonitrile and analyzing the extracts on a Waters 510 HPLC with UV detector, using hexanophenone as an internal standard. It is important to note that pups were removed from their dams early on the morning prior to milking, prior to that day's dosing, and the pups were held separately until killed by decapitation 1–2 h later. Thus, although dams were dosed on the day of milking, the most recent pup exposure through the dam was 25–26 h previously.

Immunotoxicological assessment. Two subsets of F_1 rats (n = 6/dose/sex) were evaluated at 8 weeks of age for changes in the function of the immune system. The first subset of rats was used to evaluate splenic lymphoproliferative (LP) responses to T- and B-cell mitogens [i.e., concanavalin A (ConA), phytohemagglutinin (PHA), and *Salmonella typhimurium* mitogen (STM)] using *in vitro* ^{3}H-thymidine incorporation. This subset of rats was also used to measure splenic natural killer (NK) cell activity using an *in vitro* ^{51}Cr-release assay (Smialowicz *et al*., 1991). The second subset was used to measure the primary antibody plaque-forming cell (PFC) response to sheep red blood cells (SRBC). In addition, splenic lymphocytes from these same rats were evaluated using dual label flow cytometry for subpopulations of CD4 helper (phycoerythrin [PE] conjugated W3/25, PharMingen, San Diego, CA) and CD8 cytotoxic/suppressor (fluorescein isothiocyanate [FITC] conjugated 341, PharMingen) T lymphocytes, CD5 pan T lymphocytes (PE conjugated OX-19, PharMingen), and B lymphocytes and plasma cells (FITC conjugated OX-12, Serotec, Kidlington, Oxford, UK; Smialowicz *et al.*, 1994). Body, spleen, and thymus weights were measured in both sets of rats.

Data were analyzed by one-way analysis of variance (ANOVA), with *post hoc* analysis using Dunnett's multiple comparison *t*-test (RS/1, 1988). Differences between control and treatment groups were reported as being statistically significant when $p < 0.05$.

Neurotoxicological assessment. A subset of rats (n = 10/dose/sex; n = 8/sex at the high dose) was evaluated for neurological and behavioral alterations using a functional observational battery (FOB), an automated measure of motor activity, passive avoidance, and a Morris water maze. These tests were conducted after maturation and took place at scheduled times (described below) over the next several months in order to detect potential long-term or persistent effects. The rats were also pharmacologically challenged with triadimefon (another triazole fungicide) to detect alterations in the biological response (hyperactivity) to that chemical.

Testing was conducted using the FOB and motor activity on PND49/50 and again on PND70/71. Testing took place over 2 days (half of the rats tested each day), with the treatment and sex counterbalanced across squads of rats. The FOB is a series of observational and manipulative tests designed to assess the neurological integrity of the test subject (Moser *et al.*, 1988). Motor activity is an apical measure of neurobehavioral function. Procedural details and scoring criteria for the FOB protocol are provided in McDaniel and Moser (1993). Following brief home-cage assessments for any unusual postures or activities, hand-held evaluations were made while the rat was gently held around the chest. While holding the rat, the observer ranked its reactivity and any changes in general appearance, including lacrimation, salivation, ptosis, and piloerection.

Open-field observations took place while the rat explored the top of a laboratory cart (60 × 30 cm with a 6.5-cm perimeter barrier) for 3 min. During this time, the observer ranked the rat's arousal and activity level and recorded the number of rears as well as any tremors, convulsions, and abnormal postures. Next, sensorimotor responses were ranked in response to a variety of stimuli (approach of a pen, touch of the pen to the posterior flank, click stimulus using a metal clicker, pinch on the tail using forceps, and constriction of the pupil to a penlight stimulus). Aerial righting was also ranked. Finally, forelimb and hindlimb grip strength, landing foot splay, rectal temperature, and body weight were quantified using appropriate devices. The same observer conducted all tests and was blind with respect to the dose levels.

Motor activity data were collected shortly after FOB testing, using an automated chamber shaped like a figure-eight (Reiter, 1983). Activity was measured as interruptions (counts) of any of the eight photocell beams distributed around the maze. Counts were recorded in 12 five-minute intervals, for a total session length of 1 h.

Rats were tested using a passive avoidance learning and memory task on PND56–69. In this simple one-trial learning, rats were placed in a shuttle box with an electrified grid floor connected to a precision-regulated shocker (Coulbourn Instruments, Allentown, PA). One side of the chamber was lined on the outside with black poster board; the other side was illuminated with a high-intensity lamp. On the training trial, the rat was placed in the light side and after 30 s the door between the chambers was opened. When the rat crossed into the dark side, the door was shut and a 3-s, 0.4-mA shock was delivered. For the retention trials, the rat was again placed in the light side, but there was no shock if they passed into the dark side. For all trials, the latency to cross was recorded. Retention tests were limited to 5 min. Training took place on PND56 and 57, with a 24-h retention test on PND57 and 58 (half of the rats on each day). In the same rats, a 2-week retention test was conducted on PND68 and 69. Treatment and sex were counterbalanced across squads of rats and across days.

On PND62, a challenge dose of triadimefon was administered to all rats. Triadimefon is another triazole fungicide that has been shown to produce hyperactivity in rats (Crofton, 1996; Moser *et al*., 1989). Triadimefon (100 mg/kg) was administered intraperitoneally, and 1 h later, rats were placed in the motor activity chambers for a 1-h determination of activity.

A Morris water maze was used to evaluate spatial and working memory, beginning on PND74. The water maze was a round galvanized steel tank 140 cm in diameter, filled to a depth of 44 cm with water. A round plexiglass platform (9 cm in diameter) was placed 2 cm beneath the surface of the water, which was made opaque using a black Tempra® paint. Water temperature was maintained at 25–27°C, and the water was changed daily. The rats were

videotaped, and the image was digitized for computer analysis using water maze tracking software (HVS Image, Ormond Crescent, Hampton, UK). For the spatial training, the platform was placed in either of two positions. While the platform position was the same for each rat, it was counterbalanced across treatment and sex. Training took place during two trials each day. The rat was placed into the water at one of four starting points, which were varied such that the rat never started at the same place within 2 days. Each trial continued until the rat mounted the platform, or for a maximum of 60 s. If the rat did not find the platform within the 60 s, it was guided there by the observer. After 15 s on the platform, the rat was replaced in the cage, and after 5 min the second trial began. Spatial training took place over 2 weeks: on Friday of the second week, a probe trial was conducted in which the platform was removed and the rat's tendency to search in the correct quadrant was measured over 60 s.

A variation of this water maze task was continued for the next 2 weeks, in which the platform was moved to a new location each day. Starting positions again varied across the four possible positions. Working memory was assessed by the degree of reduction in search time on the second trial compared to the first trial each day.

Data analysis for the behavioral test measures depended on the type of data generated. In general, two-way ANOVAs were conducted with a grouping factor of dose and time as a repeated (within-subject) factor. Data from males and females were considered as separate experiments. When significant overall effects were obtained, step-down analyses were then conducted. Where the overall dose-by-time interaction was significant, analyses at each time point were then conducted to determine which time points were significant, and at those times, which dose groups differed from control, using Dunnett's *t*-test (for continuous data) or *t*-test contrasts (for rank-order data). Continuous data were analyzed by a general linear model (GLM; SAS, 1990). Rank-order data were analyzed using a categorical modeling procedure (CATMOD; SAS, 1990) that fits linear models to functions of response frequencies, which is then analyzed by weighted regression. In all cases, resulting probability values < 0.05 were considered significant.

PND46 necropsy. One subset of F_1 rats (n = 11–12/sex/dose) was killed, in a random order, by CO_2 asphyxiation on PND45-46 to identify any histopathological effects present at the end of the dosing period. Terminal body weights were recorded, and the following organs were weighed and examined histologically: liver, thymus, spleen, kidneys, adrenals, ovaries, uterus/vagina, testes, epididymides, seminal vesicles/coagulating glands, ventral prostate, and dorsolateral prostate. Both absolute organ weight and relative weight (ratio organ weight:body weight) were considered dependent variables. Statistical analyses are described below.

