Two Generation Reproduction Study of Styrene by Inhalation in Crl-CD Rats

George Cruzan, ToxWorks, 1153 Roadstown Road, Bridgeton, New Jersey 08302, toxworks@aol.com

Willem D. Faber, Willem Faber Consulting, 1338 Strong Road, Victor, New York 14564, wfaber@msn.com

Keith A. Johnson, The Dow Chemical Company, Midland, Michigan, 48674, KAJohnson@Dow.com

Linda S. Roberts, ChevronTexaco Energy Research and Technology Company, 100 Chevron Way, Richmond, California 94802, Lroberts@chevrontexaco.com

Juergen Hellwig, BASF Aktiengesellschaft, 67056 Ludwigshafen, Germany, juergen.hellwig@basf-ag.de

Ed Carney, The Dow Chemical Company, Midland, Michigan, 48674, ecarney@Dow.com

John T. Yarrington, WIL Research Laboratories, LLC, 1407 George Road, Ashland, Ohio 44805, Yarrington@wilresearch.com

Donald G. Stump, WIL Research Laboratories, LLC, 1407 George Road, Ashland, Ohio 44805, dstump@wilresearch.com

Abstract

This study was conducted to evaluate the potential adverse effects of styrene on reproductive capability from whole-body inhalation exposure of F_0 and F_1 parental animals. Assessments included gonadal function, estrous cyclicity, mating behavior, conception rate, gestation, parturition, lactation and weaning in the F_0 and F_1 generations, and F_1 generation offspring growth and development. Four groups of male and female Crl:CD(SD)IGS BR rats (25/sex/group) were exposed to 0, 50, 150 and 500 ppm styrene for six hours daily for at least 70 consecutive days prior to mating for the F_0 and F_1 generations. Inhalation exposure for the F_0 and F_1 females continued throughout mating and gestation through gestation day 20. Inhalation exposure of the F_0 and F_1 females was suspended from gestation day 21 through lactation day 4. On lactation days 1 through 4, the F_0 and F_1 females received styrene in virgin olive oil via oral gavage at dose levels of 66, 117 and 300 mg/kg/day (divided into three equal doses, approximately two hours apart). These oral dosages were calculated to provide similar maternal blood peak concentrations as provided by the inhalation exposures. Inhalation exposure of the F_0 and F_1 females was re-initiated on lactation day 5.

Styrene exposure did not affect survival or clinical observations. Rats in the 150 and 500 ppm groups in both parental generations gained weight more slowly than the controls. There were no indications of adverse effects on reproductive performance in either the F_0 or F_1 generation. Male and female mating and fertility indices, pre-coital intervals, spermatogenic endpoints, reproductive organ weights, lengths of estrous cycle and gestation, live litter size and postnatal survival were similar in all exposure groups. Additionally, ovarian follicle counts and corpora lutea counts for the F_1 females in the high-exposure group were similar to the control values. No adverse exposure-related macroscopic pathology was noted at any exposure level in the F_0 and F_1 generations. A previously characterized pattern of degeneration of the olfactory epithelium that lines the dorsal septum and dorsal and medial aspects of the nasal turbinates occurred in the F_0 and F_1 generation animals from the 500 ppm group. In the 500 ppm group, F_2 birthweights were reduced compared to the control and F_2 offspring from both the 150 and 500 ppm exposure groups gained weight more slowly than the controls.

Based on the results of this study, an exposure level of 50 ppm was considered to be the NOAEL for F_0 and F_1 parental systemic toxicity; the NOAEL for F_0 and F_1 reproductive toxicity was 500 ppm or greater.

Introduction

Styrene (CAS # 100-42-5) is a commercially important monomer, which is used in the manufacture of polystyrene products (packaging, insulation, etc.), acrylonitrile-butadiene-styrene (ABS) products (appliance cases, automotive parts, etc.), synthetic rubber, and reinforced plastics. Exposure to the general population occurs at levels of micrograms per day from ambient air and intake of food (IARC, 2002).

The reproductive and developmental effects of styrene have been extensively reviewed by Brown et al. (2000). Reports of styrene-related effects on human reproduction are limited and conflicting. A large study of US women concluded that styrene exposure did not affect menstrual cycle (Lemasters et al., 1985); however, Cho et al. (2001) concluded that exposure to styrene increased the risk of menstrual cycles longer than 35 days. One study (Jelnes, 1998) suggested increased sperm abnormalities in workers exposed to high levels of styrene in the reinforced plastics industry. A later study of 23 workers (Kolstad et al., 1999) found no effect on sperm abnormalities, but reported a decrease in sperm density during the first 6 months of exposure to styrene in the reinforced plastics industry. In a study of 220 male reinforced plastics workers exposed to high levels of styrene, there was no relationship between exposure and time to pregnancy of their partners (Kolstad et al., 2000). A study of female reinforced plastics workers reported a possible decrease (4%) in birth weight of offspring of mothers exposed to styrene above 80 ppm and other solvents (Lemasters, 1989). Birth weights were taken from mothers' memory, not birth records, and the difference was not statistically significant.

No effects on fertility or reproduction were found in three generations of male and female Sprague-Dawley rats exposed to 125 or 250 ppm styrene in their drinking water (Beliles et al., 1985). The concentration was limited by the solubility of styrene in water (approximately 300 ppm). Water consumption was significantly reduced in both groups compared to controls, indicating taste aversion. While this study demonstrated no effects on fertility, gestation, or reproduction, its value for risk assessment is limited due to the low doses achieved (<25 mg/kg/day).

No effects on ovarian or testicular pathology have been reported in several of the subchronic or chronic toxicity studies in rats [500 to 2000 mg/kg/day gavage, 50-1500 ppm inhalation] and mice [150-300 mg/kg/day gavage, 20 to 200 ppm inhalation] (NCI, 1979; Cruzan et al., 1997, 1998, 2001; Roycroft et al., 1995). On the other hand, testicular pathology and decreases in sperm count were reported in rats treated with 400 mg/kg/day styrene by gavage for 60 days (Srivastava et al., 1989). Decreased free testosterone in plasma was reported in pre-pubertal male C57BL/6 mice exposed to 50 mg/l styrene in drinking water for 4 weeks (12 mg/kg/day). There were no effects on body weight, testis weight, plasma cortisone, or plasma luteinizing hormone (Takao et al., 2000).

Because data on the reproductive effects of styrene were limited, a two-generation reproduction study was conducted via whole-body inhalation according to current regulatory guidelines. Developmental neurotoxicity evaluation of selected offspring from the second generation are reported in an accompanying article (Cruzan et al., in press). In most reproduction studies conducted by inhalation, exposure is stopped on day 20 of gestation and reinstated on lactation

day 5 to minimize stress on the offspring from the more than six-hour separation that would occur during inhalation exposure of the dam. Because high concentrations of styrene may cause central nervous system (CNS) depression and significant development of the CNS occurs during the first few days after birth in rats, F_0 and F_1 dams were treated orally during lactation days 1-4 at doses estimated by physiologically based pharmacokinetic (PBPK) modeling to mimic a six-hour inhalation exposure.

