Supporting Information

Effects of a Chlorinated Drinking Water Concentrate in a Multigenerational Reproductive Toxicity Study in Rats

U.S. EPA's Four Lab Study

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Table of Contents

Background of EPA's Four Lab project.
The multiple objectives of the Integrated Disinfection By-products Mixtures Research Project (also known as the Four Lab Study) have been detailed elsewhere[1-3]. The overarching purpose of this integrated multi-disciplinary research is to address concerns related to potential health effects from exposure to DBPs that cannot be addressed directly from toxicological studies of individual DBPs or simple DBP mixtures. Factors motivating the investigation of complex mixtures of DBPs include: 1) exposure to DBPs are ubiquitous across the U.S. population, 2) a substantial amount of the material that makes up the total organic halide and total organic carbon portions of the DBPs has not been identified, and 3) epidemiologic data, though not conclusive, are suggestive of potential developmental, reproductive, or carcinogenic health effects in humans exposed to DBPs. The goal of the Four Lab Study is to provide sound, defensible, experimental data on environmentally relevant mixtures of DBPs and improve estimation of the potential health risks of exposure to the DBP mixtures formed during disinfection of drinking water. Due to the concerns raised by some epidemiological studies[4-9], a phased research plan was developed with a particular focus on reproductive and developmental endpoints.

In earlier phases of the Four Lab project, water was obtained from East Fork Lake, OH, and disinfected by either chlorination or ozonation/postchlorination[10] and then concentrated by reverse osmosis[11]. After lost volatiles were spiked back, these water concentrates were evaluated for toxicity in vitro[12, 13] and in vivo. For the in vivo evaluation, concentrates were provided as drinking water to Sprague-Dawley rats in a developmental toxicity screen[14]. These toxicity evaluations were conducted in concert with thorough chemical characterizations[15]. This series of papers also includes discussions of research issues[3] and risk assessment[16] of complex DBP mixtures.

In a more recent effort[17], using water from a different source, we implemented an alternate approach for preparing concentrate in order to reduce concentrations of sulfate and sodium to acceptable levels. Water concentrate was prepared by first concentrating the water by reverse osmosis, and then chlorinating. Before chlorination, however, barium hydroxide, rather than sodium hydroxide was used for pH adjustment, thus reducing the amount of sodium added to the water. In addition, the subsequent formation and precipitation of barium sulfate, which is extremely insoluble, facilitated the removal of sulfate[18]. This latter methodology was used to prepare the concentrate for the multigenerational study described here.

Supplemental methodological information
Body weights and water consumption. Body weights and water consumption were recorded at least twice per week throughout the experiment.

Parturition. Beginning on GD 20, dams were observed periodically to determine the time of parturition. The stage of parturition (completed, in progress, first pup delivered) was also recorded.

Necropsies. Full necropsies were conducted on P_0 females at 21 days postpartum (upon weaning of their litters), on F_1 A-males (i.e., breeder males; see Table S1) at PND 89-93, and on F_1 A-females at PND 95-103 (after PND-6 examinations of the F_2 litters). Animals were weighed and killed by decapitation; trunk blood was collected and sera prepared. Sera were frozen at -80ºC for hormone analysis. Cranial, thoracic, abdominal, and pelvic viscera were examined grossly. Organ weights were recorded for the brain, kidneys, spleen, ovaries, testes, thymus, liver, lung, adrenal glands, pituitary, uterus with oviducts and cervix, epididymides, prostate, and seminal vesicles with coagulating glands (and fluids). Uterine implantation sites were counted. Uteri from nonparous females were stained with 2% ammonium sulfide to detect cases of full-litter resorption[19]. Brains were frozen at -80ºC for measurement of hypothalamic catecholamines. Tissues from liver, lungs, kidneys, adrenals, thymus, spleen, stomach, duodenum, ileum, cecum, colon (proximal, middle, distal), mesenteric lymph nodes, trachea, esophagus, thyroid, pituitary, urinary bladder, prostate, seminal vesicle and coagulating gland, vagina, and ovaries, were fixed in buffered formalin. Due to technical error, thyroids were not collected from F_1 males.

For males, the left testis and epididymis were fixed in Bouin's fluid whereas the right testis was frozen at -30ºC for subsequent enumeration of testicular spermatids. The right caput epididymis was sampled for assessment of sperm motility and morphology while the cauda and remaining caput tissue was frozen at -30ºC for determination of sperm counts.

Histology. For P_0 females, F_1 A-males, and A-females, fixed tissues from 10 randomly selected rats per sex per group were embedded in paraffin blocks, sectioned, stained with hematoxylin and eosin, and examined microscopically. If results from this initial examination suggested a treatment effect, the specified tissue of the remaining rats was also processed and examined.

For F_1-A females, the lactational ovaries were examined quantitatively for primordial and primary follicles by examining 20 cross sections (5 microns thick) per ovary. Routine histopathological examination of the ovaries was conducted in conjunction with the enumeration of follicles.

Hormone measurements. Estradiol, progesterone, and testosterone were analyzed by antibody-coated tube radioimmunoassay using Coat-a-Count® kits (Diagnostic Products, Los Angeles, CA). Intra-assay coefficients of variation for quality control samples were 2.67, 1.91, and 4.70, respectively. Estradiol and progesterone samples were each quantified in single assays whereas testosterone required multiple assays. The inter-assay coefficient of variation for testosterone was 8.01.