Reproductive assessment and necropsy. In the reproductive toxicity set of F_1 rats (n = 15 litters/dose/sex), prepuce separation (PS) was evaluated beginning PND35, and vaginal opening (VO) was evaluated beginning PND25. As adults, these animals were mated with an untreated mate and allowed to rear the first litter to PND10 to check for normal maturation of the pups. End points examined in these litters were number of pups, weight, sex, and external malformations in the young. The F_2 young were removed on PND10, and the treated adult animals were re-paired with an untreated mate. The resulting pregnant females were killed on GD19, and the fetuses were evaluated for malformations of the visceral organs and skeletons. Treated F_1 adult females were subject to necropsy, with weights and histology collected on liver, kidneys, spleen, adrenals, and reproductive organs. The adult treated F_1 males were killed shortly thereafter, and the organs listed above were removed and weighed and evaluated microscopically. Blood was collected and analyzed for standard measures of hematology and clinical chemistry. In addition, a testis and epididymis were processed for sperm count and motility (from the epididymis, as in Chapin *et al.*, 1997b). Testes and epididymides were immersion-fixed in Bouin's, embedded in paraffin, sectioned, and stained with PAS-H.

For reproductive assessments, organ weights, and organ-to-body weight ratios, linear regression was used to assess dose-response trends (Neter *et al.*, 1985); otherwise, analysis of variance methods were used to determine significant difference between dose groups. These were followed by *post hoc* comparisons with the control group using Dunnett's *t*-test. Data for preweaning end points, as well as PS and VO, were averaged over litter and analyzed using the litter as the unit of measure. Due to the maturing of these offspring and decreasing sample size of the original litters, this analysis was not used for measures made after PND46. A detailed description of the analyses is found in Chapin *et al*. (1997a). Probability values < 0.05 were considered statistically significant, and all data are presented as mean $\pm$ SEM.

Terminal assessment of brain neuropathology. These same F_1 male and female rats from the reproductive group (n = 8–15/dose/sex) were assessed for persistent morphological alterations in the structure of the brain. Rapid immersion fixation of the brain was employed in this necropsy, as perfusion fixation was not compatible with some of the reproductive end points being examined in the same animals. Brains were rapidly and carefully removed, in a random order, from adults (PND152–154) and immediately immersed in chilled 10% buffered formalin (pH = 7.4) for 2 weeks at 4°C prior to processing and embedding in paraffin to avoid histological artifacts due to drying. Brains were cut sagittally at 6 μm, mounted on slides, and Nissl-substance stained with cresyl violet. The regions of the brain examined included olfactory bulbs, striatum, thalamus, neocortex, hippocampus, brainstem, and cerebellum. Six sections per animal, which were representative of the above brain regions, were examined. Animals (n = 134) from all dose groups were uniquely coded, and slides of sections from all animals were read blind for a qualitative assessment of morphological alterations. Qualitative assessments included incidence data and description of regional findings for all sections of each animal. This assessment was performed on all sections at low magnification ($\times$40) and high magnification ($\times$100 and $\times$200 nominally) on a Vanox AH2 microscope (Olympus). Incidence data were statistically analyzed using the nonparametric analysis of Kruskal-Wallis (SAS, 1990).

Following qualitative examinations, a quantitative assessment was performed that included simple morphometry of three cortical regions of the brain: neocortex, hippocampus, and cerebellum. These regions were the focus of morphometric analyses for two reasons. First, the *a priori* rationale was that these are representative regions that undergo rapid development during prenatal, perinatal, and postnatal periods that were coincident with exposure to this pesticide. The second reason for looking at these regions was based on the results of the qualitative assessment in which the neocortex and hippocampus appeared to have morphological changes in a large number of animals. All morphometric analyses were performed blind to the treatment code with a computer-based image analysis system (Stereoinvestigator®, Microbrightfield, Colchester, VT) interfaced via a high-resolution digital color camera (3CCD, model D330, Dage-MTI, Michigan City, IN) attached to a Vanox AH-2 Microscope. All measures were performed on corresponding homologous sagittal sections from animals from all dose groups. The measures included mean layer I width of neocortex, total cortical width (layer I–VI of somatosensory cortex), mean width of the corpus callosum (midway between splenium and genu), mean width of molecular layer of dentate gyrus, mean width of CA1 *stratum radiatum*, mean layer of the white matter of the alveus of the dorsal hippocampus, and mean molecular layer width of lobule IV of the cerebellum.

RESULTS

Table 1 presents a summary of tebuconazole effects on most end points addressed in this study. Details are presented below.

Maternal and Pup Assessment

F_0 maternal body weight gain during gestation was depressed in the high-dose group, but otherwise the dams looked healthy. On GD21, the high-dose group weighed 341.5 $\pm$ 5.0 g (having gained 74.0 $\pm$ 2.8 g; mean $\pm$ SEM), compared to control values of 354.8 $\pm$ 6.3 g (gain of 87.8 $\pm$ 3.3 g).

Table 2 presents pre- and postweaning data for offspring in this study. Control dams had an average of 11.2 $\pm$ 0.6 live

TABLE 1
Significant Effects and Effective Doses of Perinatal Tebuconazole

	Male	Female	M/F
Maternal indices			
Depressed weight gain		60	
Milk analyses		—	
Preweaning assessments (males and females combined)			
Increased number dead/litter			60
Lowered birth weight, weight gain			60
Eye opening, anogenital distance			—
Postweaning assessments			
Increased liver weight (PND46 necropsy only)	60	60	
Decreased kidney weight (both necropsies)	60	6[a]	
Increased spleen Weight (both necropsies)	60	—	
Decreased epididymal weight (postmating necropsy)	60		
Decreased uterine weight (postmating necropsy)		6[a]	
Body weight, PS	—	—	
Accelerated VO		20[a]	
Immune organ weights			
Increased spleen (immunized rats)	60	60	
Thymus, spleen (nonimmunized)	—	—	
Immune responses			
Increased splenic leukocyte number	60	60	
Altered percentage phenotypes	60	—	
Lymphoproliferation, PFC response, NK activity	—	—	
Cognition			
Passive avoidance	—	—	
Altered habituation in MA chambers	—	60	
Delayed spatial acquisition in water maze	60	60	
Probe, swim speed, working memory	—	—	
FOB/motor activity			
Increased handling reactivity	60	—	
Increased approach response	—	20	
All other functional end points	—	—	
Triadimefon challenge	—	—	
Neuropathology			
Increased hippocampal pyknotic cells	6	6	
Reproductive function			
First mating	—	—	
Vaginal cyclicity		—	
Second mating, decreased litter size		6[a]	

Note. M/F, males and females combined. Numbers indicate the lowest doses that were significantly different from controls, and '—' indicates no significant effect.

[a]No dose response; high dose not significant.

pups/litter; this was not statistically altered by tebuconazole exposure, although there was a trend ($p = 0.07$) toward fewer pups in the high-dose group (9.7 ± 0.8 pups/litter; see Table 2). The number of dead pups per litter on PND0 was significantly increased at 60 mg/kg/day (Table 2). No treatment-related mortality occurred after PND0. Pup weight at birth was reduced only at 60 mg/kg/day (an average of 7.6 g in controls, 6.6 g at 60 mg/kg/day); this reduction was maintained out to weaning. There was no difference in the effect on body weight between males and females; Figure 1 presents weight gain for both sexes from PND1–21.

Developmental landmarks were mostly unchanged by tebuconazole exposure; these data are listed in Table 2. Eye opening appeared accelerated in the high-dose group, but the difference was not statistically significant. Anogenital distance for males and females on PND1 was unchanged by tebuconazole exposure. PS was likewise unchanged. Vaginal opening (VO) was significantly accelerated in the middle-dose group only.

Tissue Levels

Tebuconazole was detected in all tissues tested from the high-dose group, with the mean concentration (± SD) as follows: maternal liver (6.4 ± 3.1 μg/g; $n = 8$) > maternal plasma (1.0 ± 0.5 μg/ml; $n = 8$) ≥ milk (0.6 ± 0.6 μg/ml; $n = 4$, undetectable in three, and insufficient volume in the fourth). The mid-dose group had detectable levels only in liver (2.1 ± 0.5 μg/g). Tebuconazole was not detected in plasma from any F_1 pups at PND7, before the start of direct dosing (LOD = 0.16 μg/ml). Pup livers had an interfering peak that could not be resolved with changes in run time or buffer; however, this peak showed no dose-related increase. Thus, there was measurable tebuconazole in maternal liver (top two doses) and plasma and milk (top dose only).

