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The Effects of 4-Nonylphenol in Rats: A Multigeneration Reproduction Study

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The alkylphenol breakdown products of alkylphenol ethoxylates have been shown in *in vitro* studies to be weakly estrogenic, but few *in vivo* data address this issue in mammals. Because estrogens have been found to be most potent during developmental/perinatal exposures, this study maximized developmental exposure to nonylphenol (NP) by treating 3.5 generations of Sprague-Dawley rats to NP in diet at 200, 650, and 2000 ppm to determine the range and severity of any toxicity. Dose rate was higher for younger rats; calculated dose ranges were 9–35, 30–100, and 100–350 mg/kg/d for the low (200NP), middle (650NP), and high (2000NP) dose groups, respectively. There were adult (F_0, F_1, F_2) and postnatal day (pnd) 21 (F_1, F_2, F_3) necropsies; the oldest F_3 rats were killed on pnd 55–58. Body weight gain was reduced by 8–10% in the 650NP and 2000NP groups. Vaginal opening was accelerated by ≈ 2 days (650NP) and ≈ 6 days (2000NP) in F_1, F_2, and F_3 generations. Uterine weights at pnd 21 were increased in 650NP (14%) and 2000NP (50%) F_1 females, but not in other generations. Testis descent, anogenital distance, and preputial separation were not consistently changed. No consistent changes were seen in pup number, weight or viability, litter indices, or other functional reproductive measures. Relative ovary weight in F_2 adults was decreased at 650NP and 2000NP by 12%; relative ovary was unchanged in other generations. Follicle counts were unchanged in F_2 adults. Sperm indices, including CASA measures, were unchanged in F_0 and F_1 males. In F_2 rats, epididymal sperm density was reduced by 8% and 13% at 650NP and 2000NP, respectively. Testicular spermatid count was reduced by 13% in 2000NP F_2 males; testis and epididymis weights were unchanged. Erosion of gastric and duodenal mucosa was monitored grossly and microscopically, and never found. Kidney weights were increased in 650NP and 2000NP males, and renal medullary tubular dilatation and cyst formation were noted in all generations of males, and often at the lowest dose tested. These data show that NP had limited effects on the reproductive system in the presence of measurable nephrotoxicity. The F_2 sperm effects are either statistical/biological "noise," or imply heretofore unknown pharmacokinetics or toxicodynamics. These sperm data should be interpreted cautiously until the findings are repeated.

Key words: **alkyphenols; gestational exposure; multigeneration; nonylphenol ethoxylate; reproduction study.**

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Alkylphenol ethoxylates are widely used as surfactants; >360,000 tons were produced in 1988 (Ahel *et al.* in Nimrod and Benson, 1996). Of these, nonylphenol ethoxylate (NPE) comprises ≈ 80% of production (Naylor, 1995). NPE, like other alkylphenols, enters the hydrologic cycle after use; during anaerobic wastewater treatment, the polyethoxylate side chain is successively cleaved back, leaving nonylphenol (NP) (rev. in Nimrod and Benson, 1996). During the more common aerobic process of treating wastewater, both NPE and NP are degraded to biologically inactive molecules. Because of its hydrophobic nature, in anaerobic conditions NP adsorbs to sewage sludg. When present in rivers, NP tends to concentrate in bottom sediments, where it may be found at levels up to 3 μg/g sediment.

NP was found to stimulate the growth of estrogen-dependent MCF-7 cells *in vitro* (Soto *et al*., 1991), and to stimulate vitellogenin production in cultured trout hepatocytes (White *et al*., 1994), a process that is normally estrogen dependent (Pelissero *et al*., 1993). NP was positive in the uterotrophic bioassay in mice (Shelby *et al*., 1996), and was found to bind to and stimulate ligand-dependent gene transcription from transfected estrogen receptors in both yeast and mammalian cells (Shelby *et al*., 1996; White *et al*., 1994), although the potency appears to be 1000–1,000,000 times less than 17β estradiol.

Estrogenic compounds appear to produce their greatest mammalian effects when administered gestationally (e.g., Gray and Kelce, 1996). In addition, it has been proposed (Sharpe and Skakkebaek, 1993) that developmental exposure to estrogenic chemicals in the environment may be responsible for the proposed reduction in human sperm counts (Carlsen *et al*., 1992) and in the better-documented increase in malformations of the reproductive system (rev. in Toppari *et al*., 1996).

Although numerous *in vitro* and piscine studies demonstrated the estrogenic activity of NP, there were few data on long-term exposure in mammals. This study was intended to fill this gap. To provide the most opportunities to observe adverse sequelae, this study maximized gestational exposure to NP: three generations of pups were produced, and measures of reproductive system development were closely monitored, in addition to evaluating adult reproductive performance and necropsy endpoints.

MATERIALS AND METHODS

Animals and housing. Crl-CD® rats were purchased from the Portage, MI facility of Charles River Labs. Thirty each males and females were obtained for the pilot study, and 132 each were obtained for the main study; quarantine was ≈ 8 days, and age at study start was ≈ 11 wks. Animals were identified by tail tattoo, housed in polycarbonate cages with BetaChip hardwood animal bedding, and allowed *ad libitum* access to feed and filtered tap water. Animals were individually housed unless they were cohabiting or delivering or nursing a litter. Light cycle was 12:12; temperature was 68–74°F, relative humidity was 30–70%. Water quality was monitored prior to study start to ascertain that contaminants were below levels set forth in the Safe Drinking Water Act. Powdered NIH-07 feed was obtained from Ziegler Bros (Gardners, PA) and monitored for contaminants by the vendor. All animal procedures met standards set for by the National Academy's *Guide for the Care and Use of Laboratory Animals* and AAALAC, and approved by the appropriate IACUC.