Materials and Methods

Study Design

Four groups of male and female Crl:CD rats (25/sex/group) were exposed to vapor atmospheres of styrene at 0, 50, 150 or 500 ppm for six hours daily for at least 70 consecutive days prior to mating. Daily vaginal smears were performed for assessment of estrous cyclicity, beginning 21 days prior to pairing. Females were paired with males on a 1:1 basis for 14 days or until evidence of mating was observed. The F_0 and F_1 females continued inhalation exposure throughout mating and gestation through gestation day 20. On lactation days 1 through 4, the F_0 and F_1 females received styrene in virgin olive oil via oral gavage at dose levels of 66, 117 and 300 mg/kg/day (divided into three equal doses, approximately two hours apart) at a dose volume of 1 ml/kg/dose. The doses were calculated to mimic the peak maternal blood level of styrene during a six-hour inhalation exposure at the target concentration based on the PBPK model of Sarangapani et al. (2002). Inhalation exposure of the F_0 and F_1 females was re-initiated on lactation day 5 and continued through the day prior to euthanasia. Offspring were weaned on lactation day 21; exposure of F_1 pups began on postnatal day (PND) 22. Inhalation exposure of the F_0 and F_1 males continued throughout mating, and through the day prior to euthanasia. Spermatogenic endpoints were recorded for all F_0 and F_1 males. Ovarian primordial follicle counts and the corpora lutea counts were recorded for all F_1 females in both the control and high-exposure groups and for F_1 females in the other exposure groups that did not mate or produce offspring.

Test Material

Styrene monomer (inhibited from self-reaction by 10 ppm t-butylcatechol), CAS No. 100-42-5, was provided by Chevron Phillips Chemical Company LLP, St. James, LA. The purity and stability of the styrene were verified by gas chromotography with flame ionization detection. When present in the chromatograms, the percentage of benzene, ethylbenzene, styrene oxide and styrene dimers was also determined. Results obtained indicated the styrene was at least 99.9% pure.

Animals and Animal Husbandry

One hundred fifteen male and 116 female Crl:CD®(SD)IGS BR rats from different colonies (to avoid sibling matings) were received from Charles River Laboratories, Inc., Raleigh, North Carolina, on July 24, 2001. The animals were 37 and 38 days old upon receipt, respectively. At the conclusion of the acclimation period, all available F_0 animals were weighed and examined in detail for physical abnormalities. Animals judged to be in good health and meeting acceptable body weight requirements were randomized into treatment groups by a computerized program to ensure homogeneity of treatment groups.

Until pairing, all F_0 and F_1 parental test animals were individually housed in clean, wire-mesh cages suspended above cage-board. During cohabitation, the animals were paired for mating in the home cage of the male. Following positive evidence of mating, the males were housed in suspended wire-mesh cages until the scheduled necropsy of the parental generations, and the females were transferred to plastic maternity cages with nesting material (Bed-O'Cobs®; The Andersons, Industrial Products Division, Maumee, Ohio). The dams were housed in these cages

until weaning on lactation day 21. Animals were housed in accordance with the "Guide for the Care and Use of Laboratory Animals." The animal care program including animal facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

Animals were fed PMI Nutrition International, Inc., Certified Rodent LabDiet® 5002 *ad libitum*. Municipal water was reverse-osmosis-treated (on-site) and delivered by an automatic watering system to individual cages *ad libitum*, except by water bottles when water consumption was measured during gestation and lactation. No water was available during inhalation exposure. Animals were housed throughout the acclimation period and during the study in an environmentally controlled room.

Parental Observations

All animals were observed twice daily (at least seven hours apart) for moribundity and mortality, appearance, behavior and pharmacotoxic signs (prior to exposure/gavage dosing for the F_0 and F_1 animals). During inhalation exposures, approximately 50% of the F_0 and F_1 animals in each group were visible through the chamber windows; the visible animals were observed for appearance and behavior at the mid-point of exposure. The F_0 and F_1 animals were also observed within one hour following exposure/gavage dosing. Detailed physical examinations were recorded weekly for all F_0 and F_1 parental animals throughout the study period. F_0 and F_1 females expected to deliver were also observed twice daily during the period of expected parturition and at parturition for dystocia or other difficulties.

Individual F_0 and F_1 male body weights were recorded weekly throughout the study and prior to the scheduled necropsy. Individual F_0 and F_1 female body weights were recorded weekly until evidence of copulation was observed. Once evidence of mating was observed, female body weights were recorded on gestation days 0, 4, 7, 11, 14 and 20 and on lactation days 1, 4, 5 (pre-exposure), 7, 14 and 21.

Individual F_0 and F_1 male food consumption was measured on a weekly basis (except during the mating period) until the scheduled necropsy. Individual F_0 and F_1 female food consumption was measured on a weekly basis until the start of the mating period. Female food consumption was recorded on gestation days 0, 4, 7, 11, 14 and 20 and lactation days 1, 4, 5, 7, 14 and 21. Water consumption was recorded daily during gestation and lactation for the F_0 and F_1 females.

Assessment of Reproductive Performance

Vaginal smears were prepared daily to determine the stage of estrus for each female, beginning 21 days prior to pairing and continuing until evidence of mating was observed. For females with no evidence of mating, smearing was continued until termination of the mating period. The average cycle length was calculated for complete estrous cycles.

After a minimum of 70 days of exposure, each female was housed overnight in the home cage of a randomly chosen male from the same exposure group, avoiding sibling matings. Each mating pair was examined daily for the presence of a copulatory plug or the presence of sperm in a vaginal smear. The day when evidence of mating was identified was termed day 0 of gestation. The animals were then separated and the female was housed in an individual plastic cage with

nesting material. When evidence of mating was not apparent after 14 days, the female was placed for an additional seven days with another male of the same exposure group that had successfully mated. If no evidence of copulation was obtained after 21 days, the animals were separated without further opportunity for mating, and the female was placed in a plastic cage containing nesting material. Following the second mating period, the females were euthanized on gestation day 15 (females that mated with the second male) or postcohabitation day 15 (females that did not mate with the second male). Pre-coital intervals were calculated according to the number of 12-hour dark cycles prior to evidence of mating.

All females were allowed to deliver naturally and rear their young to weaning (PND 21). During the period of expected parturition, the females were observed twice daily for initiation and completion of parturition and for signs of dystocia. On the day parturition was judged complete (PND 0), the sex of each pup was determined and each was examined for gross malformations; the numbers of stillborn and live pups were recorded. Individual gestation lengths were calculated using the date delivery started.

Offspring Evaluations

All pups were individually identified by application of tattoo markings on the digits on PND 0. Each litter was examined twice daily for survival and signs of toxicity. Intact offspring dying from PND 0 to 4 were necropsied using a fresh dissection technique (Stuckhardt and Poppe, 1984). A detailed gross necropsy was performed on any pup dying after postnatal day 4 and prior to weaning and for all F_1 pups dying between PND 22 and 28.