There were no dose-related differences in milk lactose level (control value: 28.2 ± 1.8 mg/ml), protein (control: 9.6 ± 0.4 g/dl), or total lipids (control: 12.4 ± 0.5 % v/v).

PND46 Necropsy

At the PND46 necropsy (see Table 3), female terminal body weight was unchanged (control: 155.7 ± 4.0 g, shown in Fig. 1), and relative (but not absolute) liver weight was increased by 12% in the high-dose females; tissue histology was unchanged. There was also a significant decrease (9%) in female relative kidney weight at only the middle-dose level. The PND46 male necropsy data showed few differences; body weight was unchanged from a control value of 192.7 ± 4.1 g (Figure 1). The high-dose group had a 26% increase in relative spleen weight, a 10% increase in relative liver weight, and an 8% decrease in relative kidney weight. In addition, the middle-dose group had a lower absolute kidney weight.

Immunological Assessment

Immunological changes produced by tebuconazole are summarized in Table 1. Splenic NK cell activity, mitogen-stimulated LP responses, and the PFC response to SRBC were not affected by tebuconazole. The only immunological changes observed were an increase in spleen weight and splenic leukocyte cellularity in the SRBC-immunized high-dose group. The

TABLE 2
Developmental Indices Following Tebuconazole Treatment

	Tebuconazole dose (mg/kg/day)			
	0	6	20	60
Neonate				
No. of litters	35	30	34	37
PND0				
No. of live/litter	11.2 ± 0.6	10.7 ± 0.6	10.9 ± 0.5	9.7 ± 0.8
No. of dead/litter	0.4 ± 0.2	0.2 ± 0.1	0.6 ± 0.3	2.2 ± 0.6*
Eye opening (day)				
Right	14.0 ± 0.1	14.1 ± 0.1	13.8 ± 0.1	13.7 ± 0.1
Left	14.0 ± 0.1	14.1 ± 0.1	13.8 ± 0.1	13.6 ± 0.2
PND1 anogenital distance (mm)				
Male	3.7 ± 0.1	3.8 ± 0.1	3.7 ± 0.1	3.7 ± 0.1
Female	1.3 ± 0.02	1.3 ± 0.03	1.3 ± 0.02	1.3 ± 0.03
Postweaning				
No. of litters	25	25	25	17
No. of female rats	88	91	80	70
Vaginal opening (day)	35.9 ± 0.3	35.7 ± 0.5	34.0 ± 0.3*	34.7 ± 0.4
No. of litters	18	21	23	15
No. of male rats	36	42	46	41
PS (day)	41.2 ± 0.25	40.9 ± 0.30	41.2 ± 0.24	41.2 ± 0.26

Note. All data are presented as mean ± SEM.
*Indicates statistically significant compared to control.

number of splenic leukocytes was significantly increased in males (i.e., 29.5 ± 0.7 × 10^6 cells for control vs 36.8 ± 1.95 × 10^6 cells for the high dose), and in females (i.e., 23.8 ± 1.2 × 10^6 cells for control vs 33.6 ± 2.8 × 10^6 cells for the high dose). The relative spleen weight was significantly increased 28 and 24% in the high-dose males and females, respectively.

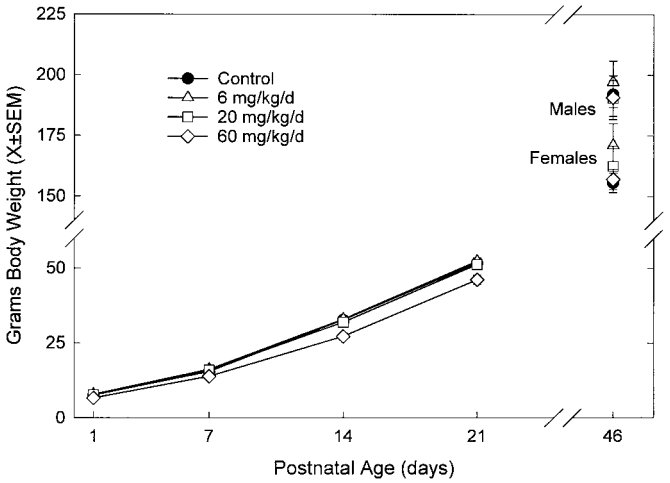

FIG. 1. Body weight of rats treated with tebuconazole from GD14 to PND42. On PND1–21, data are combined for males and females, as there was no difference between the sexes at that time (X ± SEM; error bars, where not evident, are within the size of the symbol). At PND46, data for each sex are presented separately. The high-dose group had significant effects on weight preweaning, but at PND46 there were no treatment-related group differences.

In the high-dose male rats, there was a significant increase in the percentage of $CD4^+CD8^-$ (T cells) and a decrease in the $CD4^-CD8^-$ (presumably primarily B cells, but also some macrophages); these data are presented in Table 4. There was no change, however, in the $CD4^-CD8^+$ T cells (data not shown). In addition, $OX12^+OX19^-$ (B cells) were also decreased and $OX12^-OX19^+$ (T cells) were increased, which corroborates the pattern observed for the CD4/CD8 markers. No such effects were detected in the high-dose females. These alterations in splenic lymphocyte subpopulations did not translate into any overall immune function deficits, in that antibody production was unchanged.

Neurotoxicological Evaluations

A summary of the neurotoxicological effects of tebuconazole exposure is presented in Table 1. Few alterations were detected using the FOB. There were no overall effects on the end points evaluating neuromuscular or autonomic function, and no involuntary movements were observed. High-dose males showed an increased reactivity to being handled at both test times (mean score: controls = 1.5; high-dose group = 1.9); this was not observed in females. No other measures of general reactivity or activity were altered. Females showed an overall increased response to the approach stimulus (mean score: controls = 1.8, mid-dose group = 2.2); however, this occurred only in the middle-dose group (20 mg/kg) and was not observed in males. In addition, no other changes in sensorimotor responsiveness were seen.

TABLE 3
Organ Weights (Absolute and/or Relative to Body Weight) from PND46 and Adult Necropsy in Tebuconazole-Treated Rats

	Tebuconazole dose (mg/kg/day)			
	0	6	20	60
PND46 necropsy				
Males				
No. of litters	27	25	25	23
No. of rats	11	12	12	11
Body weight	192.7 ± 4.1	197 ± 55.7	180.9 ± 6.1	189.7 ± 6.1
Relative liver weight	4.87 ± 0.1	4.84 ± 0.1	5.07 ± 0.1	5.38 ± 0.1*
Relative spleen weight	0.35 ± 0.01	0.36 ± 0.01	0.38 ± 0.01	0.44 ± 0.01*
Kidney weight				
Absolute	1.68 ± 0.04	1.65 ± 0.04	1.50 ± 0.05*	1.53 ± 0.04
Relative	0.88 ± 0.02	0.84 ± 0.01	0.83 ± 0.01	0.81 ± 0.02*
Relative epididymis weight	0.14 ± 0.004	0.13 ± 0.004	0.15 ± 0.008	0.13 ± 0.007
Females				
No. of litters	25	25	25	17
No. of rats	12	7	12	12
Body weight	155.7 ± 4.0	171.2 ± 8.8	162.5 ± 8.2	157.1 ± 4.2
Relative liver weight	4.75 ± 0.1	4.92 ± 0.2	4.79 ± 0.1	5.32 ± 0.1*
Relative spleen weight	0.36 ± 0.01	0.36 ± 0.02	0.37 ± 0.02	0.40 ± 0.01
Relative kidney weight	0.86 ± 0.02	0.90 ± 0.02	0.78 ± 0.02*	0.83 ± 0.02
Relative uterus weight	0.19 ± 0.03	0.18 ± 0.03	0.15 ± 0.01	0.19 ± 0.02
Adult necropsy (postmating)				
Males				
No. of rats	15	15	16	16
Body weight	548.5 ± 21.8	553.6 ± 14.0	536.8 ± 12.2	496.4 ± 8.9*
Relative liver weight	3.84 ± 0.08	3.79 ± 0.08	3.69 ± 0.10	3.82 ± 0.08
Relative spleen weight	0.18 ± 0.01	0.19 ± 0.01	0.19 ± 0.005	0.21 ± 0.004*
Absolute kidney weight	3.50 ± 0.13	3.32 ± 0.09	3.19 ± 0.16	2.95 ± 0.07*
Left epididymis absolute weight	0.263 ± 0.007	0.250 ± 0.008	0.246 ± 0.009	0.218 ± 0.016*
Pregnant females				
No. of rats	12	11	10	13
Corrected body weight[a]	401.1 ± 14.0	393.2 ± 9.1	430.1 ± 11.1	426.0 ± 9.0*
Relative liver weight	4.53 ± 0.10	4.40 ± 0.11	4.20 ± 0.13	4.60 ± 0.10
Relative spleen weight	0.27 ± 0.01	0.25 ± 0.01	0.22 ± 0.01*	0.26 ± 0.01
Relative kidney weight	0.58 ± 0.01	0.53 ± 0.01*	0.51 ± 0.02*	0.55 ± 0.01
Absolute empty uterus	5.42 ± 0.24	4.41 ± 0.26*	4.38 ± 0.37*	4.01 ± 0.14