Chemical. Branched 4-nonylphenol (CAS# 84852–15–3) was provided by Schnectady International. Identity as 4-NP was confirmed using high-performance liquid chromatography (HPLC); the chromatogram contained 29 peaks, each accounting for 0.12–19.5% of the total integrated area. Stability studies showed that the chemical and formulations were stable for up to 7 days under animal room conditions, and for 3 weeks at room temperature in the dark. Thus, bulk mixes were performed every 1–2 weeks, and feed holders were replaced every week or as necessary.

NP concentrations in feed were 75, 250, 800, and 3000 ppm for the dose range–finding (DRF) study, and 200, 650, and 2000 ppm for the main study. NP was mixed with NIH-07 feed by weighing the appropriate amount of NP, adding to a small amount of powdered feed in a beaker, mixing thoroughly, and then adding this premix to a Patterson-Kelly blender, and blending for 10 min with, 30 min without, and 10 min with the intensifier bar. Archival samples were retained from each mix; samples from the first, second, seventh, and twelfth mixes were analyzed for NP concentrations by HLPC. Formulations were 91–113% of target concentration.

Study design. DRF study animals (6 each males and females per dose level) were given NP in powdered feed at 0, 75, 250, 800, or 3000 ppm for 4 weeks, with no mating or cohabitation. Feed and water consumption were measured twice weekly. Clinical signs were observed twice daily, and body weights were obtained at study start, weekly during exposure, and at termination after 4 weeks of exposure. At termination, animals were euthanized with CO_2, and a gross necropsy performed. The stomach and intestines were opened and examined carefully for evidence of erosion; no evidence of erosion was found macroscopically or microscopically. Weights were collected on liver, spleen, right kidney, testis, and epididymis. Based on data generated from the DRF study, concentrations for the main study were set at 0, 200, 650, and 2000 ppm NP.

Main study. There were 30 pairs/dose level. Exposure was continuous following the first F_0 dose until the necropsy of the F_3 animals. Mated females produced a single litter in each generation. Because of the focus on effects after gestational exposure, adult F_0 exposure premating was relatively short: adult F_0 pairs consumed NP for 6 weeks, and females were subject to vaginal lavage for 3 weeks., prior to the 2-week cohabitation. Mating was confirmed by vaginal lavage; females were removed from the males cage after 2 weeks or a smear revealed vaginal sperm, whichever came first. The females delivered; pups were counted, weighed, sexed, and anogenital distance measured on pnd 1 and 21. Pups were weighed and counted also on pnd 4, 7, and 14. F_0 male exposure continued for 15 weeks prior to termination.

At pnd 21, one male and female pup were randomly selected from each of 20 litters per dose level, killed by CO_2 asphyxiation, and necropsied. Weight was collected on liver, kidneys, spleen, ovaries, vagina/cervix/uterus, right whole and cauda epididymis, and right testis. Reproductive organs were fixed in Bouin's; gross lesions were preserved in 10% neutral buffered formalin (NBF).

One rat/sex/litter was selected to form the F1 breeding pairs, and the remaining nonselected, animals were sacrificed by CO_2 asphyxiation and discarded. On the selected animals, vaginal opening was assessed daily; testis descent and preputial separation was assessed daily on the males after pnd 16 and 35, respectively. Vaginal opening assessments began pnd 30 for F_1 rats, and pnd 21 for F_2 and F_3 rats.

The F_0 adults were killed by CO_2 asphyxiation (pnd 154 $\pm$ 7) after the F_1 pups were weaned at pnd 21; necropsy was performed on 20 randomly chosen F_0 adult rats/sex/group. After terminal body weight and asphyxiation, sperm motility was determined in phosphate-buffered saline/bovine serum albumin using a CellSoft system (Cryo Resources, Ltd., NY). Briefly, the right vas deferens was placed into 37°C media, cut into three pieces, and incubated for $<$ 5 min until cloudy. A sample was loaded into a MicroCell® slide (Conception Technologies, San Diego, CA), checked for proper dilution ($<$ 40 cells per visual field), and then $\geq$ 15 fields were videotaped for 3–8 sec each. At analysis, a lab reference videotape was first evaluated to assure proper calibration, and then $\geq$ 200 sperm/rat were analyzed for motility. The mean values from each rat were used to generate a mean group value for each end point. Cauda epididymal sperm density was determined using a hemacytometer, and two slides were prepared for each animal for assessment of sperm morphology. Weights were collected on body, liver, kidneys, spleen, ovaries, vagina/cervix/uterus, right whole and cauda epididymis, right testis, ventral prostate, and seminal vesicles (with coagulating glands). Liver, kidney, spleen, stomach, jejunum, duodenum, ileum, ventral prostate, seminal vesicles, female reproductive tract, and gross lesions were preserved in NBF. In F_0 males, only ventral prostate was weighed, whereas dorsolateral and ventral prostate lobes were dissected and weighed in the necropsies for the F_1 and F_2 adults. Stomach and duodenum were processed through paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and evaluated for NP-induced erosion. Because of increased kidney weights, kidneys from the first 10 F_0 animals/sex/group were processed through paraffin as above and evaluated. Right testis was frozen for subsequent determination of testicular spermatid head counts (TSHC); frozen testes were approximately bisected, divested of tunica and easily removed blood vessels, weighed, and homogenized in 10% dimethyl sulfoxide in saline using a VirTis Cyclone® homogenizer. Resistant spermatid heads were counted with a hemacytometer. The data are expressed as both spermatid heads per total testis, and heads per milligram testis.