To reduce variability among litter size, 10 F_1 and F_2 pups from each litter were randomly selected of equal sex distribution, if possible, on PND 4. The remaining F_1 and F_2 offspring were weighed, euthanized by intraperitoneal injection of sodium pentobarbital and discarded on PND 4.

Pups were individually sexed on PND 0, 4, 7, 14 and 21. F_1 pups were individually weighed on PND 1, 4, 7, 14 and 21; F_2 pups were individually weighed on PND 1, 4, 7, 11, 13, 17 and 21. The following investigations were used to assess the maturation of the selected F_1 and F_2 pups: pinna detachment, surface righting response, hair growth, incisor eruption, eye opening, balanopreputial separation, and vaginal patency. Individual pups were weighed on the day of sexual maturation. These data are presented in the companion developmental neurotoxicity study (Cruzan et al., in press)

Weaning and Selection for F_1

Each F_0 dam and its litter remained housed together (except during inhalation exposures of the dams 6 hrs/day on lactation days 5-20) until weaning on lactation day 21. On PND 21, a computerized randomization procedure was used to select two F_1 male and two F_1 female weanlings per litter. These pups were exposed to the test article for six hours per day beginning on PND 22. Between PND 22 and 28, 6 male and 6 female control pups died; 0 from the 150 ppm group; 1 male and 2 females from the 150 ppm group, and 1 male and 2 females from the 500 ppm group died. One male and one female from each litter, when available, were randomly selected on PND 28 to comprise the F_1 generation (16, 23, 23, and 22 males and females for 0, 50, 150 and 500 ppm, respectively). Additional rats (9, 2, 2, and 3 males and females for 0, 50,

150 and 500 ppm) were randomly chosen from the remaining male and female from each litter to make 25 male and 25 female in each F_1 exposure group (0, 50, 150, and 500 ppm). The remaining pups were euthanized on PND 28

Ovarian and Spermatogenic Endpoint Evaluations
A bilateral evaluation of one section of each ovary was performed for F_0 females. A quantitative histologic evaluation of five sections (at least 100µm apart) from the inner third of each ovary was conducted on all F_1 females in the control and high dose group. This examination included enumeration of the total number of primordial follicles and corpora lutea according to the methods of Bolon et al. (1997) and Bucci et al. (1997). The primordial follicles were defined as small oocyte with a nucleus surrounded by a partial or unbroken single layer of flattened to cuboidal follicular/granulosa cells. Due to the size of corpora lutea (much larger than primordial follicles) each corpus luteum was possibly sectioned and counted multiple times, resulting in a value that was larger than would be expected.

Spermatogenic endpoints were evaluated using the methods described by Nemec et al. (2004). Immediately upon euthanasia, the right epididymis of each F_0 and F_1 male was exposed, excised, and weighed. Sperm motility was assessed using the Hamilton-Thorne HTM-IVOS Version 10 (Beverly, MA) computer-assisted sperm analysis (CASA) system. Sperm morphology was evaluated by light microscopy via a modification of the wet-mount evaluation technique (Linder et al., 1992).

The left testis and cauda epididymis from all F_0 and F_1 males from all exposure groups were weighed and stored frozen (approximately -20°C). These tissues from the control and 500 ppm groups were then thawed, homogenized and a sample was evaluated for determination of homogenization-resistant spermatid count and sperm production rate (Blazak et al., 1985).

Pathology

All F_0 and F_1 adult animals were euthanized by isoflurane inhalation and exsanguination. Vaginal smears were performed on all females on the day of euthanasia to determine the stage of estrous cycle. All surviving males were euthanized approximately 3 weeks following completion of the parturition period. All surviving females that delivered were euthanized between 6 and 10 days after weaning of their litters. Females that mated but did not give birth were euthanized on presumed gestation day 25. Females that experienced total litter loss were euthanized within 24 hours. All surviving F1 weanlings not selected for styrene exposure were euthanized on PND 21. All F1 weanlings exposed to styrene PND 22-27 but not chosen to become F1 parents, were euthanized on PND 28. All F2 weanlings not selected for behavioral evaluation were euthanized on PND 21. A complete necropsy was conducted, selected organs were weighed and selective histopathologic examination was performed.

Statistical Methods

Analyses were conducted using two-tailed tests (except as noted otherwise) for a minimum significance level of 5%, comparing each test article-treated group to the differences. Parental mating and fertility indices were analyzed using the Chi-square test with Yates'correction factor

(Hollander and Wolfe, 1999). Mean parental (weekly, gestation and lactation) and F_2 offspring body weight data, food consumption and food efficiency data, organ weight data, maternal estrous cycle data, pre-coital intervals, gestation lengths, implantation sites, unaccounted sites, ovarian primordial follicle counts, mean number of pups born, live litter size, epididymal and testicular sperm numbers, and sperm production rates were analyzed for heterogeneity of variance (Levene, 1960) and normality (Royston, 1982). If the data were homogeneous and normal, a parametric one-way analysis of variance (ANOVA) was used to determine intergroup differences (Snedecor and Cochran, 1980). If the results of the ANOVA were significant ($p<0.05$), Dunnett's (1964) test was applied to compare the control group versus all treatment groups. If the data were not homogeneous and normal, the data were analyzed by the Kruskal-Wallis (1952) nonparametric ANOVA test to determine the intergroup differences. If the ANOVA revealed statistical significance ($p<0.05$), the Mann-Whitney U-test (Kruskal and Wallis, 1952) was used to compare the test article-treated groups to the control group. Pup weights through weaning were analyzed separately by sex by a nested analysis of covariance (ANCOVA). The number of pups born was used as the covariate. The following assumptions were made regarding the ANCOVA: homogeneity of regression slopes, linear relationship between the pup weights and number of pups born, and additive group and regression effects. Histopathologic findings in the test article-treated groups were compared to the control group using a two-tailed Fisher's Exact test (Steel and Torrie, 1980).

Results

Exposures

Gas chromatographic analyses of chamber atmospheres demonstrated mean daily styrene exposure concentrations of 0, 50, 151, and 499 ppm for F_0 generation and 0, 50, 153, and 501 ppm for F_1 generation. Standard deviation/mean concentration never exceeded 3.5%. Low levels of styrene oxide were detected in exposure chambers on nearly 50% of analyses. Except for 5 occasions, the styrene oxide level was less than 1 ppm, was never greater than 2.3 ppm, and was not proportional to the chamber styrene concentration. Styrene dimer was detected in chamber atmospheres of ~20% of the samples tested; levels were less than 2 ppm.

Analyses of the oral dosing formulations used during lactation days 1-4 showed homogeneity and stability; analyzed concentrations were within 12% of target.

Parental Evaluations

No exposure-related clinical findings were noted at the weekly detailed physical examinations or at the observations made prior to, at the midpoint, or one hour following exposure in either F_0 or F_1 animals. Findings noted in the treated groups occurred infrequently, at similar frequencies in the control group, and/or in a manner that was not exposure-related.