Note. All data presented as mean ± SEM; weights are in grams.
[a]Terminal body weight minus uterine contents.
*Indicates statistically significant compared to control.

Total activity levels in the figure-eight chambers were not altered in tebuconazole-treated rats, but the high-dose group did show a difference in the change of activity during the session, i.e., habituation. This habituation of activity, a form of within-session learning, was altered (higher activity at the end of the session) on both test days in high-dose female rats; males showed a nonsignificant trend toward this effect.

At no time was there a difference in the dose groups with respect to the passive avoidance task. On the training trial, almost all rats crossed into the dark side in less than a minute (median latencies ranging from 7 to 22 s for both males and females). At the 24-h retention time, median latencies showed a wide range (males, 63–300 s; females, 136–292 s), but there were no significant group differences. This was also the case for the 12-day test (median latencies: males, 106–300 s; females, 70–194 s). Unfortunately, high variability in the data for this task may have precluded the ability to detect a small change.

A challenge dose of triadimefon produced the expected hyperactivity (about 150%) in all dose groups when compared to the activity data collected on PND49/50 and PND70/71. There were, however, no treatment-related differences in activity levels.

Spatial training in the Morris water maze was significantly altered by tebuconazole, and both males and females in the high-dose group learned the position of the platform at a slower rate compared to controls. Acquisition in females was delayed during the first week only, whereas males were slower than controls in the second week of training as well. Figure 2 shows that all treatment groups had similar latencies on the first day

TABLE 4
The Percentage of Splenic Leukocytes Following Immunization with SRBC in Rats Treated with Tebuconazole (60 mg/kg/day Group Only)

Dose	$CD4^+CD8^-$	$CD4^-CD8^-$	$OX12^+OX19^-$	$OX12^-OX19^+$
Males				
Control	25.3 ± 0.3	57.3 ± 1.6	44.0 ± 1.6	38.7 ± 1.1
60 mg/kg/day	29.8 ± 1.7*	49.7 ± 1.3*	38.1 ± 1.1*	45.9 ± 0.8*
Females				
Control	31.0 ± 1.9	49.4 ± 2.6	36.4 ± 1.8	47.9 ± 1.5
60 mg/kg/day	33.0 ± 1.3	46.0 ± 1.9	34.3 ± 0.8	50.9 ± 1.4

Note. High-dose males showed significantly greater T cells and lower B-cell indicators.
*Indicates statistically significant compared to control; n = six/dose group.

of training (control males, 50.6 ± 5.2 s; females, 54.4 ± 5.0), and the control groups (both sexes) learned the task such that average latencies of 10–15 s were achieved by the end of the first week. In contrast, this level of performance was not attained in the high-dose groups, even at the end of 2 weeks (average latencies: males, 17.7 s; females, 21.4 s). Data for the path length measure mirrored the findings with latency; path length for males and females controls was 161 ± 40 cm and 181 ± 40 cm on the last trial, whereas data for the high-dose groups were 252 ± 67 cm and 389 ± 63 cm. There were no differences in swim speed (male and female controls, 18.5 and 20.1 cm/s; high-dose, 17.9 and 20.6 cm/s). Spatial analysis of the paths across three concentric zones indicated that the high-dose group spent more time circling in the outer zone of the tank (the platform was located in the middle zone). Although all rats spent most of the swim time in the outer zone early in training, as training progressed, the control rats ventured into the middle and inner zone significantly more than did the high-dose group.

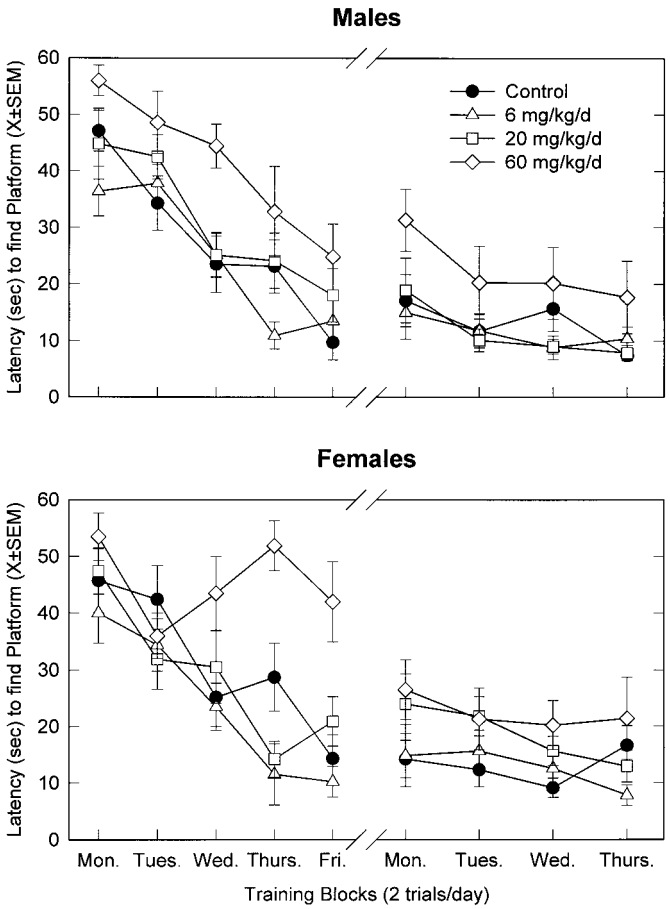

FIG. 2. Spatial acquisition in a Morris water maze. Training took place in two daily trials (60-s maximum), with the start position varying between four points in the tank. Average latencies for each daily block (X ± SEM) are shown for males (top panel) and females (bottom panel). The high-dose males had significantly greater latencies throughout the 2 weeks of training, whereas the high-dose females showed significant effects only in the first week.

Memory was apparently not affected; there were no significant group differences in the probe trial (recall of the platform position); these data are shown in Figure 3. All rats spent more time in the correct quadrant than would be expected by chance, and much less time in the opposite quadrant. There was, however, a trend toward less time in the correct quadrant for the high-dose males, as shown in Figure 3. Another measure of performance in the probe trial, the number of crossings of the platform position, also showed no treatment-related differences.

Working-memory training indicated that rats could learn the new platform position each day from the first to the second trial. The performance of this task appeared to stabilize after the first day, but no treatment-related differences between the groups emerged. Over the week of training, rats had higher latencies on the first trial (control males, 25.7 s; females, 26.6) compared to the second trial (control males, 13.1 s; females, 19.0 s; high-dose males, 17.9 s; females, 21.2 s).