Catecholamine measurements. Methods for preparation and measurement of hypothalamic tissue for catecholamine measurement have been previously described in detail[20]. Briefly, brains were carefully excised and frozen at -80ºC. While partially frozen, anterior and posterior hypothalamic areas, along with caudate nuclei, were dissected out. Norepinephrine, dopamine, and 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations were quantified by high-performance liquid chromatography and electrochemical detection.

Immunotoxicity testing. Six F_1 litters per group each provided two males and two females for evaluation of immune responses. After weaning, these rats were continuously exposed to their designated waters; because of limited availability of the customized water-delivery system, these rats were exposed via amber glass water bottles with Teflon®-lined caps. From each of these litters, one rat/sex/group was evaluated for antibody-mediated immunity whereas one rat/sex/group was evaluated for cell-mediated immunity. An additional six litters provided two females, one each for testing antibody- and cell-mediated immunity, were similarly evaluated except treatment was halted at weaning; these "recovery" animals were maintained on purified water. Methods for evaluating immune responses have been described previously[21]. Briefly, primary (IgM) and recall (IgG) antibody responses to a standard antigen (sheep erythrocytes) were evaluated. Primary immunizations occurred on PND 43 and sera were collected on PND 48 for ELISA measurement of IgM titers. Booster immunizations occurred on PND 57 and sera were collected on PND 63 for ELISA of IgG titers. Cellular immunity was evaluated by measuring the delayed-type hypersensitivity response; sensitization to bovine serum albumin (BSA) occurred on PND 43, challenge injection of BSA to a footpad (with saline control administered to the opposite footpad) was on PND 50, and footpad thickness was measured 24 hr later, on PND 51.

Neurobehavioral toxicity testing. Male and female F_1 littermates (10/sex/group) were evaluated for neurobehavioral alterations using motor activity and a battery of functional and observational measures [22]. For these animals, treatment was discontinued at weaning (PND 21). On PND 27 and 41, rats were tested in figure-eight motor activity chambers during 1-hr sessions. On PND 62, these rats were tested with the functional observational battery (FOB) followed by 1 hr in the activity chambers. At the younger ages, rats were tested in the chambers that only tabulate locomotor activity counts; however, on PND 62 they were tested in the chambers with attached rearing collars that measure the number of vertically directed activity counts as well as horizontal (locomotor) activity.

Table S1. Disposition of F_1 weanlings on PND 21. From each litter, one A-male and one A-female were selected to breed, and one B-male and one B-female were killed at puberty (males on PND 55, females on the day of vaginal opening). From 20 litters per group, one C-male was used for artificial insemination of untreated females and one D-male was killed on PND 55 for epididymal sperm count determination. From 6 litters per group, one rat per sex was evaluated for antibody response and one rat per sex was evaluated for delayed-type hypersensitivity (DTH); these rats were exposed postweaning using water bottles rather than the water-delivery system. From an additional 6 litters per group, one female was evaluated for antibody response and another was evaluated for DTH; these were "recovery" animals in that exposure was halted at weaning. From an additional 10 litters, one rat per sex was evaluated for neurobehavioral effects on motor activity and in a functional observational battery (FOB).

Fate	**Litters per group**	**Post-weaning exposure**	**Males**	**Females**
A	All	Yes	Breed (produce F_2)	Breed (produce F_2)
B	All	Yes	Pubertal necropsy, PND 55	Pubertal necropsy
C	20	Yes	Artificial insemination	
D	20	Yes	Pubertal necropsy, PND 55 Sperm counts	
Immunotoxicity				
Exposed	6	Via bottles	1/litter: antibody response 1/litter: DTH	1/litter: antibody response 1/litter: DTH
Recovery	6	No		1/litter: antibody response 1/litter: DTH
Neurotoxicity	10	No	Activity, FOB	Activity, FOB

Table S2. Summary of reproductive and developmental data for P_0 dams and their F_1 litters.

	Control	Treated
Number of females		
Pregnant	79	118
Delivered GD 21	48	71
GD 22	31	47
With live litters PND 0	79	118
PND 6	79	117
Mean ± S.E. per litter		
Implantation sites	14.1 ± 0.2	14.3 ± 0.2
Live pups PND 0	13.3 ± 0.2	13.3 ± 0.2
PND 6	13.1 ± 0.2	13.1 ± 0.2
Males (%)	51.5 ± 1.4	48.3 ± 1.3
Prenatal loss (%)	5.7 ± 0.9	6.7 ± 0.9
Loss GD 0-6 (%)	1.5 ± 0.6	2.1 ± 0.9
Perinatal loss (%)	7.1 ± 1.0	8.6 ± 1.2
Loss GD 6-21 (%)[a]	0.0 ± 0.0	0.7 ± 0.4
Pup weight (g) PND 0	6.5 ± 0.1	6.6 ± 0.1
PND 6	13.7 ± 0.2	13.8 ± 0.2
PND-0 Anogenital distance (mm)[a,b]		
Males	3.06 ± 0.04	3.76 ± 0.06*
Females	1.50 ± 0.03	1.51 ± 0.02

*Significantly different from control value ($p<0.05$).
[a]Block 1 only.
[b]n=12 litters per group. Anogenital distance (AGD) reflects the influence of androgens on development of the male phenotype; androgen agonists will increase female AGD and have no affect on males whereas androgen antagonists will decrease male AGD and have no affect on females. The significant increase in male AGD fits neither profile and is unlikely related to endocrine disruption.