Left testis and epididymis were fixed in 4% paraformaldehyde/3% glutaraldehyde, embedded in glycol methacrylate (GMA), sectioned at 3–5 microns, and stained with PAS-H. Motility videotapes (CASA endpoints and percent motility), morphology slides, and TSHC were analyzed for controls and high-dose males first, and if a change was seen, then the low- and middle-dose groups were also evaluated. Epididymal sperm density was determined in all groups.

The F_1 females were subject to daily vaginal lavage and estrous cyclicity evaluation for 3 weeks prior to mating at pnd 86 ($\pm$ 10) (Cooper *et al*., 1993).

The same sequence of steps and measurements described above was pursued for the mating of the F_1 adults, delivery and the evaluation of the F_2 neonates, selection of F_2 rats for pnd 21 necropsy and animals to be reared to mating, necropsy of F_1 adults, evaluation of F_2 reproductive development, cohabitation of F_2 adults, evaluation of F_3 neonates, selection of F_3 pups for pnd 21 necropsy, and monitoring the reproductive development of F_3 animals. Necropsy for F_1 and F_2 adults occurred at pnd 147 $\pm$ 10. Due to logistical

constraints, the F_3 animals were not reared to adulthood, but were killed and necropsied at pnd 55–58.

Ovaries were fixed in Bouin's for 48 h, then routinely processed through paraffin and evaluated for the F_0 and F_1 females. For 10 control and high-dose F_2 females/group, both ovaries were step-sectioned every 100 μm, for a total of 12 sections per ovary, and stained with hematoxylin and eosin (Bolon *et al*., 1997). Follicle counts were made in these 10 F_2 rats/group. This generation was chosen based on changes in ovary weights (below).

Statistics. Most hypotheses were tested using the nonparameteric multiple comparisons procedure of Dunn (1964) or Shirley (1977) as modified by Williams (1986). Jonckheere's test (1954) was used to identify those comparisons necessitating Shirley's test. For comparisons with only two groups (sperm data using only control and high-dose groups, for example), Wilcoxon's test was used.

For proportionate data, the Cochran-Armitage was used (Armitage, 1971).

Because litter size can affect individual pup weight, litter size was used as a covariate in the pup weight analyses, using parameteric covariance procedures developed by Neter *et al.* (1985).

The relative time spent in estrous cycle stages was analyzed using multivariate analysis of variance to test for the simultaneous equality of measurements across doses (Morrison, 1976). Estrous cycle data were arcsine transformed prior to analysis, to bring the data into closer conformance with normality assumptions.

Feed consumption was evaluated both as per animal per day, and per kilogram body weight per day. Organ weights were analyzed as both absolute and relative to body weight.

For convenience and efficiency, we will refer to animals that consumed 200 ppm NP as 200NP, those that consumed 650 ppm NP will be referred to as 650NP, and the high-dose group will be 2000NP.

RESULTS

Because of the wealth of data generated by this design, this report will focus primarily on those changes that were statistically significant. Data appearing in the text and tables with an asterisk are significantly different from controls. Negative data may be mentioned in the text to provide context. Because not all data will be presented here, the reader is referred to our Web home page (http://www.niehs.nih.gov/dirtb/dirrtg), where the text tables may be found and examined. The Web site contains all the nonappendix tables from the report. In addition, the entire report of the study has been filed with the National Technical Information Service and is available for a nominal fee.[2]

Dose Range–Finding Study

Males and females at 3000NP gained 38% and 61% less weight than controls, respectively. Feed consumption was unchanged for males, but was decreased for 3000NP females by up to 60%. Terminal body weight differences were not significant by the end of the 28-day study; no changes in organ weight or gross appearance were noted at necropsy. It was estimated from these data that 2000 ppm NP would be sufficient to slightly reduce body weight gain over the course of a generation; concentrations for the main study were set at 200, 650, and 2000 ppm in feed.

Main Study, F_0

Table 1 provides an overview of the effects seen, a summary of the end points measured, and an indication of the direction of all statistically significant changes that were observed. Specific data for each of these changes are presented in the succeeding tables and text below.

Adult F_0 feed consumption was slightly reduced initially, but recovered after the first 1–2 weeks. Estimated consumed doses are shown in Table 2. The dose ranges account for two differences: females consumed $\approx$ 25% more than males on a body weight basis, and the doses decreased over time as the animals grew. Weanling animals consumed nearly three times more NP than adult animals on a body weight basis in all generations. In this and subsequent generations, there were no NP-related clinical signs or symptoms.

Indices of mating behavior or libido were unchanged in the F_0 rats. Litter size was reduced by *9% at 2000NP (Table 1); proportion liveborn, absolute or adjusted pup weight, and gestation length were unchanged at all doses.