Neuropathology Assessment in Adults

The qualitative regional analysis of the brain indicated that the morphology was relatively normal across all regions (olfactory bulbs, striatum, thalamus, brainstem, and cerebellum),

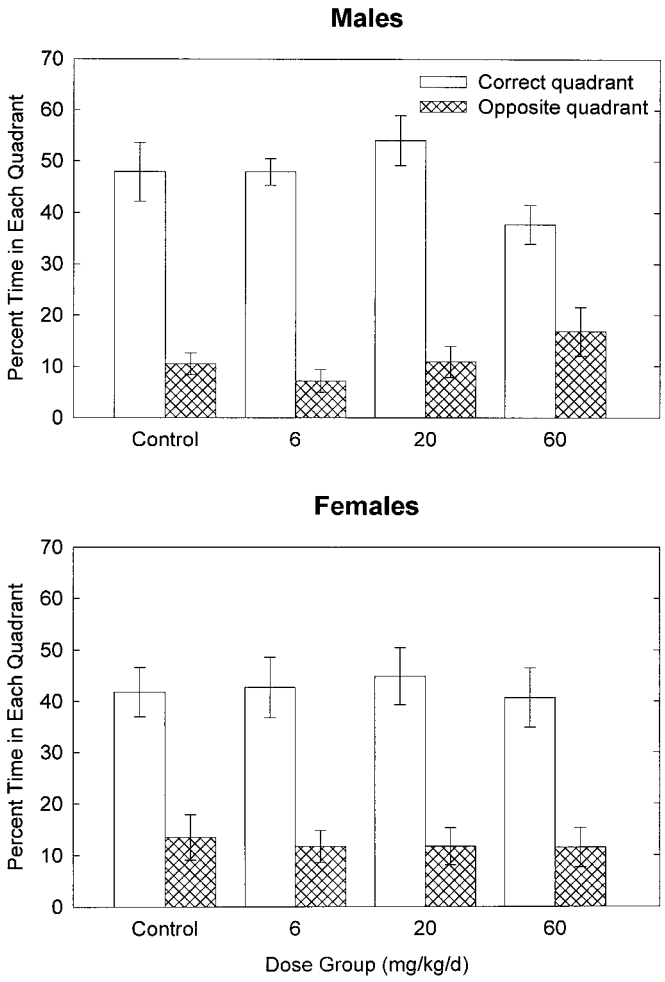

FIG. 3. Spatial memory in a Morris water maze. A probe trial was conducted on the Friday of the second week of training. The platform was removed and the rats allowed to swim freely for 60 s. The percent of time spent in each imaginary quadrant was recorded; times in the correct and opposite quadrant (X ± SEM) are shown here for males (top panel) and females (bottom panel). There was no significant overall effect of tebuconazole on the spatial bias for the correct quadrant.

with the exception of the neocortex and hippocampus. In control tissues there was a higher than normal background incidence of pyknotic cells in the hippocampal cells fields, particularly in the dentate gyrus (9 out of 34 brains; 26%). This was probably due to the lack of perfusion fixation and is consistent with previous findings of fixation artifacts produced by immersion fixation of brains (Cammermeyer, 1960; Fix and Garman, 2000; Garman, 1990; Krinke and Landes, 1995). The apparent fixation artifacts were characterized by nuclear condensation and perineuronal spaces around granule cells in the dentate gyrus and some cortical pyramidal cells, and some corkscrew-like dendrites of neocortical pyramidal cells in controls. However, there was an increase in the incidence of pathological indications across all doses of the tebuconazole treatment groups above this background incidence, and the appearance of pyknotic cells were most often not accompanied by perineuronal spaces or corkscrew dendrites. In addition, the pyknotic and darkly stained cells in treated animals were not uniform in their hyperchromatosis. The incidence of animals with pathological findings in the low-, medium-, and high-dose groups were 17 of 29 (59%), 26 of 34 (76%), and 25 of 37 (68%), respectively; this incidence of pathological findings was significantly different from control in all tebuconazole treatment groups.

Although the number of animals with indications of neuropathology across the mid- and high-dose groups appeared similar, the severity of pyramidal cell loss and pyknotic cells across subfields of the hippocampal formation did follow a dose-related trend. This trend included more pyknotic cells across hippocampal cell fields with increasing dose. Representative micrographs of the hippocampal formation are shown in Figure 4. The highest incidence of pyknotic cells was observed in dentate gyrus and progressed from CA4 to CA1 pyramidal cell fields in the two higher-dose groups. In general, there were more pyknotic cells in the ventral hippocampus than the dorsal hippocampus. However, the involvement of subfields of the hippocampus and extension of pyknotic cells into the neocortex increased in incidence with increasing doses of tebuconazole. Although these pyknotic cells are an indication of sick and possibly dying cells, there was also an indication of overt cell loss within pyramidal cells of the CA3-4 cell field of the hippocampus and layer V of the neocortex that was not seen in controls. This apparent cell loss in CA3-4 of the hippocampus was observed in the lowest dose group but at a low incidence (3 of 29 brains; 10%) and did not involve overt findings in the neocortex. The amount of apparent cell loss was more extensive in the intermediate-dose group and extended into the neocortex (6 of 34; 18%). In the highest dose group, both the number of animals with overt cell loss in cortical structures and extent of cell loss increased in the hippocampus and neocortex (10 of 37; 27%). There were no robust gender-related differences across dose groups in any of the pathological findings.

There were no significant effects of tebuconazole exposure on any of the simple morphometric measures in neocortex, hippocampus of cerebellum (Table 5). This indicates that there were no gross differences in shrinkage of the structures examined.

Reproductive Assessment and Necropsy

Reproductive function was marginally changed by tebuconazole. In the first mating of treated females, there were no tebuconazole-related differences in estrous cycle length, the number of females becoming pregnant or delivering a litter, or in litter size (control: 11.2 ± 1.2 pups). For the second litter, mean litter size was 15.2, 11.7, 11.9, and 14.1 for the control to high-dose groups, respectively; only the low- and middle-dose groups were significantly lowered. The middle-dose group also had fewer resorptions and implants per dam, al-