Table S3. Summary of F_1 Day-13 litter examinations of eye opening and nipple retention

F_1 PND-13 Examination	Control	Treated
Number of litters	39	58
Mean ± S.E. per litter		
Both eyes fully closed (%)	71.5 ± 5.4	62.3 ± 4.7
Both eyes fully open (%)	8.0 ± 2.7	12.3 ± 2.6
Number of nipples		
Males	2.2 ± 0.2	1.8 ± 0.2
Females	12.1 ± 0.0	12.0 ± 0.0
Males with nipples (% affected)	30.1 ± 4.4	42.0 ± 4.1[a]
Supernumerary nipple - females (% affected)	4.7 ± 1.5	5.6 ± 1.5

[a]Marginal significant difference from control (p=0.0576).

Table S4. Summary of pubertal data for F_1 progeny.

F_1 Pubertal Data	Control	Treated
Number of litters	39	57[a]
Mean ± S.E. per litter		
Males		
Age at Puberty (preputial separation)		
Days since GD 22	45.8 ± 0.3	46.2 ± 0.2
Days since day of birth	46.3 ± 0.3	46.7 ± 0.3
Body weight (g) on day of puberty	267.8 ± 3.2	267.2 ± 2.5
Total Testosterone (ng/ml)		
Serum	3.28 ± 0.45	2.72 ± 0.28
Interstitial Fluid	174.7 ± 22.8	128.9 ± 15.0
Total Testost. (ng/mg Testis, Interstitial Fluid)	0.113 ± 0.014	0.085 ± 0.009
Females		
Age at Puberty (vaginal opening)		
Days since GD 22	33.5 ± 0.3	34.3 ± 0.3*
Days since day of birth	34.1 ± 0.3	34.9 ± 0.3*
Body weight (g) on day of puberty	128.2 ± 2.2	132.5 ± 2.1
Serum Estradiol (pg/ml)	30.0 ± 1.5	32.2 ± 1.1
Serum Progesterone (ng/ml)	13.1 ± 1.5	14.3 ± 1.3

*Significantly different from control value ($p<0.05$).

Note: Age at puberty is presented as a conception-based (days since GD 22) as well as a birth-based value.

[a]Does not include one litter euthanized at weaning due to dental malocclusion in all pups.

Table S5. Summary of reproductive and developmental data for F_1 breeding pairs and their F_2 litters maintained to PND 6.

	Control	Treated
Number of F_1 females		
Bred	39	57
Mated days 1-4	36	56
day 5	1	0
days 6-10	1	0
Did not mate	1	1
Pregnant	37	55
Delivered GD 21	21	31
GD 22	16	24
With live litters PND 0	37	55
PND 6	37	54
Mean ± S.E. per litter		
Implantation sites	17.4 ± 0.4	17.0 ± 0.3
Live pups PND 0	16.2 ± 0.4	16.0 ± 0.3
PND 6	15.7 ± 0.3	15.2 ± 0.4
Males (%)	49.3 ± 2.5	51.4 ± 1.8
Prenatal loss (%)	6.6 ± 1.0	5.9 ± 1.0
Postnatal loss (%)	2.8 ± 0.8	4.6 ± 1.9
Perinatal loss (%)	9.2 ± 1.2	10.3 ± 2.0
Pup weight (g) PND 0	6.1 ± 0.1	6.3 ± 0.1*
PND 6	12.5 ± 0.3	12.7 ± 0.2

*Significantly different from control value ($p<0.05$).

Table S6. Brain catecholamines. Dopamine, norepinephrine, and dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were measured in the hypothalamus and the caudate nucleus in F_1 females. Values (pg/µg protein) represent the mean ± S.E. of 10 animals per group. No significant differences between groups were observed.

	Control	Treated
Dopamine		
Anterior hypothalamus	7.27 ± 1.23	7.53 ± 1.16
Posterior hypothalamus	2.12 ± 0.17	2.20 ± 0.11
Caudate nucleus	65.41 ± 5.47	66.59 ± 2.91
Norepinehrine		
Anterior hypothalamus	16.37 ± 0.91	16.35 ± 1.09
Posterior hypothalamus	13.09 ± 0.68	12.87 ± 0.76
Caudate nucleus	1.53 ± 0.77	1.36 ± 0.02
DOPAC		
Anterior hypothalamus	7.22 ± 1.12	4.89 ± 0.89
Posterior hypothalamus	0.22 ± 0.16	0.14 ± 0.08
Caudate nucleus	38.11 ± 3.66	32.28 ± 4.10

Table S7. Mean ± S.E. organ weights of F_1 adults at necropsy.