At the necropsy of 20 representative adult F_0 males and females per dose level, male body and organ weights were unchanged and grossly normal except for an increase in relative kidneys weights. Weights of paired kidneys corrected for body weight was increased by *7% and *10% at 650NP and 2000NP, respectively (Table 2). Female body weight was reduced at 2000NP by *$\approx$ 9%; organ weights were unchanged across dose groups (not shown). Estrous cycle length prior to cohabitation was unchanged by NP. Sperm indices, including CASA, were similar in controls and 2000NP groups; epididymal sperm density and morphology were unchanged.

There was no dose-related histopathologic indication of gastric erosion or duodenal lesions. Of 10 animals/sex/dose level examined for renal lesions, there was a slight increase in the incidence of renal tubular mineralization in males, starting at the lowest concentration. (Table 2). This consisted of deeply basophilic granular deposits of mineral in tubules near the corticomedullary junction, which in males may be suggestive of renal tubular degeneration/necrosis with subsequent mineralization. Of 10 males examined per dose level, granular renal casts, suggestive of previous tubular degeneration/necrosis, were seen in no controls and one male in each dosed group; none were seen in any female. Renal tubular mineralization was common in females of all groups including controls. Hydronephrosis (mild) was seen in one female each in the middle- and high-dose groups, and not in controls (not shown). No microscopic lesions were noted in left testis or epididymis.

F_1 Generation

In F_1 pups, the absolute or relative-to-body weight anogenital distance (AGD) on pnd 1 or 21 was unchanged, as was the

[2] National Technical Information Service, 5285 Port Royal Rd., Springfield, VA, 22161, telephone (703) 487-4650. Ask for report number PB97210900, "Nonylphenol: multigenerational reproductive effects in Sprague-Dawley rats when exposed to nonylphenol in the diet."

TABLE 1
Multigenerational Reproductive Effects in Sprague-Dawley Rats When Exposed to Nonylphenol in the Diet

Generation:	F_0			F_1			F_2			F_3		
Treatment Group (ppm):	200	650	2000	200	650	2000	200	650	2000	200	650	2000
General Toxicity Parameters												
Adult Terminal Body Weights	—	—	↓F	—	↓F	↓M,F	—	↓M	↓M,F	—	↓F	↓M,F
Feed Consumption (g/animal/day)	—	—	—	—	—	—	—	—	—			
Mortality	1M,1F	0M,1F	0M,0F	0M,0F	0M,1F	0M,0F	0M,1F	0M,0F	0M,2F	0M,0F	0M,0F	1M,0F
Reproductive Parameters												
Estrous Cycle Length	—	—	—	—	—	↑	—	—	↑			
Mating Index	—	—	—	—	—	—	—	—	—			
Pregnancy Index	—	—	—	—	—	—	—	—	—			
Fertility Index	—	—	—	—	—	—	—	↓	—			
Gestation Length	—	—	—	—	—	—	↑	↑	↑			
Live Pups per Litter	—	—	↓	—	—	—	—	—	—			
Proportion of Pups Born Alive	—	—	—	—	—	—	—	—	—			
Pup Sex Ratio		—	—	—	—	—	—	—	—			
Adjusted Live Pup Weight[a]	—	—	—	—	—	—	—	—	↑M			
Pup Survival	—	—	—	—	—	—	—	—	—			
Developmental Parameters												
Anogenital Distance (PND 1 and 21)				—	—	—	—	—	—	—	—	—
Day of Vaginal Opening				—	↓	↓	—	↓	↓	—	↓	↓
Day of Testicular Descent				—	—	—	↓	—	↓	—	↑	—
Day of Prepuce Separation				—	—	↑	—	—	—	—	—	—
Sperm Parameters												
Vas Deferens Sperm Motion Data			—			—			—			
Epididymal Sperm Density	—	—	—	—	—	—	—	↓	↓			
Epid. Sperm Morph., % Abnormal			—			—	↑	↑	↑			
No. Spermatids per mg Testis			—			—	—	—	↓			
Total Spermatid Heads per Testis			—			—	—	—	↓			
PND 21 Weights												
Terminal Body Weight				—	—	—	—	—	—	—	↓M,F	—
Kidneys (Relative)				—	—	—	—	—	↑F	—	—	—
Liver (Relative)				—	—	—	—	—	—	—	—	—
Spleen (Relative)				—	—	—	—	—	—	—	—	—
Ovaries (Absolute)				—	—	—	—	—	—	—	—	—
Vagina/Uterus (Relative)				—	↑	↑	—	—	—	—	—	—
Right Testis (Absolute)				—	—	—	—	—	—	—	—	—
Right Epididymis (Absolute)				—	—	—	—	—	—	—	—	—
Right Cauda Epididymis (Absolute)				—	—	—	—	—	—	—	—	—
Adult/PND 55–58 Organ Weights/Histopathology												
Kidneys (Relative)	—	↑M	↑M	—	—	↑M,F	—	↑M	↑M	—	—	—
Liver (Relative)	—	—	—	—	—	—	—	—	—	—	—	—
Spleen (Relative)	—	—	—	—	—	—	—	—	—	—	—	—
Ovaries (Absolute)	—	—	—	—	—	↓	—	↓	↓	—	—	↓
Vagina/Uterus (Relative)	—	—	—	—	—	—	—	—	—	—	—	—
Right Testis (Absolute)	—	—	—	—	—	↓	—	—	—	—	—	—
Right Epididymis (Absolute)	—	—	—	—	—	—	—	—	—	—	↓	↓
Right Cauda Epididymis (Absolute)	—	—	—	—	—	—	—	—	—	—	—	—
Ventral Prostate (Relative)	—	—	—	—	—	—	—	—	—	—	—	—
Dorsolateral Prostate (Relative)				—	—	—	—	—	—			
Seminal Vesicles w/C.G. (Relative)	—	—	—	—	—	—	—	—	—	—	—	—
Renal Tubule Histopathology	↑M	↑M	↑M	↑M	↑M	↑M,F	↑M	↑M,F	↑M,F	↑M,F	↑M,F	↑M,F