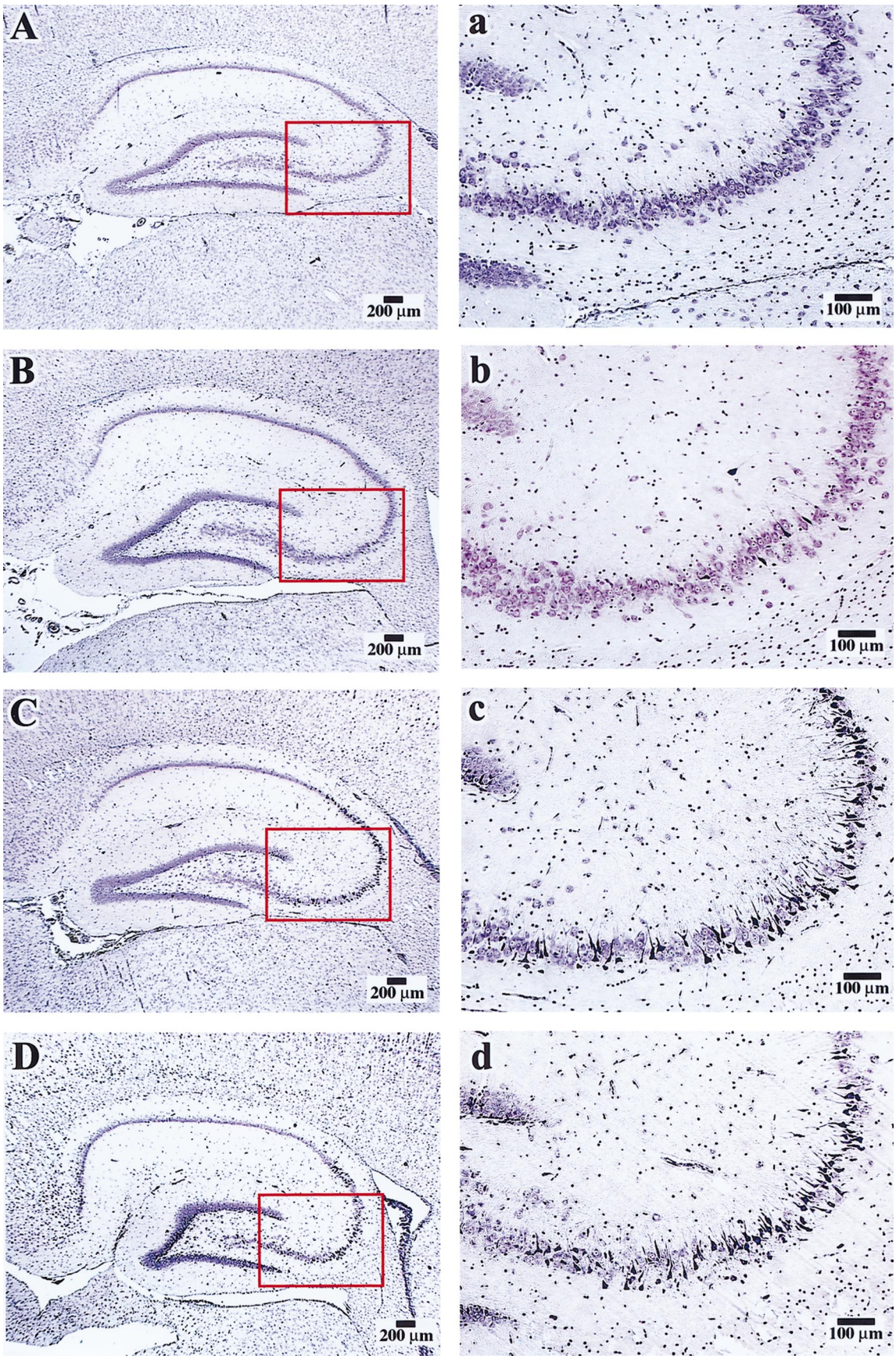

TABLE 5
Regional Brain Morphometric Measures in Microns (X ± SEM)

		Tebuconazole dose (mg/kg/day)			
		0	6	20	60
Neocortex					
Layer I	Female	158.90 ± 5.06 (12)	176.38 ± 8.15 (8)	162.97 ± 9.50 (10)	159.89 ± 5.22 (13)
	Male	162.24 ± 7.04 (14)	166.88 ± 5.93 (15)	168.08 ± 3.88 (15)	159.89 ± 5.22 (12)
Layer I–VI	Female	1939.22 ± 19.58 (12)	2056.89 ± 78.72 (8)	2034.33 ± 61.68 (10)	1961.23 ± 39.05 (13)
	Male	2017.62 ± 35.28 (14)	2121.42 ± 48.45 (15)	2034.50 ± 32.64 (15)	2052.85 ± 52.65 (12)
Corpus callosum	Female	362.89 ± 37.16 (12)	421.47 ± 29.18 (8)	400.75 ± 30.82 (10)	380.47 ± 34.54 (13)
	Male	467.87 ± 27.53 (14)	425.65 ± 24.31 (15)	445.26 ± 38.18 (15)	380.33 ± 23.61 (12)
Hippocampus					
Molecular layer	Female	240.37 ± 9.33 (12)	247.62 ± 10.05 (8)	271.88 ± 14.08 (10)	251.08 ± 7.34 (12)
	Male	257.10 ± 5.73 (13)	262.97 ± 7.13 (14)	263.39 ± 5.48 (15)	247.98 ± 8.57 (13)
CA1 *stratum radiatum*	Female	388.87 ± 13.99 (12)	364.62 ± 19.12 (8)	369.14 ± 12.38 (10)	359.56 ± 11.40 (12)
	Male	364.34 ± 14.38 (13)	381.84 ± 10.82 (14)	380.41 ± 8.97 (15)	355.58 ± 10.29 (13)
Alveus	Female	117.15 ± 8.11 (12)	108.87 ± 10.31 (8)	132.90 ± 7.10 (10)	113.29 ± 5.35 (12)
	Male	130.31 ± 5.09 (13)	134.96 ± 5.67 (14)	136.72 ± 5.03 (15)	134.93 ± 3.90 (13)
Cerebellum					
Lobule IV	Female	170.82 ± 14.79 (10)	171.15 ± 10.89 (8)	167.48 ± 8.07 (10)	158.16 ± 6.59 (12)
	Male	156.30 ± 4.07 (13)	167.39 ± 4.41 (14)	168.97 ± 7.45 (14)	156.50 ± 7.46 (13)

Note. Number of animals in each group in parentheses.

though arithmetically the same number of corpora lutea (16.1/dam). Necropsy data for these groups are presented in Table 3. The pregnant females had decreased relative kidney weight (9% in the low-dose and 12% in the middle-dose groups), decreased relative spleen weight (20%, middle-dose group only), and decreased empty uterus weight, both absolute and relative (20–25%, low-dose and middle-dose groups).

With treated males, there were no tebuconazole-related differences in the number of live pups per litter in either the first or second mating. For the first mating, the mean litter sizes were 12.8, 13.0, 12.9, and 12.3 live pups for the control to high-dose groups, respectively. For the second mating, the litter sizes were 13.0, 13.3, 12.4, and 11.4 live fetuses/dam. At necropsy, high-dose males weighed significantly less than the control (Table 3). The only significant organ weight changes were seen at the high dose, which showed a 17% increase in relative spleen weight, a 16% decrease in absolute kidneys weight, and a 17% decrease in left cauda epididymal weight. Sperm counts in cauda epididymis or left testis were statistically unchanged either on a weight or whole-organ basis. There were no changes in the standard hematology or clinical chemical values.

Histology of liver, kidney, spleen, adrenals, and reproductive organs of males and females was unremarkable, and there were no treatment-related microscopic alterations in tissue structure.

DISCUSSION

Essentially all of the organ systems had some tebuconazole-related effects in the high-dose group (60 mg/kg/day). In studies submitted by the manufacturer to the U.S. EPA (summarized in U.S. EPA, 1999), the reported LOELs (lowest observable effect levels) ranged from 50 to 60 mg/kg/day. Thus, the present data agree well with the effective dose range from earlier studies. Reported effects in those studies included changes in organ weights, developmental effects, and teratological anomalies. Many of the alterations observed in this study were also reported in those studies. Specifically, a two-generation reproductive study indicated decreased pup weight at birth, which persisted for 3–4 weeks (LOEL: 50 mg/kg/day); we also report an effect on pup weight and litter size at 60 mg/kg/day. Decreased liver and kidney weight were also reported in two studies (LOELs: 50 or 60 mg/kg/day); we also found a decrease in kidney weight (at 60 mg/kg/day in males and females at the PND46 necropsy, but only at 6 and 20

FIG. 4. Neuropathological examination of the brain revealed dose-related increase in the incidence of pyknotic cells in the hippocampal formation Cresyl violet–stained sagittal sections. The vehicle group is illustrated in panel A and a (top row). Representative micrographs from the low-, medium-, and high-dose group are displayed in panels B, C, and D, respectively. Higher magnification of the CA3 area of the hippocampus is depicted in panels a, b, c, and d (right column) of the corresponding dose groups from the above micrographs. Note the loss of pyramidal cell bodies in the CA3 region of the hippocampus (panel d). Scale bars are noted in each micrograph.

mg/kg/day in females at the later necropsy), but liver weight was increased in the present study (LOEL: 60 mg/kg/day, both sexes). We also report increased spleen weight (LOEL: 60 mg/kg/day in males only). In the previous unpublished studies (U.S. EPA, 1999), splenic hemosiderosis was reported at similar dose levels; however, this was not noted in the present study. There have been no previous neurotoxicity or immunotoxicity studies with tebuconazole; however, a developmental study in mice reported frank malformations of skull, brain, and spinal cord at 100 mg/kg/day (U.S. EPA, 1999).

In this study, the high dose produced maternal toxicity (depressed weight gain) and perinatal toxicity (increased pup deaths, lower birth weight). Body weights of pups were lower throughout lactation, but these weights attained control values by PND46. Note that the weights recovered even in the face of continued dosing (until PND42). No overt toxicity was observed around PND7, when the pups started being dosed directly with the test compound. Maternal toxicity has been shown to influence the outcome of subsequent evaluations in the offspring (reviewed in Rogers, 1987; Schardein, 1987), although the magnitude of overt toxicity in this study was relatively small (depressed weight gain, no other signs of toxicity). Nonspecific or generalized changes were not evident on any of the end points. Given this lack of overt systemic toxicity, the relative specificity of the findings reported here is interesting. Furthermore, the neuropathological effects were observed in the lower-dose groups where maternal effects were not observed. According to current guidance on neurotoxicity risk assessment, it is inappropriate to assume that developmental effects at doses that caused minimal maternal toxicity resulted only from that maternal toxicity, and thus these effects of tebuconazole may be considered as adverse neurotoxic effects until shown otherwise (U.S. EPA, 1998).