	Control	Treated
Males		
Terminal body weight (g)	536.4 ± 8.7	536.9 ± 6.1
Brain (g)	2.05 ± 0.02	2.05 ± 0.01
Pituitary (mg)	13.31 ± 0.26	13.71 ± 0.21
Thymus (g)	0.53 ± 0.03	0.53 ± 0.02
Lung (g)	2.07 ± 0.08	2.07 ± 0.06
Kidneys (g)	3.96 ± 0.06	4.20 ± 0.05*
Liver (g)	22.69 ± 0.60	21.18 ± 0.34*
Spleen (g)	0.97 ± 0.03	0.95 ± 0.02
Adrenals (mg)	60.0 ± 2.9	61.2 ± 2.1
Relative weights (per 100 g body weight)[a]		
Brain (g)	0.385 ± 0.006	0.384 ± 0.004
Pituitary (mg)	2.48 ± 0.04	2.56 ± 0.04
Thymus (g)	0.099 ± 0.005	0.098 ± 0.004
Lung (g)	0.386 ± 0.013	0.386 ± 0.011
Kidneys (g)	0.735 ± 0.010	0.785 ± 0.008***
Liver (g)	4.21 ± 0.06	3.94 ± 0.04***
Spleen (g)	0.180 ± 0.005	0.177 ± 0.003
Adrenals (mg)	11.1 ± 0.5	11.4 ± 0.4
Females		
Terminal body weight (g)	359.6 ± 5.1	363.0 ± 4.1
Brain (g)	1.87 0.01	1.89 ± 0.01
Pituitary (mg)	17.3 ± 0.4	15.9 ± 0.4*
Thymus (g)	0.42 ± 0.01	0.43 ± 0.01
Lung (g)	1.44 ± 0.02	1.56 ± 0.04*
Kidneys (g)	2.38 ± 0.04	2.63 ± 0.03***
Liver (g)	16.15 ± 0.28	16.55 ± 0.23
Spleen (g)	0.80 ± 0.03	0.80 ± 0.02
Adrenals (mg)	95.2 ± 4.9	104.4 ± 7.2
Relative weights (per 100 g body weight) [a]		
Brain (g)	0.524 ± 0.007	0.523 ± 0.006
Pituitary (mg)	4.83 ± 0.11	4.39 ± 0.11**
Thymus (g)	0.116 ± 0.004	0.120 ± 0.004
Lung (g)	0.402 ± 0.006	0.429 ± 0.009*
Kidneys (g)	0.663 ± 0.007	0.726 ± 0.009***
Liver (g)	4.50 ± 0.06	4.56 ± 0.05
Spleen (g)	0.225 ± 0.007	0.222 ± 0.005
Adrenals (mg)	26.7 ± 1.4	28.9 ± 2.2

[a]Inferential statistical analysis of relative organ weights used absolute weights with body weight as a covariate.

*Significantly different from control (p<0.05).

***Significantly different from control (p<0.001).

Table S8. Mean ± S.E. sperm-count data for F_1 pubertal and adult males.

F_1 Males – Epididymal Sperm Counts	Control	Treated
Pubertal males (PND 55)		
No. Examined	20	20
Counts		
Caput epididymis	39.1 ± 1.1	39.5 ± 1.4
Cauda epididymis	16.9 ± 1.3	15.9 ± 1.2
Epididymis (caput + cauda)	56.0 ± 2.1	55.4 ± 2.3
Epididymis count per gram tissue	201.4 ± 6.8	200.6 ± 7.7
Adult males (PND 89-93)		
No. Examined	38	57
Caput epididymis		
Count	88.0 ± 1.8	81.8 ± 2.0*
Count/g tissue	300.4 ± 5.8	276.5 ± 7.3*
Cauda epididymis		
Count	205.4 ± 4.5	205.1 ± 5.9
Count/g tissue	629.9 ± 11.3	614.3 ± 15.9
Epididymis (caput + cauda)		
Count	293.4 ± 5.5	286.9 ± 7.1
Count/g tissue	473.8 ± 7.9	454.9 ± 11.0

*Significantly different from control value ($p<0.05$).

Table S9. Immunotoxicity data. Results are presented as mean ± S.E. for six F_1 rats per group. Treatment was discontinued at weaning for "recovery" females. For the remaining animals, treatment was continued after weaning with water bottles. No differences between groups were observed.

	Control	Treated
Serum IgM Titer (Log2)		
Males	5.9 ± 0.7	6.0 ± 0.7
Females	6.4 ± 0.2	5.7 ± 0.5
Females – recovery	6.3 ± 0.8	7.0 ± 0.3
Serum IgG Titer (Log2)		
Males	11.9 ± 0.3	14.9 ± 1.4
Females	14.1 ± 1.1	11.8 ± 0.8
Females – recovery	12.4 ± 0.6	13.0 ± 0.5
Delayed-type hypersensitivity (footpad-thickness change, mm)		
Males	0.771 ± 0.125	0.657 ± 0.156
Females	0.740 ± 0.240	0.652 ± 0.222
Females – recovery	1.035 ± 0.033	0.826 ± 0.402

Table S10. Motor activity data. Results are presented as mean ± S.E. for 10 F_1 rats per group. Treatment was discontinued at weaning. There was a trend towards increased activity at PND 27 (p=0.0747).