Body weight gain was slightly reduced in F_0 males and females at 500 ppm as evidenced by decreased mean body weights during premating weeks 3-10 (Table 1). In F_0 males exposed at 150 ppm, mean weight gain was reduced during the first week (31 vs. 39 g in the control group); body weight was significantly reduced at week 7. Body weights of F_1 males and females at 150

and 500 ppm were reduced compared to controls after exposure on PND 22-27 and remained reduced through the F_1 exposure period (Table 1). There was no effect on bodyweight or bodyweight gain at 50 ppm in either the F_0 or F_1 exposure periods.

Only very minor differences in food consumption between exposed and control groups were reported (data not shown).

Gestation

Styrene exposure had no effect on body weight gain or food consumption during gestation in either the F_0 or F_1 dams (Table 1). Mean body weight reductions observed in the 500 ppm group F_1 females during gestation were attributed to the reduced body weight gains observed during the pre-mating period. At 500 ppm water consumption during gestation was increased slightly, but statistically significantly, in both F_0 and F_1 dams (Figure 1). There was no effect at 150 or 50 ppm.

The mean lengths of gestation were unaffected by test article exposure in the F_0 (Table 2) and F_1 (Table 3) treated rats. No signs of dystocia were noted at any exposure level.

Lactation

There was no effect of styrene exposure on body weight gain or food consumption during lactation in either the F_0 or F_1 dams (data not shown). Water consumption increased during lactation days 5-14 in F_0 dams exposed to 150 or 500 ppm, but not in F_1 dams. No effect was noted in F_0 or F_1 dams at 50 ppm.

Reproductive Performance

Exposure of F_0 females had no effect on mean estrous cycle length (Table 2). The means for all treated groups (4.2 to 5.1 days) were within the historical range (4.1 to 5.1 days), while the mean for the control females (5.8 days) was abnormally high due to 3 control females with abnormally long estrus cycles. Exposure of F_1 females had no effect on mean estrous cycle length (Table 3). No effects from exposure of F_0 or F_1 rats were observed on the mean numbers of days between pairing and coitus. Styrene exposure had no effects on F_0 or F_1 spermatogenic endpoints (Table 4). Exposure to styrene did not affect F_0 or F_1 male or female mating index, male or female fertility index, mean number of pups born, the number of former implantation sites, or the number of unaccounted sites (Table 2, 3).

Pathology

At the scheduled F_0 and F_1 male and female necropsies, no macroscopic findings attributed to styrene were observed. Increases in relative (to final body weight) liver weights were observed in the 150 ppm and 500 ppm group F_0 and F_1 males (data not shown). Other organ weights (absolute and relative to final body weight) were unaffected by the test article at all exposure levels.

Microscopic evaluations were performed only on tissues from all F_0 and F_1 parental animals in the 0 and 500 ppm groups and for those adult animals in the 50 and 150 ppm exposure groups that were found dead or were euthanized *in extremis.* In the 500 ppm males and females exposure-related microscopic findings were confined to the nasal cavity. Increased occurrences

of minimal to mild degeneration of the olfactory epithelium that lined the dorsal septum and dorsal medial aspects of the dorsal turbinates (ethmoturbinates) primarily at nasal levels II, III and IV (Young, 1981) were found compared to the control group. The olfactory epithelial degeneration was characterized by disorganization and generally one or more of the following features: regenerative hyperplasia, individual cell necrosis, atrophy, and increased presence of Bowman's glandular elements and cysts within olfactory epithelium. Despite these olfactory epithelial changes, the exposure-induced lesions did not have any inflammatory response. The incidence of nasal lesions was less in high-exposure F_1 rats than in F_0 rats. No other exposure-related microscopic findings were noted in the 500 ppm group.

At the scheduled necropsy of the F_1 females, mean numbers of primordial follicles per section (Bolon et al., 1997) were 5.0 and 5.1 for females in the control and 500 ppm groups, respectively. The mean numbers of corpora lutea per section (Bucci et al., 1997) were 9.2 and 10.1 for females in the same respective groups. The mean numbers of primordial follicles and corpora lutea for all examined animals were unaffected by test article exposure.

In addition, reproductive tract organs for low- and mid-exposure group adult animals that did not mate or produce offspring were examined microscopically. No treatment-related effects were found.

Offspring Evaluations (Tables 2, 3)

The mean number of F_1 and F_2 pups born, live litter size, percentage of males per litter at birth and postnatal survival between PND 0 (relative to number born), 0-1, 1-4 (pre-selection), 4 (post-selection)-7, 7-14, 14-21, and from birth to PND 4 (pre-selection) and PND 4 (post-selection) to PND 21 were unaffected by styrene exposure. The numbers of F_1 and F_2 pups found dead, euthanized *in extremis* and/or missing, as well as the general physical condition of all F_1 pups in this study were unaffected by styrene exposure.

Mean F_1 male and female pup body weights were unaffected by parental exposure to styrene. Mean F_2 pup body weight gains and mean body weights in the 500 ppm group were decreased (6.8%-13.3%) throughout the pre-weaning period (PND 0-21). The mean male and female F_2 pup body weight changes in the 150 ppm group were similar to the control group during PND 1-4, but were decreased PND 4-21 (only males significantly different from control). Mean body weights and mean body weight gains in the 50 ppm group F_2 males and females were unaffected by maternal exposure to styrene (Figure 2).

Offspring pathology

No macroscopic findings that could be attributed to parental exposure with the test article were noted at the scheduled necropsy of F_1 or F_2 pups euthanized on PND 21. Mean organ weights (absolute and relative to final body weight) in the styrene exposed F_1 males and females examined at the PND 21 necropsy were similar to the control group.

Statistically significant ($p<0.01$) reductions in mean absolute pituitary, thymus and uterine weight occurred in F_2 female offspring of dams exposed to 500 ppm. Because these pups had reduced body weight, compared to controls, and the relative organ weights were not reduced, the reductions in female pituitary, thymus and uterine weight were attributed to growth retardation, not to direct effects on these organs. Similarly, a reduction in absolute, but not relative, pituitary weight in F_2 female offspring of dams exposed to 150 ppm styrene was considered due to growth retardation. Statistically significant ($p<0.01$) reductions in mean absolute and relative pituitary weight occurred in F_2 male offspring of dams exposed to 500 ppm (Table 5). Because the relative pituitary weight was decreased in males, this was attributed to a test article effect on the pituitary, not just growth retardation.

Discussion

The parental systemic toxicity of styrene reported in this study was similar to that previously reported in rats following long-term inhalation exposure (Cruzan et al., 1997, 1998). Findings included degeneration of the olfactory epithelium that lines the dorsal septum and dorsal and medial aspects of the nasal turbinates of F_0 and F_1 animals in the high-exposure group (500 ppm; nasal tissue was not examined in the low- and mid-exposure groups), decreased mean body weights in the mid-exposure group (F_0 and F_1 males and F_1 females) and high-exposure group (F_0 and F_1 males and females), and increased water consumption during gestation in the high-exposure group (F_0 and F_1 females) and during lactation in the mid- and high-exposure groups (F_0 females). In the previous chronic study, nasal lesions increased in incidence and severity with dose. A NOAEL was not found, but effects at 50 ppm were slight and not all animals were affected even after 24 months. Decreased body weight and increased water consumption were found at 500 and 1000 ppm for 2 years.