Tebuconazole produced neurobehavioral alterations, mostly affecting measures of learning (water maze acquisition in both sexes, activity habituation in females). Most other neurological and behavioral end points were not changed, indicating that deficits in motor, performance, or other functions could not account for these findings. Overall motor activity levels were not altered at any time point, and swim speed in the water maze was also not affected by tebuconazole. Increased handling reactivity was observed in the high-dose males, as well as increased approach response in females at the middle dose only. Even though these changes were significant across all test times, there were no other correlative changes to support the biological significance of these findings. Tebuconazole did not affect either the passive avoidance test, which measures a form of associative learning, or the working memory paradigm of the water maze test, which requires short-term memory. This indicates that tebuconazole may produce specific effects on spatial learning. The Morris water maze has been used extensively in neurobiological research, and has been used to evaluate the effects of neurological lesions and environmental manipulations (reviewed in Brandeis *et al*., 1989; Poucet and Benhamou, 1997). Relatively few studies have used this test in the context of developmental neurotoxicity, although the effects of a few persistent environmental chemicals have been evaluated (e.g., Kuhlmann *et al*., 1997). The data from the present study suggest that the water maze may be a sensitive tool to identify deficits in spatial memory produced by pesticides.

There was no influence of tebuconazole exposure in response to an acute challenge with triadimefon, a structurally similar triazole. For logistical reasons, the drug challenge was conducted between the two FOB evaluations and before water maze testing. Prior studies with triadimefon (*e.g*., Allen and MacPhail, 1991; Moser and MacPhail, 1989) indicate no persistent effects and no influence on subsequent dose-response determinations with other chemicals. In addition, the effects on FOB end points and motor activity were consistent across the pre- and postchallenge evaluations, and for all measures the control values were similar in these studies to our historical control database. Thus, while the possibility cannot be dismissed, it is unlikely that the acute triadimefon challenge influenced subsequent testing in these rats.

Neuropathological findings in the hippocampal formation and posterior cortex of the high-dose group may be related to the functional deficits observed in the water maze spatial learning task, as previous studies have demonstrated that hippocampal damage results in spatial learning deficits in the water maze (see for example, Brandeis *et al.*, 1989; Poucet and Benhamou, 1997). Neuropathological effects were observed in both of the lower-dose groups but with less severity. These findings illustrate that perinatal dosing with tebuconazole may have persistent effects on the structure of the nervous system but do not provide information on critical windows of exposure that may have resulted in these pathological findings.

Some caveats should be discussed in light of the immersion fixation and the histological artifacts that can result from this type of tissue preparation. It is well documented that hyperchromatosis and perineuronal spaces resulting from retraction can occur in neurons following immersion fixation (Cammermeyer, 1960; Garman, 1990). This does not, however, obviate the findings of the current study in which both the incidence and severity of neuropathological signs (e.g., pyknotic cells and cell loss) were observed above background levels and were dose related. The degree of hyperchromatosis varied between and within regions in tebuconazole-exposed animals, suggesting ongoing death and dying rather than punctate damage associated with histological artifacts of tissue handling. In addition, the appearance of pyknotic cells was not generally accompanied by perineuronal retraction or corkscrewlike formations in the dendrites of pyramidal cells. It should also be noted that the morphometric data were negative with regard to treatment, and thus these data indicate that there was no gross shrinkage of structures between dose groups due to fixation or handling of the brains. There are two questions to be considered in interpreting these data: can the histological artifact be

falsely interpreted as being the result of treatment, and does the background artifact mask treatment-related damage. We believe the answer to both of these considerations is no, based on several lines of evidence. This study included assessment of sections from a large number of animals, with all slides being read in a coded fashion. This reduces the likelihood of bias for spurious findings in a few animals. Although perfusion fixation is well documented as preferable for tissue preservation of cytoarchitecture of the nervous system to detect effects, as it reduces background histological artifacts (Cammermeyer, 1960; Cammermeyer, 1968; Fix and Garman, 2000; Garman, 1990; Krinke and Landes, 1995), it does not reduce the concern raised in a hazard identification study when the effects are observed above background and are dose related. Future studies, which should involve perfusion fixation, could resolve the time course of dosing that produces the neuropathological findings, determine a possible NOEL, and also determine if exposure during a finite critical windows is essential for producing these effects. These neuropathological findings and treatment-related alterations in spatial learning also suggest that synaptic transmission should be examined in future studies, because of the occurrence of greater damage in the ventral hippocampus than dorsal hippocampus, the progressive increase of pyknotic cells from dentate gyrus through CA cells fields, and the overt cell loss associated with increasing dose of tebuconazole. The lack of an NOEL and the combined structural and functional alterations observed in this study warrant future replication and extension of the present study.

In the immunological evaluations, tebuconazole produced interesting changes in the percentage of T and B cells in the high-dose groups. The decrease in the percentage of B cells in the high-dose males might be indicative of a depressed antibody response to SRBC; however, as this did not occur, the physiological relevance of this decrease is questionable. The strength of phenotypic analysis of cells using flow cytometry is predicated on linkage to an alteration in an immune function, but to extrapolate immune dysfunction from an observation of a change in cell population(s) is speculative. Thus, the immunological significance of these changes is not known.

Assessments of the F_1 adults indicated that although tebuconazole altered some organ weights, especially liver and kidney, there were no postweaning body weight differences or evidence of compromised health. Increases in liver or kidney weight in the absence of visible microscopic pathology are not uncommon and may reflect either adaptive metabolic responses or real and substantive ultrastructural changes in cellular composition. In the absence of ultrastructural studies, these outcomes are indistinguishable (Haschek and Rousseaux, 1998).

Tebuconazole-induced changes in reproductive function were seen only in the low- and middle-dose groups, and the high dose was not different from controls, making it difficult to ascribe any biological significance to these findings. Although changes in epididymal weight have been associated with reduced fertility in mice (Chapin *et al.*, 1997b), the lack of any detectable change in sperm number (which comprise a significant proportion of epididymal weight) and the lack of change in fertility at the high dose do not signal meaningful reproductive toxicity for tebuconazole. Indeed, the reproductive data *in toto* suggest that perinatal exposure to tebuconazole, even at a maternally toxic dose, is without lasting effect on the reproductive system.

In summary, perinatal exposure to tebuconazole produced adverse effects, altered learning in a spatial cognitive task, and hippocampal and neocortical neuropathology. In contrast, there were no overall effects on the immunological or reproductive systems of the rats. The nature of the effects suggests that further assessments should be performed to determine the specificity and magnitude of cognitive alterations and neurotoxicity produced by tebuconazole.

ACKNOWLEDGMENTS

The authors would like to acknowledge the excellent technical assistance of Eric Haskins and Willis Purdie (NIEHS) for animal handling and necropsy skills, Kathy McDaniel and Pam Phillips (U.S. EPA) for behavioral testing, and Wanda C. Williams and Carey B. Copeland (U.S. EPA) for immunological evaluations. We also thank Dr. Cynthia Smith (NIEHS) for help with the chemistry issues and Jean Orlein of ASI for statistical support. Tebuconazole verification and tissue levels were analyzed by Midwest Research Institute (Kansas City, MO) under contract to NIEHS (N01-ES-55385).

REFERENCES

Allen, A. R., and MacPhail, R. C. (1991). Effects of triadimefon on a multiple schedule of fixed-interval performance: Comparison with methylphenidate, *d*-amphetamine, and chlorpromazine. *Pharmacol. Biochem. Behav.* **40,** 775–780.

Bammer, G. (1982). Pharmacological investigations of neurotransmitter involvement in passive avoidance responding: A review and some new results. *Neurosci. Biobehav. Rev.* **6,** 247–296.

Benjamins, J. A., and McKhann, G. M. (1981). Development, regeneration, and aging of the brain. In *Basic Neurochemistry* (G. J. Siegel, R. W. Albers, B. W. Agranoff, and R. Katzman, Eds.), 3rd ed., pp. 445–469. Little, Brown and Co., Boston.

Brandeis, R., Brandys, Y., and Yehuda, S. (1989). The use of the Morris water maze in the study of memory and learning. *Int. J. Neurosci.* **48,** 29–69.