Note. M = male, F = female. ↑ = statistically significant increase in the parameter; ↓ = statistically significant decrease in the parameter; — = no effect; C.G. = coagulating gland.
[a]Pup weight adjusted for litter size by ANCOVA.

TABLE 2
Effects from Male and Female F_0 Rats during Nonylphenol Exposure

	Nonylphenol (ppm in feed)				
	0	200	650	2000	Trend[a]
Mean Daily Dose Range, mg/kg/d	0	8–19	28–63	88–185	
Lactational Dose Range, mg/kg/d	0	15–36	51–127	145–375	
Number Live Pups/Litter[b] (26–28)	15.8 ± 0.7	15.6 ± 0.5	15.1 ± 0.4	14.4 ± 0.4*	$p = 0.004$
Female Terminal Body Wt, g (20)	360.0 ± 7.0	371.3 ± 5.3	353.5 ± 6.9	327.8 ± 5.2*	$p < 0.001$
Male Terminal Body Wt, g (20)	645.4 ± 10.8	616.8 ± 12.0	641.4 ± 9.5	622.4 ± 9.9	$p = 0.451$
Absolute Kidneys Wt. g (20)	4.52 ± 0.10	4.46 ± 0.13	4.73 ± 0.11	4.79 ± 0.08	$p = 0.033$
Relative Kidneys Wt, g[b]	7.0 ± 0.12	7.2 ± 0.13	7.5 ± 0.14*	7.7 ± 0.12*	$p < 0.001$
Right Testis Wt, g (20)	1.87 ± 0.03	1.86 ± 0.03	1.92 ± 0.03	1.86 ± 0.03	$p = 0.783$
Relative Rt. Epididymis Wt, g (20)[b]	1.20 ± 0.03	1.20 ± 0.02	1.20 ± 0.02	1.20 ± 0.02	$p = 0.464$
M/F Renal Tubular Mineralization (10M/10F)	0/9	3/10	3/10	4/9	

Note. Mean ± SEM (*n*).
[a]Trend = linear regression.
[b]Mean ratio (g organ wt/kg body wt) ± SEM.
*$p < 0.05$ compared to controls.

growth and survival of the pups to pnd 21. In the 20 male and 20 female F_1 pups per concentration killed on pnd 21, there were no changes in male body or organ weights (not shown), or in female body weights or organ weights, with the exception of vagina/uterus (Table 3), which was increased by 0%, *14%,

TABLE 3
Effects from F_1 Male and Female Rats during Nonylphenol Exposure

	Nonylphenol (ppm in feed)				
	0	200	650	2000	Trend[a]
Male Anogenital Distance, pnd1, mm (27)	2.67 ± 0.08	2.70 ± 0.09	2.63 ± 0.08	2.74 ± 0.08	$p = 0.807$
Female Anogenital Distance, pnd 1, mm (27)	1.20 ± 0.04	1.23 ± 0.06	1.18 ± 0.05	1.22 ± 0.05	$p = 0.909$
Female pnd21 Body Wts, g[a] (20)	39.8 ± 1.8	39.7 ± 1.6	37.6 ± 1.0	35.1 ± 1.6	$p = 0.056$
pnd21 Relative Vagina/Uterus Wt,[b] (20)	2.1 ± 0.06	2.1 ± 0.12	2.4 ± 0.07*	3.0 ± 0.15*	$p < 0.001$
% Females with Open Vagina, pnd30	3	0	50*	93*	$p < 0.001$
Age at Preputial Separation, days (30)	42.6 ± 0.3	42.3 ± 0.3	42.2 ± 0.5	43.8 ± 0.3*	$p = 0.025$
Calculated Dose Range, mg/kg/d	0	9–35	30–108	100–353	
Lactational Dose Range, mg/kg/d	0	17–37	55–135	176–401	
Number Live F2 pups (27–29)	15.2 ± 0.6	15.0 ± 0.6	15.7 ± 0.4	13.1 ± 0.9	
Male Terminal Body Wt., g (20)	646.1 ± 10.9	648.4 ± 13.3	648.2 ± 13.0	585.1 ± 17.0*	$p = 0.027$
Absolute Kidneys Wt., g (20)	4.57 ± 0.08	4.50 ± 0.12	4.74 ± 0.09	4.92 ± 0.11	$p = 0.007$
Relative Kidneys Wt.	7.1 ± 0.10	7.1 ± 0.16	7.3 ± 0.11	8.5 ± 0.24*	$p < 0.001$
Absolute Rt. Testis Wt., g (20)	1.91 ± 0.02	1.81 ± 0.04	1.87 ± 0.05	1.80 ± 0.03*	$p = 0.038$
Relative Rt. Epididymal Wt (20)	1.16 ± 0.04	1.13 ± 0.04	1.13 ± 0.03	1.20 ± 0.04	$p = 0.420$
Absolute Ventral Prostate Wt., mg (20)	1001.1 ± 51.5	910.0 ± 54.8	975.9 ± 58.6	789.8 ± 37.1*	$p = 0.016$
Relative Ventral Prostate Wt.	1.55 ± 0.08	1.43 ± 0.08	1.51 ± 0.09	1.38 ± 0.08	$p = 0.255$
Female Terminal Body Wt, g (20)	356.3 ± 7.7	350.2 ± 5.2	332.2 ± 3.3*	313.1 ± 5.2*	$p < 0.001$
Absolute Kidneys Wt., g (20)	2.48 ± 0.06	2.51 ± 0.07	2.30 ± 0.04	2.42 ± 0.04	$p = 0.161$
Relative Kidneys Wt.	6.97 ± 0.11	7.15 ± 0.14	6.94 ± 0.12	7.77 ± 0.17*	$p = 0.003$
Estrous Cycle Length, d	4.14 ± 0.06	4.30 ± 0.08	4.40 ± 0.09	4.71 ± 0.07*	
Female Renal Tubular Regeneration/Dilatation/Cysts/Fibrosis (10)	3/0/0/0	2/0/0/0	3/0/0/0	8/8/4/2	
Male Renal Tubular Casts/Mineralization/Hydronephrosis (10)	0/1/1	0/4/1	3/7/3	4/7/6	