	Control	Treated
Horizontal Counts		
Males – PND 27[a]	261.1 ± 19.4	287.7 ± 20.6
PND 41	316.8 ± 24.3	332.2 ± 20.4
PND 62	296.6 ± 19.7	331.6 ± 12.1
Females – PND 27[a]	243.0 ± 20.5	300.3 ± 12.5
PND 41	298.3 ± 21.6	318.2 ± 15.9
PND 62	326.0 ± 28.8	359.0 ± 18.5
Vertical Counts (PND 62)		
Males	82.1 ± 10.3	83.9 ± 11.3
Females	66.9 ± 9.1	67.4 ± 8.4

[a]There were no significant interactions of sex and dose; follow-up analyses with sexes combined suggested increased activity on PND27 but this did not reach statistical significance (p=0.0747).

Table S11. Functional observational battery data. Results are presented as mean ± S.E. (for continuous data) or as median (range; -- indicates no range of scores) for ordinal-scored data for 10 F_1 rats per group. Tested at PND 62, treatment was discontinued at weaning. The following endpoints had identical responses (score=1) for all animals tested and are not listed in the table: righting reflex, proprioceptive response, toe-pinch response, pinna response, palpebral response, tremors, convulsions, pupil response, lacrimation, and salivation.

	Control	Treated
Neuromuscular Domain		
Gait score – Males	1 (1-2)	1 (1-2)
Females	1 --	1 (1-2)
Forelimb grip strength[a] – Males	1.386 ± 0.054	1.278 ± 0.067
Females	0.977 ± 0.057	1.063 ± 0.050
Hindlimb grip strength[b] – Males	1.289 ± 0.061	1.114 ± 0.068
Females	0.857 ± 0.051	0.795 ± 0.031
Landing foot splay – Males	111.2 ± 5.3	99.8 ± 6.6
Females	84.7 ± 5.9	82.3 ± 7.6
Sensorimotor Domain		
Click response[c] – Males	3 (2-4)	3 (2-3)
Females	3 (2-3)	3.5 (2-4)
Tail-pinch response – Males	3 (2-4)	3.5 (3-4)
Females	4 (3-4)	4 (3-4)
Activity Domain		
Open-field rearing – Males	10.5 ± 1.3	10.7 ± 0.9
Females	14.2 ± 1.7	15.0 ± 1.8
Open-field activity – Males	4 (3-5)	4 (4-5)
Females	4 (4-5)	4.5 (4-5)
Excitability Domain		
Handling reactivity – Males	3 (2-3)	3 (2-3)
Females	2.5 (2-3)	2 (2-3)
Arousal – Males	4 --	4 --
Females	4 --	4 --
General Health		
Body temperature – Males	38.0 ± 0.1	37.8 ± 0.1
Females	38.1 ± 0.2	38.4 ± 0.2
Body weight – Males	386.5 ± 6.3	391.3 ± 7.5
Females	243.2 ± 7.3	241.5 ± 7.6
Clinical signs – Males	1 altered gait	1 altered gait
Females	1 miosis	1 miosis, 1 altered gait

[a]Forelimb grip strength: significant dose-by-sex interaction (p=0.0090) but analyses of each sex separately did not show any treatment effect.
[b]Hindlimb grip strength: trend towards lower hindlimb grip strength (overall dose p=0.0833), no interaction with sex); combined data indicated 11% decrease in average strength.
[c]Click response: significant dose-by-sex interaction (p=0.0026), but analyses of each sex separately did not reach significance (male p=0.0565; female p=0.0642).

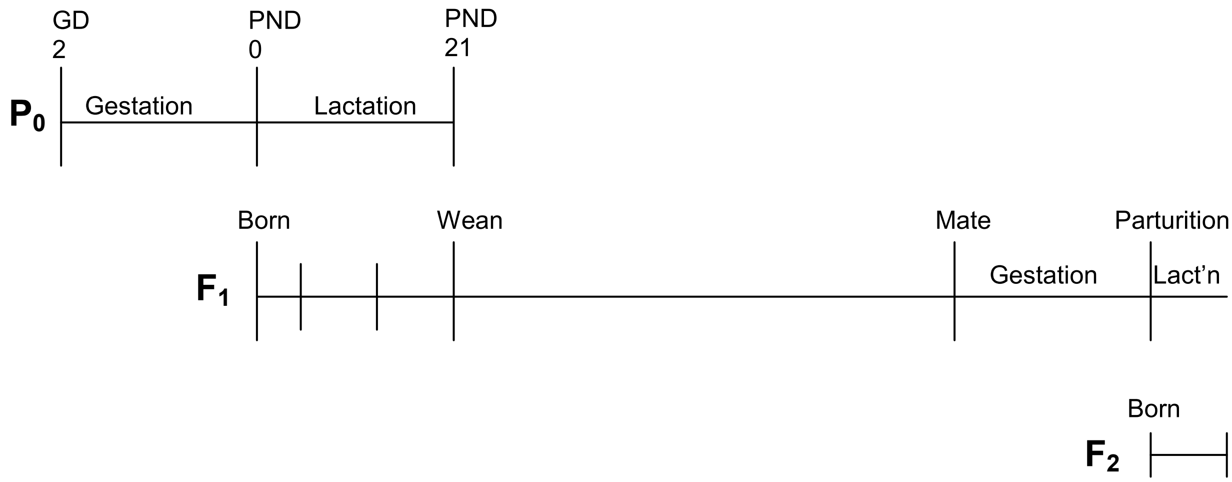


Figure S1. General time-line of the multigenerational bioassay. Parental females (P_0 generation) were obtained timed-pregnant on gestation day (GD) 0. Treatment began on GD 2 and continued for the remainder of the experiment. The P_0 dams delivered their litters (F_1 generation) which were examined at postnatal day (PND) 0 (birth), 6, 13, and 21. Litters were weaned at PND 21 and select F_1 offspring were maintained to adulthood and bred to produce the F_2 generation. F_2 litters were examined at PND 0 and 6.