Byskov, A. G., and Høyer, P. E. (1994). Embryology of mammalian gonads and ducts. In *The Physiology of Reproduction* (E. Knobil and J. D. Neill, Eds.), 2nd ed., pp. 487–531. Raven Press, New York.

Cammermeyer, J. (1960). The post-mortem origin and mechanism of neuronal hyperchromatosis and nuclear pyknosis. *Exp. Neurol.* **2,** 379–405.

Cammermeyer, J. (1968). Nonspecific changes of the central nervous system in normal and experimental material. In *The Structure and Function of the Nervous Tissue* (G. H. Bourne, Ed.), Vol. 6, pp. 131–251. Academic Press, New York.

Chapin, R. E., Harris, M. W., Shelby, M. D, Smialowicz, R. J., Moser, V. C., Padilla, S., MacPhail, R. C., Barone, S., Lockhart, A. C., and Mauney, M. A. (1996). The effects of perinatal/juvenile pesticide exposure on adult CNS, immune, and reproductive function in rats. *Toxicologist* **30,** 52.

Chapin, R .E., Harris, M. W., Davis, B. J., Ward, S. M., Wilson, R. E., Mauney, M. A., Lockhart, A. C., Smialowicz, R. J., Moser, V. C., Burka, L. T., and Collins, B. J. (1997a). The effects of perinatal/juvenile methoxy-

chlor exposure on adult rat nervous, immune, and reproductive system function. *Fundam. Appl. Toxicol.* **40,** 138–157.

Chapin, R. E., Sloane, R. A., and Haseman, J. K. (1997b). The relationships among reproductive endpoints in Swiss mice, using the reproductive assessment by continuous breeding database. *Fundam. Appl. Toxicol.* **38,** 129–142.

Crofton, K. M. (1996). A structure-activity relationship for the neurotoxicity of triazole fungicides. *Toxicol. Lett.* **84,** 155–159.

Dostal, L. A., Weaver, R. P., and Schwetz, B. A. (1990). Excretion of high concentrations of cimetidine and ranitidine into rat milk and their effects on milk composition and mammary gland nucleic acid content. *Toxicol. Appl. Pharmacol.* **102,** 430–442.

Fix, A. S., and Garman, R. H. (2000). Practical aspects of neuropathology: A technical guide for working with the nervous system. *Toxicol. Pathol.* **28,** 122–131.

Garman, R. H. (1990). Artifacts in routine immersion fixed nervous tissue. *Toxicol. Pathol.* **18,** 149–153.

Haschek, W. M., and Rousseaux, C. G. (1998). Hepatobiliary system. In *Fundamentals of Toxicologic Pathology*, pp. 127–152, Academic Press, San Francisco.

Hayakawa, K., Tarlinton, D., and Hardy, R. R. (1994). Absence of MHC class II expression distinguishes fetal from adult B lymphopoiesis in mice. *J. Immunol.* **152,** 4801–4807.

Krinke, G. J., and Landes, C. (1995). The dorsal fascia dentata as a probe of fixation quality in the rodent brain. *Cesk. Patol.* **31,** 28–30.

Kuhlmann, A. C., McGlothan, J. L., and Guilarte, T. R. (1997). Developmental lead exposure causes spatial learning deficits in adult rats. *Neurosci. Lett.* **233,** 101–104.

Kwok, I. M.-Y., and Leoffler, R. T. (1993). The biochemical mode of action of some newer azole fungicides. *Pestic. Sci.* **39,** 1–11.

Lamb, D. C., Kelly, D. E., Manning, N. J., Hollomon, D. W., and Kelly, S. L. (1998). Expression, purification, reconstitution and inhibition of Ustilago maydis sterol 14 alpha-demethylase (CYP51; P450(14DM)). *FEMS Microbiol. Lett.* **169,** 369–373.

Luster, M. I., Portier, C., Pait, D. G., White, K. L., Jr., Gennings, C., Munson, A. E., and Rosenthal, G. J. (1992). Risk assessment in immunotoxicology I. Sensitivity and predictability of immune tests. *Fundam. Appl. Toxicol.* **18,** 200–210.

McDaniel, K. L., and Moser, V. C. (1993). Utility of a neurobehavioral screening battery for differentiating the effects of two pyrethroids, permethrin and cypermethrin. *Neurotoxicol. Teratol.* **15,** 71–83.

Moser, V. C. (1989). Screening approaches to neurotoxicity: A functional observational battery. *J. Am. Coll. Toxicol.* **8,** 85–93.

Moser, V. C., Cheek, B. M., and MacPhail, R. C. (1995). A multidisciplinary approach to toxicological screening. III. Neurobehavioral toxicity. *J. Toxicol. Environ. Health* **45,** 173–210.

Moser, V. C., McCormick, J. P., Creason, J. P. and MacPhail, R. C. (1988). Comparison of chlordimeform and carbaryl using a functional observational battery. *Fundam. Appl. Toxicol.* **11,** 189–206.

Moser, V. C., and MacPhail, R. C. (1989). Neurobehavioral effects of triadimefon, a triazole fungicide, in male and female rats. *Neurotoxicol. Teratol.* **11,** 285–293.

Narotsky, M. G., and Kavlock, R. J. (1995). A multidisciplinary approach to toxicological screening. II. Developmental toxicity. *J. Toxicol. Environ. Health* **45,** 145–171.

NRC (1993). *Pesticides in the Diets of Infants and Children*, National Academy Press, Washington, D.C.

Neter, J., Wasserman, W., and Kutner, M. H. (1985). *Applied Linear Statistical Models*, 2nd ed. Richard D. Irwin, Inc., Homewood, IL.

Owens, J. J. T., and Raff, M. C. (1970). Studies on the differentiation of thymus-derived lymphocytes. *J. Exp. Med.* **132,** 1216–1232.

Poucet, B., and Benhamou, S. (1997). The neuropsychology of spatial cognition in the rat. *Crit. Rev. Neurobiol.* **11,** 101–120.

Reiter, L. W. (1983). Chemical exposures and animal activity: Utility of the figure-eight maze. In *Developments in the Science and Practice of Toxicology* (A. W. Hayes, R. C. Schnell, and T. S. Miya, Eds.), pp. 73–84. Elsevier, New York.

Rogers, J. M. (1987). Comparison of maternal and fetal toxic dose responses in mammals. *Teratol. Carcinog. Mutagen.* **7,** 297–306.

RS/1. (1988). RS/1 Release 4. BBN Software Products Corp., Cambridge, MA.

Santoni, A., Riccardi, C., Barlozzari, T., and Herberman, R. B. (1982). Natural suppressor cells for murine NK activity. In *NK Cells and Other Natural Effector Cells* (R. B. Herberman, Ed.), pp. 527–533, Academic Press, New York.

Schardein, J. L. (1987). Approaches to defining the relationship of maternal and developmental toxicity. *Teratol. Carcinog. Mutagen.* **7,** 255–271.

Smialowicz, R. J., Riddle, M. M., Williams, W. C., and Diliberto, J. J. (1994). Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on humoral immunity and lymphocyte subpopulations: Differences between mice and rats. *Toxicol. Appl. Pharmacol.* **124,** 248–256.

Smialowicz, R. J., Simmons, J. E., Luebke, R. W., and Allis, J. W. (1991). Immunotoxicologic assessment of subacute exposure of rats to carbon tetrachloride with comparison to hepatotoxicity and nephrotoxicity. *Fundam. Appl. Toxicol.* **17,** 186–196

Tyan, M. L. (1981). Marrow stem cells during development and aging. In *Handbook of Immunology in Aging* (M. M-B. Kay, and T. Makinodan, Eds.), pp. 87–102. CRC Press, Boca Raton, FL.

U.S. EPA (1998). Guidelines for Neurotoxicity Risk Assessment. *Fed. Reg.* 63, 26926–26954.

U.S. EPA (1999). Tebuconazole; Pesticide Tolerance. *Fed. Reg.* 40, CFR Part 180, Vol. 64.

Woodbury, D. M. (1974). Maturation of the blood-brain and blood-CSF barriers. In *Drugs and the Developing Brain* (A. Vernadakis and N. Weiner, Eds.), pp. 259–280. Plenum Press, New York.