Reproductive performance and offspring postnatal survival prior to weaning were not adversely affected by styrene exposure. Pre-weaning pup weights were unaffected by styrene exposure for the F_1 generation. Following direct exposure of the F_1 weanlings on PND 22, body weight reductions were observed in the 500 ppm group that led to the reduced mean body weights in this group throughout the generation. In contrast to the F_1 generation, pre-weaning F_2 pup weights were reduced in both the 150 ppm and 500 ppm groups (approximately 10 to 13% on PND 21). The weights of the F_2 pups continued to be reduced following weaning in the 150 and 500 ppm groups selected for neurobehavioral evaluation in the developmental neurotoxicity component (Cruzan et al., in press). In addition, there were slight delays (generally not statistically significant) in the acquisition of developmental landmarks (Cruzan et al., in press) that were suggestive of an overall pattern of slight developmental delay in the 500 ppm group. These pre-weaning developmental endpoints are highly correlated with pup body weight (Lochry, 1987) and are consistent with the reduced body weights seen in this group. Effects may have been greater in the F_2 offspring than the F_1 because exposure of the F_1 parents was started at a younger age (PND 21 vs. PND 50) and pre-mating toxicity was more evident in F_1 parents than F_0 parents.

This study confirmed previous observations of slight body weight effects of styrene exposure at 500 ppm or greater in rats and degeneration of nasal olfactory epithelium (Cruzan et al, 1997,

1998). It further demonstrated a lack of styrene effects on gonadal function, reproductive performance and offspring survival. This enhances the conclusions of the previous 3-generation reproduction study of styrene in drinking water (Beliles et al., 1985). In addition, it supports the lack of effects on testes and ovaries reported in the subchronic studies of styrene (Cruzan et al., 1997, 1998, 2001; NCI, 1979; Roycroft et al., 1995), and disagrees with testicular pathology and decreased sperm counts reported by Srivastava et al. (1989).

The No-Observed-Adverse-Effect Level (NOAEL) for parental toxicity in this study was 50 ppm and the NOAEL for reproductive toxicity was ≥500 ppm.

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References

Adams J. 1986. Methods in Behavioral Teratology. In *Handbook of Behavioral Teratology*; Riley, E.P., Vorhees, C.V., Eds.; Plenum Press: New York, pp 67-97.

Ashby J, Lefevre PA. 2000. The peripubertal male rat assay as an alternative to the Hershberger castrated male rat assay for the detection of anti-androgens, oestrogens and metabolic modulators. J. Appl. Toxicol. 20: 35-47.

Beliles RP, Butala J H, Stock CR, Makris S. 1985. Chronic toxicity and three-generation reproduction study of styrene monomer in the drinking water of rats. Fund. Appl. Toxicol. 5: 855-868.

Blazak WF, Ernst TL, Stewart BE. 1985. Potential indicators of reproductive toxicity: testicular sperm production and epididymal sperm number, transit time and motility in Fischer 344 rats. Fundam. Appl. Toxicol. 5: 1097-1103.

Bolon B, Bucci TJ, Warbritton AR, Chen JJ, Mattison DR, Heindel JJ. 1997. Differential follicle counts as a screen for chemically induced ovarian toxicity in mice: results from continuous breeding bioassays. Fundam. Appl. Toxicol. 39: 1-10.

Brown NA, Lamb JC, Brown SM, Neal BH. 2000. A review of the developmental and reproductive toxicity of styrene. Regul. Toxicol. Pharmacol. 32: 228-247.

Bucci TJ, Bolon B, Warbritton AR, Chen JJ, Heindel JJ. 1997. Influence of sampling on the reproducibility of ovarian follicle counts in mouse toxicity studies. Reprod. Toxicol. 11: 659-696.

Cho S-I, Damokosh AI, Ryan LM, Chen D, Hu YA, Smith TJ, Christiani DC, Xu X. 2001. Effects of exposure to organic solvents on menstrual cycle length. J. Occup. Environ, Med. 43: 567-575.

Cruzan G, Cushman JR, Andrews LS, Granville GC, Miller RR, Hardy CJ, Combs DW, Mullins PA. 1997. Subchronic inhalation studies of styrene in CD rats and CD-1 mice. Fundam. Appl. Toxicol. 35: 152-165.

Cruzan G, Cushman JR, Andrews LS, Granville GC, Johnson KA, Hardy C, Combs DW, Mullins PA, Brown WR. 1998. Chronic toxicity/oncogenicity study of styrene in CD rats by inhalation exposure for 104 weeks. Toxicological Sciences 46:266-281.

Cruzan G, Cushman JR, Andrews LS, Granville GC, Johnson KA, Bevan C, Hardy CJ, Coombes DW, Mullins PA, Brown WR. 2001. Chronic toxicity/oncogenicity study of styrene in CD-1 mice by inhalation exposure for 104 weeks. J. Appl. Toxicol. 21: 185-198.

Dunnett CW. 1964. New tables for multiple comparisons with a control. Biometrics 20: 482-491.

Hollander M, Wolfe DA. 1999. *Nonparametric Statistical Methods*, 2nd ed. John Wiley and Sons, Inc.: New York, NY, p 468.

Jelnes JE. 1988. Semen quality in workers producing reinforced plastic. Reproductive Toxicology. 2: 209-212.

Kallman MJ. 1994, Assessment of Motoric Effects. In *Developmental Neurotoxicology*; Harry, G.J., Ed.; CRC Press: Boca Raton, pp 103-122.

Kolstad HA, Bonde JP, Spano M, Giwercman A, Zschiesche W, Kaae D, Larsen SB, Roeleveld N, 1999. Change in semen quality and sperm chromatin structure following occupational styrene exposure. Int, Arch. Occup. Environ, Health 72:135-141.

Kolstad HA, Bisanti L, Roeleveld N, Baldi R, Bonde JP, Joffe M. 2000. time to pregnancy among male workers of the reinforced plastics industry in Denmark, Italy and the Netherlads. Scand. J. Work Environ. Health 26: 353-358.

Kruskal WH, Wallis WA. 1952. Use of ranks in one-criterion variance analysis. J. Am. Stat. Assoc. 47: 583-621.

Lemasters GK, Hagen A, Samuels SJ., 1985. Reproductive outcomes in women exposed to solvents in 36 reinforced plastics companies. I. Menstrual dysfunction. J. Occup. Med. 27: 490-494.

Lemasters GK. 1989. Reproductive outcomes of pregnant workers employed at 36 reinforced plastics companies. II. Lowered birth weight. J. Occup. Med. 31: 115-120.

Levene H. 1960. Robust Tests for Equality of Variances. In: *Contributions to Probability and Statistics*; Stanford University Press: Palo Alto, CA, pp. 278-292.