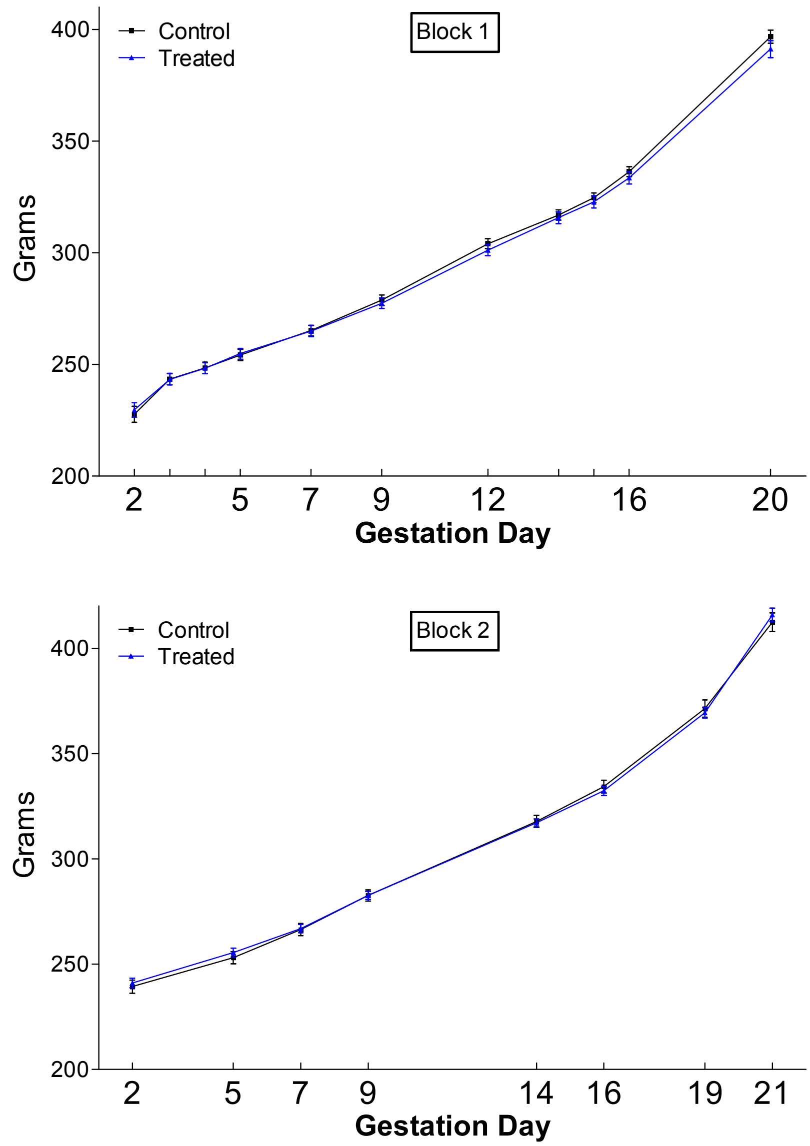


Figure S2. Mean body weights of P_0 dams (Block 1, top; Block 2, bottom) during gestation. Error bars represent standard error.