Note. Mean ± SEM (*n*). Relative organ weights: mean ratio (g organ wt/kg body wt) ± SEM.
[a] Trend = linear regression.
* $p < 0.05$ compared to controls.

and *42% from the low- to high- dose groups, respectively. No gross abnormalities of reproductive tract structure were noted.

Monitoring for vaginal opening for this generation began pnd 30, by which time one control female was open, whereas 50% and 93% of the females in the 650NP and 2000NP groups were already open (Table 3). Preputial separation was delayed by a day at 2000NP (Table 3); the day of testis descent was unchanged from the control mean ($\pm$ SEM) of 42.6 $\pm$ 0.29 days of age ($n = 30$).

Feed consumption was variably altered, but not consistently reduced by NP (not shown). In 2000NP rats, body weight during growth to mating tended to be reduced by *6–9% for males, and *8–13% for females. At delivery, 2000NP males weighed *8% less than controls, females were *13% less than controls; other dose groups were not statistically different from controls.

Indices of mating and fertility were unchanged by NP. The mean litter size of F_2 pups was reduced more at the high dose than seen in the F_1 animals, but was not statistically significant due to greater variability (Table 3). F_2 pup weight (absolute or adjusted for litter size) or viability or growth to weaning was unaffected by NP (not shown).

At necropsy of 20 representative adult F_1 rats/sex/concentration, male terminal body weight was reduced by $\approx$ 10% at 2000NP, whereas relative kidney weight was increased by *20% (Table 3). Absolute testis weight was reduced at 2000NP by *6%, though relative testis weight was increased, and number of spermatids per testis or per milligram testis was unchanged. Other male organ weights were unchanged, as were sperm measures, including CASA. Dorsolateral prostate weight was unchanged at any concentration of NP from the control mean of 701.8 $\pm$ 26.9 mg ($\pm$ SEM, $n = 20$).

Female terminal body weights were reduced at 650NP and 2000NP by *7% and *12%, respectively, compared to controls. There was a monotonic reduction in absolute ovary weight, which reached significance at 2000NP (*19%); this difference was not manifest when adjusted for body weight (not shown).

Relative kidneys weights were increased for both sexes at 2000NP (Table 3). This coincided with an increase in a variety of renal tubular lesions of minimal to mild severity. These lesions included medullary tubular dilatation, medullary cysts, focal mineralization, granular casts, and hydronephrosis. In males, the incidences of these lesions tended to be dose related, whereas in females the lesions were much more prevalent at 2000NP. The dilatation and cysts were observed at 2000NP, and were considered to be a continuum of changes relating to tubular dilatation. The fibrosis noted in two female rats was associated with renal tubular cysts.

Although no gastric lesions were noted in the 10 males per concentration that were examined, in females hyperplasia of the forestomach epithelium was noted in 0, 0, 1, and 5 females in the 0 to 2000NP groups, respectively. No lesions were observed in duodenum, or left testis or epididymis.

Finally, estrous cyclicity data collected for 3 weeks prior to cohabitation showed that cycle length was monotonically increased, significantly so at 2000NP, by $\approx$ 14%, from a control mean length of 4.14 days, to *4.71 days at 2000NP. Although this correlated with a reduced number of cycles during the evaluation period (from a control value of 4.3 to 3.6), there was no reduction in the number of females cycling normally in the treated groups.

F_2 Generation

Absolute AGD of F_2 pups was unchanged by NP exposure (control values: 2.76 mm in males, 1.32 mm in females). The AGD index (absolute value adjusted for body weight) was also unchanged. Pup survival to weaning was unaffected by NP.