Linder RE, Strader LF, Slott VL, Suarez JD. 1992. Endpoints of spermatotoxicity in the rat after short duration exposures to fourteen reproductive toxicants. Reproductive Toxicol. 6: 491-505.

Lochry EA. 1987. Concurrent use of behavioral/functional testing in existing reproductive and developmental toxicity screens: practical considerations. J. Am. Coll. Toxicol. 6: 433-439.

National Cancer Institute, 1979. Bioassay of styrene for possible carcinogenicity. NCI Technical Report #185.

Nemec MD, Pitt, JA, Topping, DC, Gingell, R, Pavkov, KL, Rauckman, EJ, Harris, SB 2004. Inhalation two-generation reproductive toxicity study of methyl isobutyl ketone in rats. Int. J. Toxicol. 23: 127-143.

Roycroft JH, Mast TJ, Ragan HA, Grumbein SL, Miller RA, Chou BJ. 1992. Toxicological effects of inhalation exposure to styrene in rats and mice. The Toxicologist 12: 397.

Royston JP. 1982. An extension of shapiro and wilk's *w* test for normality to large samples. Appl. Statist. 31: 115-124.

Sarangapani, R., Teeguarden, J.G., Cruzan, G., Clewell, H.J., and Andersen, M.E. (2002) Physiologically based Pharmacokinetic Modeling of Styrene and Styrene Oxide Respiratory Tract Dosimetry in Rodents and Humans. *Inhal. Tox.*, 14: 789-834.

Snedecor GW, Cochran WG. 1980. One Way Classifications; Analysis of Variance. In *Statistical Methods*, 7th ed.; The Iowa State University Press: Ames, IA, pp. 215-237.

Srivastava S, Seth PK, Srivastava SP. 1989. Effect of styrene administration on rat testis. Arch. Toxicol. 63: 43-46.

Steel RGD, Torrie JH. 1980. Principles and Procedures of Statistics, A Biometrical Approach, 2nd ed. McGraw-Hill Book Company: New York, NY, pp 504-506.

Stuckhardt JL, Poppe SM. 1984. Fresh visceral examination of rat and rabbit fetuses used in teratogenicity testing. Terat. Carcinogen. Mutagen. 4: 181-188.

Takao T, Nanamiya W, Nazarloo HP, Asaba K, Hashimoto K. 2000. Possible reproductive toxicity of styrene in peripubertal male mice. Endocr. J. 47: 343-347.

Young JT. 1981. Histologic examination of the rat nasal cavity. Fundam. Appl. Toxicol. 1: 309-312.

Figure legends

Figure 1. Mean water consumption of F_0 (A) and F_1 (B) females during gestation.

Figure 2. Mean bodyweights of male (A) and female (B) F_2 offspring through weaning.

Table 1. Mean body weights (g) of F_0 and F_1 rats during premating, gestation and lactation

Exposure Conc. (ppm):	0	50	150	500	0	50	150	500
F_0		Males				Females		
Week 0	266	263	264	263	190	190	190	189
1	305	301	295	287	215	210	208	203
3	378	347	359	353*	251	246	243	231*
5	437	419	413	404*	273	269	262	252*
7	483	455	454*	443*	290	288	279	269*
10	530	503	503	494	306	303	296	282*
F_0 Gestation								
Day 0					302	300	298	285
7					330	323	325	312
14					362	354	357	346
20					427	424	428	418
F_1 Males								
Week 0	110	112	103	97	97	104	93	90
1	173	173	162	151*	141	147	135	133
3	292	285	272*	262*	196	199	187	187
5	375	369	349*	336*	240	242	227	224*
7	429	421	399*	393*	267	268	253	249*
10	481	468	448	443*	293	293	278	269*
F1 Gestation								
Day 0					296	295	279	274*
7					325	324	308	307
14					356	354	340	334*
20					428	431	410	402

* Statistically significantly different from control, $p<0.05$.

Table 2. Reproductive and Offspring Parameters for F_0 Generation Rats Exposed to Styrene

	Parental Exposure Level (ppm)			
	0	50	150	500
Reproduction Parameters (mean±SD)				
No. F_0 Males/Females Assigned	25/25	25/25	25/25	25/25
Males/Females Died	0/0	0/0	0/0	0/0
Males/Females Euthanized[a]	0/7	0/2	0/2	0/3
Males/Females Completing Study	25/18	25/23	25/23	25/22
Estrous Cycle Length (days)	5.8 ± 3.1	5.1 ± 2.0	5.1 ± 2.2	4.2 ± 0.5*
Male Mating Index (%)[b]	23/25 (92)	25/25 (100)	25/25 (100)	23/25 (100)
Female Mating Index (%)[b]	25/25 (100)	25/25 (100)	25/25 (100)	25/25 (100)
Pre-coital Interval (days)	3.8 ± 3.9	2.0 ± 1.0	2.5 ± 1.3	3.6 ± 3.5
Male Fertility Index (%)[c]	21/25 (84)	24/25 (96)	24/25 (96)	23/25 (92)
Female Fertility Index (%)[c]	22/25 (88)	24/25 (96)	24/25 (96)	25/25 (100)
Fertility Determined via 2nd Male	2	0	0	2
Females Delivering Litters	20	23	24	23
Gestation Length (days)	21.9 ± 0.3	21.9 ± 0.5	21.8 ± 0.6	22.0 ± 0.3
Implantation Sites	15.2 ± 1.8	14.8 ± 3.6	15.8 ± 1.8	15.3 ± 1.8
Number Born/litter	14.5 ± 1.9	13.8 ± 3.2	14.9 ± 1.8	14.3 ± 1.6
Live Litter Size	14.3 ± 1.8	13.5 ± 3.3	14.3 ± 2.0	14.1 ± 1.6
Live Birth Index (%)[d]	99.0 ± 2.5	97.6 ± 4.5	96.1 ± 6.2	98.2 ± 3.5
Females with Surviving Pups at Weaning (PND 21)	18	23	23	22
F_1 Offspring Parameters (mean ± SD)				
Sex Distribution at Birth (% Males)	46.6 ± 14.9	51.0 ± 11.6	46.8 ± 12.1	48.9 ± 11.8
Survival (%)				
Birth to PND 4 (Pre-culling)	98.7 ± 2.8	96.1 ± 5.9	94.5 ± 8.2	92.7 ± 17.1
PND 4-21 (Post-culling)	91.1 ± 23.6	98.6 ± 4.7	98.3 ± 5.8	93.5 ± 20.8
Body Weight Gain (g)				
Males PND 1-4 (pre-culling)	2.6 ± 0.6	3.0 ± 1.1	2.9 ± 0.6	2.7 ± 1.1
Females PND 1-4 (pre-culling)	2.5 ± 0.6	3.0 ± 1.3	2.7 ± 0.8	2.5 ± 1.0
PND 21 Body Weight (g)				
Males	38.4 ± 6.3	41.4 ± 5.5	39.1 ± 5.2	38.5 ± 3.8
Females	37.6 ± 5.8	40.7 ± 7.1	37.1 ± 5.4	37.3 ± 3.9

[a] One female each from the control and 150-ppm groups were euthanized *in extremis* after the mating trial; one female each from the control and 500-ppm groups were euthanized during lactation due to total litter loss; all others were euthanized for pregnancy determination after no evidence of mating.
[b] Mating Index: Proportion of males/females showing evidence of mating relative to the number cohabited X 100; positive evidence of mating included vaginal sperm, copulatory plug and or/pregnancy.
[c] Fertility Index: Proportion of pregnancies relative to the number showing evidence of mating X 100; males were considered to have sired a litter if the paired female was gravid, regardless of delivery status. Females that did not show evidence of mating during cohabitation with the first male were paired with a second male; these outcome data were used only for calculating fertility indices.
[d] Live Birth Index: Number born live relative to the total number born X100.
Dunnett's Test, significantly different from control, * $p \leq 0.05$. No other significantly different effects.