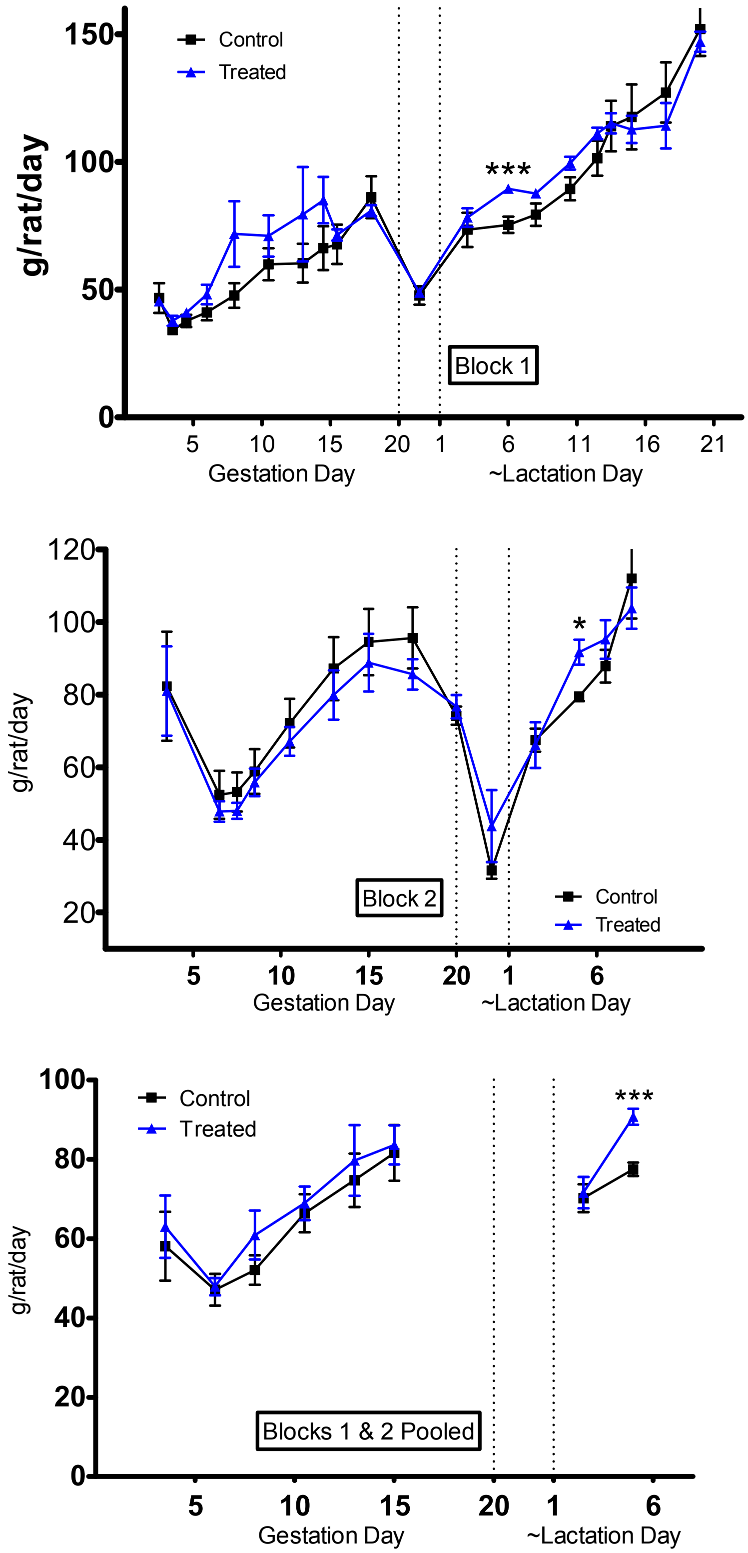


Figure S3. Mean water consumption (g/rat/day) for P_0 females. Data points represent consumption for an interval; data are plotted at the interval's midpoint. Error bars represent standard error. Top: Block 1, Middle panel: Block 2, Bottom: Blocks 1 and 2 pooled. *p<0.05, ***p<0.001.

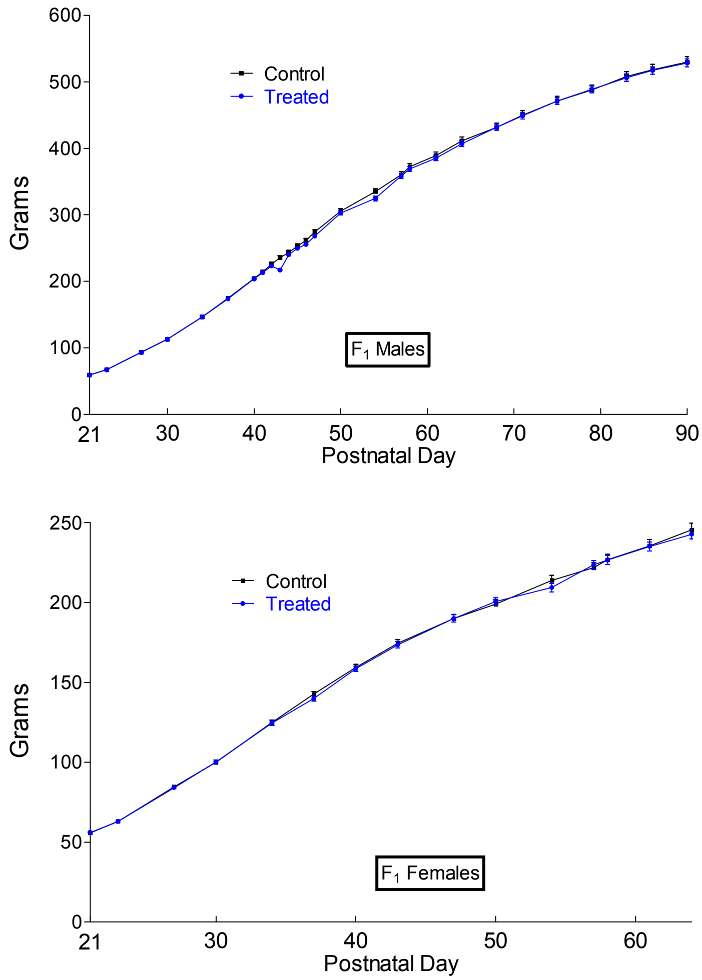
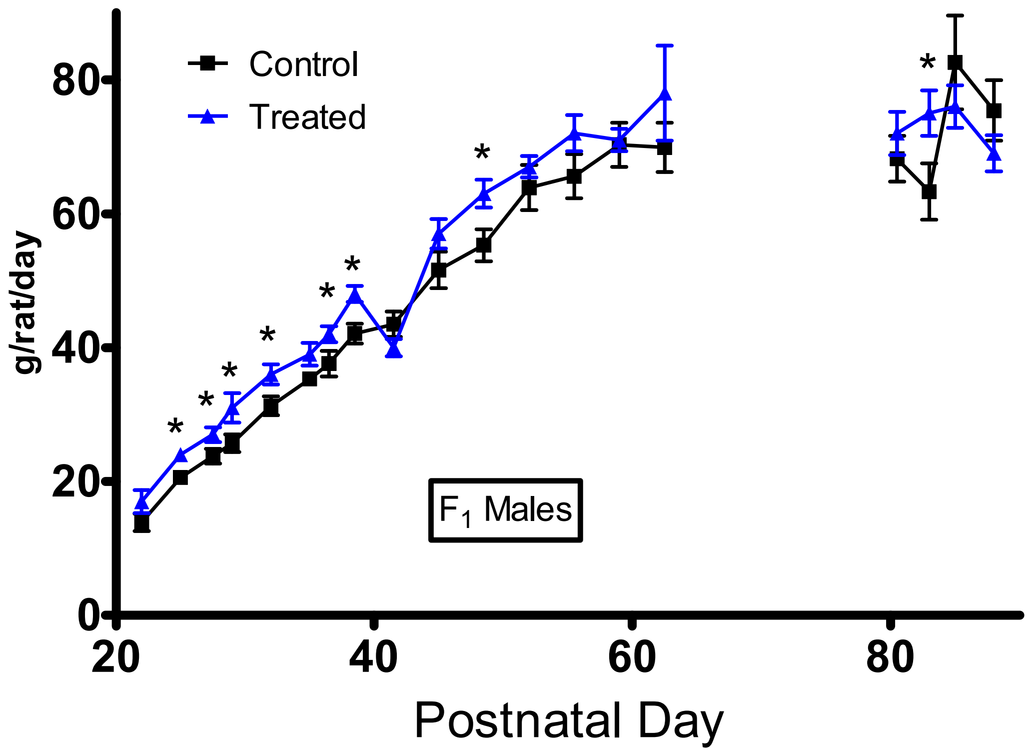