In the 20 males and females per concentration killed and necropsied at pnd 21, male body and organ weights were unchanged, and female body and absolute organ weights were unchanged, including vagina/uterus. Female relative kidneys weight was increased by *9% at 2000NP at pnd 21. No gross abnormalities of male reproductive tract structure were noted.

Monitoring for vaginal opening began at pnd 21; mean day of vaginal opening was accelerated at 650 and 2000NP by *$\approx$ 2 and *$\approx$ 6 days, respectively (Table 4). Prepuce separation was unaltered by NP, although testis descent was slightly accelerated at 200NP and 2000NP (Table 4).

Feed consumption, when expressed as grams of feed per animal per day, tended to be slightly decreased by NP. This effect became nonsignificant when expressed as grams of feed per kilogram body weight per day (not shown). At delivery of the F_3 pups, the 2000NP males weighed *7% less than controls. Treated females weighed *6%, and *12% less than controls at 650NP and 2000NP, respectively.

There were no dose-related changes in indices of mating or fertility. Mean litter size in the control through high-dose groups was 14.7, 14.7, 15.3, and 14.4 pups, respectively. Gestation length was increased in all dose groups relative to control (Table 4). This must be due at least in part to the fact that this control mean gestation length was the shortest of all control gestations in this study.

After the F_3 pups were reared and weaned, 20 representative F_2 adult males and females per concentration were killed and necropsied. Male terminal body weight was reduced by *8% and *7% at 650NP and 2000NP, respectively. Absolute male spleen weight was reduced at 650NP and 2000NP by *11 and *14%, respectively; this effect was not significant when adjusted for body weight. Relative kidneys weight was increased by *9% and *16% at 650NP and 2000NP, respectively. Male reproductive organ weights were unchanged. Dorsolateral prostate weight was unchanged at any concentration of NP from the control value of 611.2 $\pm$ 27.0 mg (mean $\pm$ SEM, $n =$ 20). Although CASA endpoints were unaffected by NP exposure, there was a monotonic reduction in epididymal sperm density, which was 2%, *8%, and *13% less than controls in the 200, 650, and 2000NP groups, respectively. The number of spermatids per milligram testis and per total testis was slightly reduced at 200 and 650NP, and was reduced at 2000NP by *13% and *12%, respectively (Table 4).

TABLE 4
Effects from F_2 Male and Female Rats during Nonylphenol Exposure

	Nonylphenol (ppm in feed)				
	0	200	650	2000	Trend[a]
Male Anogenital Distance, pnd1, mm (29)	2.76 ± 0.06	2.71 ± 0.06	2.70 ± 0.04	2.70 ± 0.06	$p = 0.353$
Female Anogenital Distance, pnd 1, mm (2)	1.32 ± 0.02	1.31 ± 0.02	1.29 ± 0.02	1.28 ± 0.02	$p = 0.276$
Female pnd21 Body Wts., g (20)	36.6 ± 1.7	39.1 ± 1.5	35.4 ± 1.1	36.9 ± 1.9	$p = 0.336$
pnd21 Relative Vagina/Uterus Wt.	2.22 ± 0.08	2.15 ± 0.17	2.82 ± 0.21	2.61 ± 0.19	$p = 0.066$
pnd21 Relative Kidneys Wts.	12.22 ± 0.18	12.09 ± 0.17	12.51 ± 0.27	13.32 ± 0.20*	$p < 0.001$
Day of First Vaginal Opening (30)	32.6 ± 0.4	32.6 ± 0.4	30.7 ± 0.5*	26.8 ± 0.3*	$p < 0.001$
Day of Testes Descent (30)	23.4 ± 0.2	22.1 ± 0.3*	22.9 ± 0.6	22.7 ± 0.6*	$p = 0.027$
Calculated Dose Range, mg/kg/d	0	10–35	31–115	109–360	
Lactational Dose Range, mg/kg/d	0	18–40	59–127	186–436	
F3 Gestational Length, d. (23–25)	21.4 ± 0.1	21.8 ± 0.08*	21.6 ± 0.1*	22.0 ± 0.1*	$p < 0.001$
Male Terminal Body Wt., g (20)	663.4 ± 13.0	646.7 ± 14.4	609.9 ± 9.6*	616.3 ± 16.0*	$p = 0.004$
Absolute Kidneys Wt., g (20)	4.52 ± 0.12	4.68 ± 0.13	4.54 ± 0.12	4.85 ± 0.10	$p = 0.100$
Relative Kidneys Wt.	6.82 ± 0.14	7.25 ± 0.17	7.45 ± 0.17*	7.94 ± 0.22*	$p < 0.001$
Absolute Ventral Prostate Wt., mg (20)	971.2 ± 61.3	924.2 ± 40.0	845.7 ± 46.5	822.5 ± 41.5	$p = 0.018$
Relative Ventral Prostate Wt.	1.47 ± 0.10	1.43 ± 0.05	1.39 ± 0.08	1.35 ± 0.07	$p = 0.327$
Relative Rt. Epididymal Wt. (20)	1.19 ± 0.02	1.18 ± 0.03	1.23 ± 0.03	1.21 ± 0.03	$p = 0.491$
Female Terminal Body Wt., g (20)	347.0 ± 5.7	340.9 ± 7.1	343.1 ± 5.3	312.0 ± 5.6*	$p < 0.001$
Absolute Ovary Wt., mg (20)	208.7 ± 5.6	195.3 ± 6.8	180.0 ± 4.6*	165.4 ± 5.5*	$p < 0.001$
Relative Ovary Wt.	0.60 ± 0.02	0.57 ± 0.02	0.53 ± 0.01*	0.53 ± 0.02*	$p < 0.001$
Estrous Cycle Length, d (28–30)	4.16 ± 0.08	4.36 ± 0.09	4.26 ± 0.10	4.91 ± 0.14*	
Female Renal Tubule Dilatation/Cysts (10)	2/0	1/0	3/1	6/2	
Male Renal Tubule Dilatation/Cysts/Casts/ Mineralization (10)	1/1/0/0	2/2/0/5	1/1/3/9	7/5/4/10	
Absolute Right Testis Wt., g (20)	1.89 ± 0.04	1.87 ± 0.04	1.81 ± 0.04	1.91 ± 0.04	$p = 0.850$
Spermatids/mg testis $\times 10^{-3}$ (20)	144.7 ± 5.1	138.6 ± 3.9	142.1 ± 6.4	125.9 ± 4.6*	$p = 0.020$
Total Spermatids/testis $\times 10^{-6}$ (20)	243.3 ± 8.0	234.6 ± 6.5	235.6 ± 9.1	214.9 ± 8.9*	$p = 0.022$
Epididymal Sperm Density[b] (20)	485.5 ± 16.1	474.0 ± 17.1	447.2 ± 14.4*	423.6 ± 17.2*	$p = 0.004$