Table 3. Reproductive and Offspring Parameters for F_1 Generation Rats Exposed to Styrene

	Parental Exposure Level (ppm)			
	0	50	150	500
Reproduction Parameters (mean ± SD)				
F_1 Males/Females Assigned (PND28)	25/25	25/25	25/25	25/25
Males/Females Died	0/1	0/0	0/0	0/0
Males/Females Euthanized[a]	0/3	0/1	0/4	0/3
Males/Females Completing Study	25/21	25/24	25/21	25/22
Estrous Cycle Length (days)	4.9 ± 1.1	4.9 ± 1.4	4.8 ± 1.4	4.5 ± 0.8
Male Mating Index (%)[b]	23/25 (92)	25/25 (100)	22/25 (88)	24/25 (96)
Female Mating Index (%)[b]	24/25 (96)	25/25 (100)	24/25 (96)	25/25 (100)
Pre-coital Interval (days)	4.7 ± 3.3	3.4 ± 1.7	4.6 ± 4.2	3.3 ± 3.5
Male Fertility Index (%)[c]	23/25 (92)	24/25 (96)	21/25 (84)	24/25 (96)
Female Fertility Index (%)[c]	23/25 (92)	24/25 (96)	23/25 (92)	25/25 (100)
Fertility Determined via 2nd Male	2	0	3	1
Females Delivering Litters	23	24	21	24
Gestation Length (days)	22.0 ± 0.6	22.0 ± 0.4	22.1 ± 0.2	21.9 ± 0.5
Implantation Sites/Litter	14.3 ± 2.6	14.6 ± 2.2	14.8 ± 2.3	14.3 ± 4.4
Number Born/litter	13.6 ± 2.5	14.1 ± 2.4	13.8 ± 2.2	13.4 ± 4.7
Live Litter Size	12.7 ± 3.6	13.7 ± 2.5	13.8 ± 2.2	13.1 ± 5.0
Live Birth Index (%)[d]	93.3 ± 21.3	97.1 ± 5.9	99.7 ± 1.4	95.0 ± 20.4
Females with Surviving Pups at Weaning (PND 21)	21	24	21	22
F_2 Offspring Parameters (mean ± SD)				
Sex Distribution at Birth (% Males)	47.1 ± 14.5	51.2 ± 12.2	47.9 ± 15.6	55.2 ± 13.8
Survival (%)				
Birth to PND 4 (Pre-culling)	92.7 ± 21.5	91.2 ± 16.0	96.9 ± 7.7	89.2 ± 28.0
PND 4-21 (Post-culling)	99.5 ± 2.2	96.9 ± 6.2	99.5 ± 2.2	97.7 ± 5.3
Body Weight Gain (g)				
Males PND 1-4 (pre-culling)	3.3 ± 0.9	3.0 ± 1.0	3.3 ± 0.7	2.9 ± 1.0
Females PND 1-4 (pre-culling)	3.0 ± 0.9	2.9 ± 0.8	3.1 ± 0.7	2.6 ± 0.7
PND 21 Body Weight (g)				
Males	42.6 ± 5.3	40.3 ± 5.2	38.2 ± 5.1*	38.0 ± 6.2*
Females	40.5 ± 4.7	39.1 ± 5.0	37.4 ± 4.8	35.4 ± 5.7*

[a] One female from the control group and two females from the 500-ppm group were euthanized during the lactation period due to total litter loss; all others were euthanized for pregnancy determination after no evidence of mating.

[b] Mating Index: Proportion of males/females showing evidence of mating relative to the number cohabited X 100; positive evidence of mating included vaginal sperm, copulatory plug and or/pregnancy.

[c] Fertility Index: Proportion of pregnancies relative to the number showing evidence of mating X 100; males were considered to have sired a litter if the paired female was gravid, regardless of delivery status. Females that did not show evidence of mating during cohabitation with the first male were paired with a second male; these outcome data were used only for calculating fertility indices.

[d] Live Birth Index: Number born live relative to the total number born X100.

Dunnett's Test: significantly different from control; *$p \leq 0.05$. No other significantly different effects.

Table 4. Spermatogenic endpoints of F_0 and F_1 males

Group (ppm):	0	50	150	500
F_0 males				
Sperm Number (millions/g tissue)				
Left Testis	79.1±17.0	ND	ND	78.6±11.2
Left Cauda Epididymis	739±148	ND	ND	727±157
Motile Sperm (%)	88.6±7.5	87.6±8.1	87.5±9.0	91.5±4.1
Progressive Motile Sperm (%)	75.6±9.9	72.4±11.3	74.8±12.1	78.4±5.7
Sperm Morphology (%)				
Normal	99.5	99.1	98.5	99.6
Normal head separated from flagellum	0.3	0.6	1.0	0.3
Head absent, normal flagellum	0.2	0.3	0.5	0.1
F_1 males				
Sperm Number (millions/g tissue)				
Left Testis	96.6±42.9	ND	ND	106.9±39.9
Left Cauda Epididymis	619±148	ND	ND	639±171
Motile Sperm (%)	84.3±12.7	80.6±19.9	76.7±25.8	86.4±8.7
Progressive Motile Sperm (%)	73.0±15.9	69.4±20.5	65.9±23.2	75.0±10.7
Sperm Morphology (%)				
Normal	99.3	98.3	98.0	98.7
Normal head separated from flagellum	0.4	1.1	1.4	0.6
Head absent, normal flagellum	0.2	0.6	0.6	0.6

ND = Not determined. No values were statistically significantly different from control.