Figure S4. Mean body weights of F_1 A-males (top) and A-females (bottom). Error bars represent standard error.



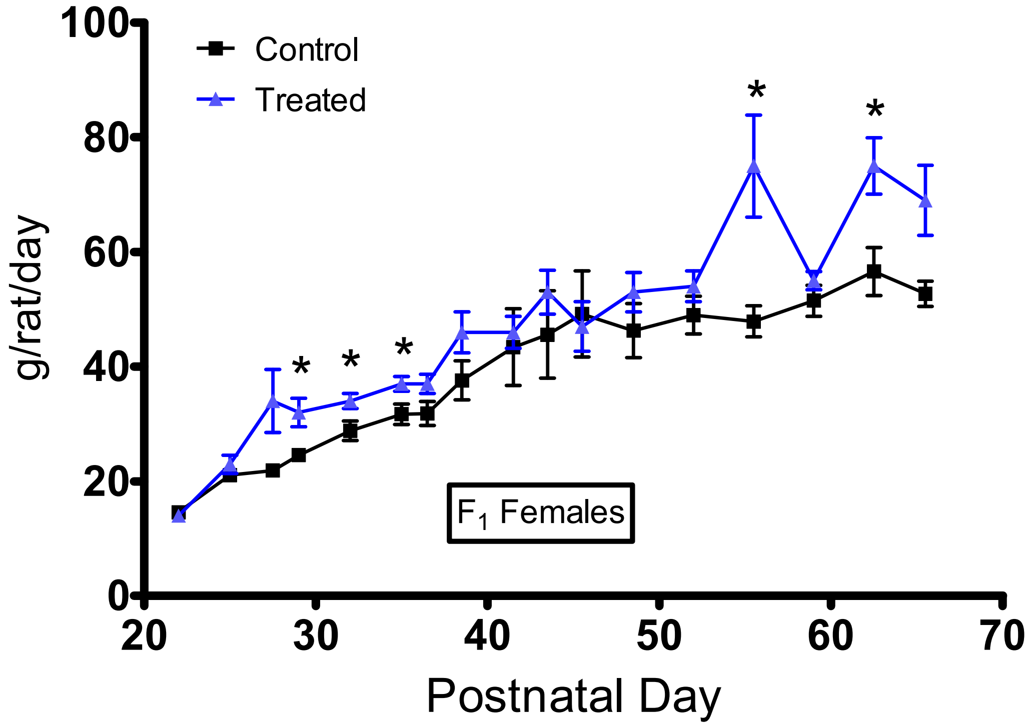


Figure S5. Mean water consumption (g/rat/day) of F_1 males (top) and females (bottom). Data points represent consumption for an interval; data are plotted at the interval's midpoint. Error bars represent standard error. *$p<0.05$.

Figure S6. Fertility assessment of F_1 males by in utero insemination of untreated females. A nonsignificantly higher percentage of treated males were infertile (left panel) and females inseminated by treated males had non-significantly reduced live embryos per corpus luteum (ovulation) compared to control males (right panel). Note: these data exclude one day's inseminations (5 males per group) that were removed from analysis due to quality control issues.

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