Table 5. Organ Weights of F_2 Offspring Not Chosen for Neurotoxicity Evaluation[a]

Group (ppm):	0	50	150	500
Males				
Brain (g)	1.441	1.418	1.376	1.378
(g/100 g BW)	3.427	3.507	3.557	3.671
Spleen (g)	0.180	0.180	0.169	0.158
(g/100 g BW)	0.418	0.432	0.426	0.406
Testis, Right (g)	0.093	0.096	0.086	0.087
(g/100 g BW)	0.220	0.232	0.230	0.226
Testis, Left (g)	0.094	0.095	0.088	0.086
(g/100 g BW)	0.221	0.231	0.226	0.221
Thymus (g)	0.193	0.182	0.163	0.169
(g/100 g BW)	0.452	0.441	0.411	0.436
Pituitary (g)	0.0038	0.0037	0.0034	0.0025
(g/100 g BW)	0.009	0.009	0.009	0.007*
Females				
Brain (g)	1.393	1.365	1.360	1.331
(g/100 g BW)	3.461	3.568	3.660	3.863*
Spleen (g)	0.173	0.176	0.165	0.149
(g/100 g BW)	0.419	0.440	0.427	0.412
Uterus (g)	0.057	0.050*	0.050	0.047*
(g/100 g BW)	0.140	0.131	0.133	0.134
Thymus (g)	0.197	0.180	0.172	0.161*
(g/100 g BW)	0.480	0.459	0.452	0.451
Pituitary (g)	0.0041	0.0039	0.0033*	0.0031*
(g/100 g BW)	0.0109	0.010	0.009	0.009

[a]Terminated PND 21, litter as experimental unit. * Significantly different from control, $p<0.05$.

Figure 1. F0 and F1 gestation water consumption

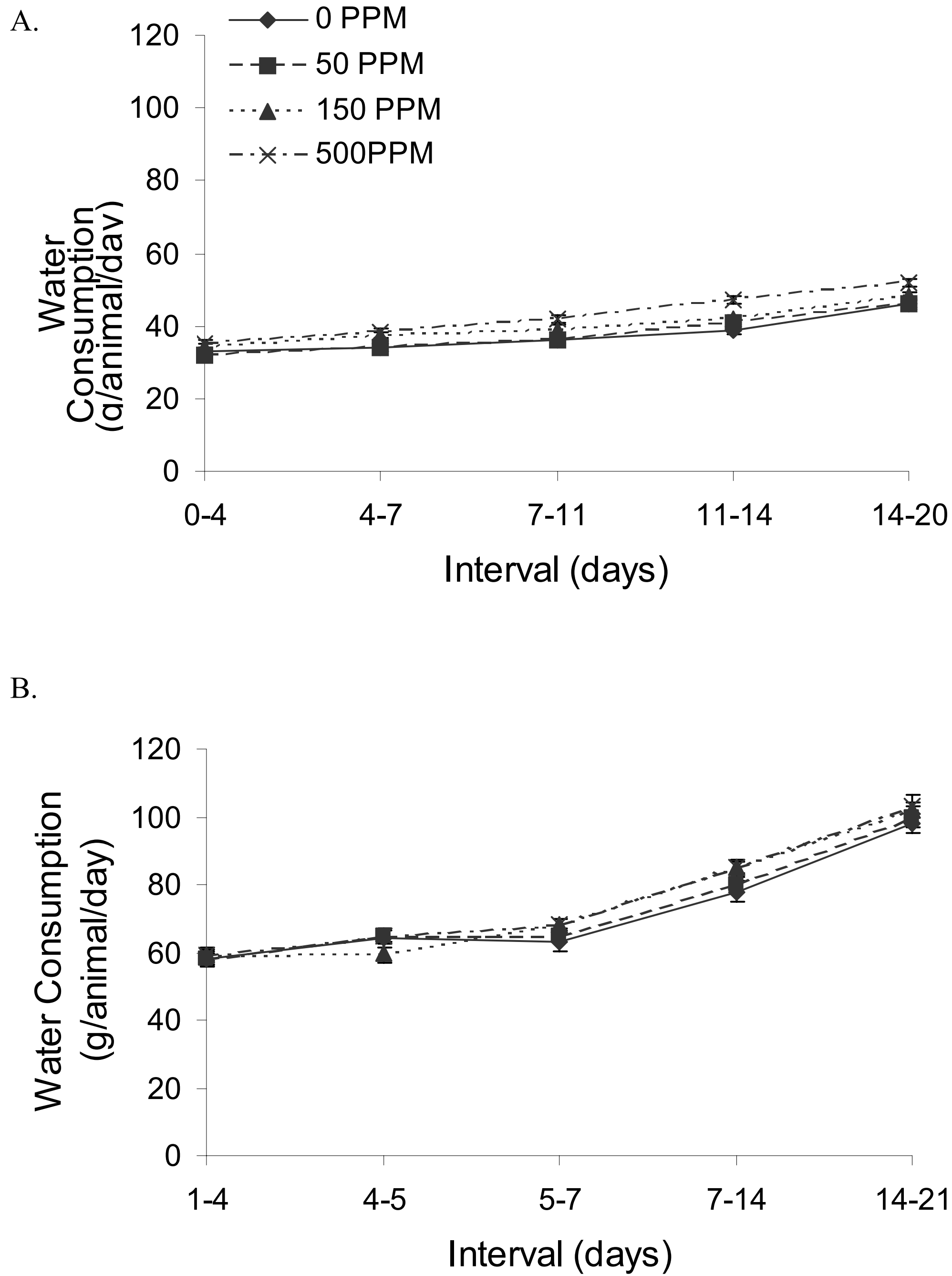
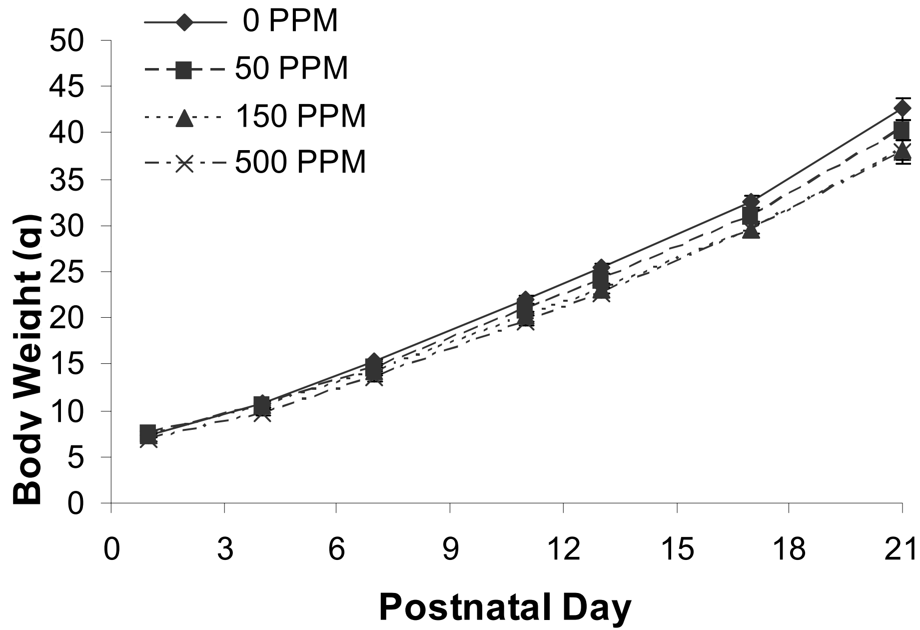


Figure 2. F2 Offspring Body weights
A.



B.