Note. Mean ± SEM (*n*). Relative organ weights: mean ratio (g organ wt/kg body wt) ± SEM.
[a] Trend = linear regression.
[b] 10^3 sperm/mg epididymis.
* $p < 0.05$ compared to controls.

There were 0.1% morphologically abnormal sperm in the F_2 controls, the lowest of any control group. The F_0 and F_1 adult controls had 0.63 and 0.48% abnormal sperm, respectively. The F_2 adult treated males had *0.43, *0.42, and *0.65% abnormal sperm, generally within historic limits but presenting a shallow dose response. It is difficult to conceive of these changes as being biologically significant.

F_2 adult female terminal body weights were reduced by *10% at 2000NP. Absolute spleen weight was also reduced at 2000NP by *13%, an effect that disappeared after correcting for body weight. Ovary weights, both absolute and relative to body weight, were reduced at 650NP and 2000NP (Table 4). The 2000NP group was selected for follicle count, because it was thought that the weight reductions might represent a gross indicator of reduced follicle number. The mean control numbers of small, medium, and large follicles were 199.1, 18.1, and 8.7, respectively; the corresponding values for the 2000NP females were 238.7, 11.5, and 4.2. All values had a coefficient of variation of ≈ 50%. There were no statistical differences between the treated animals and the control values for any size follicle. Because of this lack of difference, and the likelihood that lower groups would have smaller effect, and therefore present a smaller chance of seeing a significant difference from control, lower dose groups were not evaluated.

Premating estrous cycle length was increased at 2000NP from a control value of 4.16 days to *4.91 days. The number of cycling females and the number of females with regular cycles was unaltered.

No microscopic lesions were observed in stomachs, duodena, left testis, or epididymis. Treatment-related renal tubular effects (dilatation, cysts, casts, mineralization) were present in both sexes, and generally were more prominent in males (Table 4), with increases beginning at the lowest dose. All renal effects were minimal to mild.

F_3 Generation

Mean weight at birth for the F_3 pups was statistically unchanged, although there was a trend towards heavier pups in the treated groups. Absolute AGD was unchanged at pnd 1 and

TABLE 5
Effects from F_3 Male and Female Rats during Nonylphenol Exposure

	Nonylphenol (ppm in feed)				
	0	200	650	2000	Trend[a]
Male Anogenital Distance, pnd1, mm (29)	2.92 ± 0.07	2.97 ± 0.10	2.85 ± 0.08	2.79 ± 0.07	$p = 0.089$
Female Anogenital Distance, pnd 1, mm (26)	1.37 ± 0.05	1.43 ± 0.05	1.38 ± 0.05	1.31 ± 0.04	$p = 0.196$
Female pnd21 Body Wt., g (20)	38.0 ± 1.3	37.8 ± 1.6	32.8 ± 1.2*	36.0 ± 1.3	$p = 0.058$
pnd21 Relative Vagina/Uterus Wt.	2.16 ± 0.12	1.95 ± 0.12	2.36 ± 0.15	2.43 ± 0.16	$p = 0.073$
Day of First Vaginal Opening (30)	33.4 ± 0.3	32.9 ± 0.3	26.1 ± 0.3*	27.4 ± 0.4*	$p < 0.001$
Day of Testes Descent (30)	22.9 ± 0.1	23.2 ± 0.3	26.0 ± 0.3*	23.1 ± 0.4	$p = 0.014$
Male Terminal Body Wt., g (20)	307.1 ± 7.2	315.1 ± 9.4	288.2 ± 5.0	284.9 ± 7.3*	$p = 0.005$
Relative Kidneys Wt.	9.68 ± 0.19	9.75 ± 0.07	10.18 ± 0.25	10.20 ± 0.24	$p = 0.059$
Absolute Ventral Prostate Wt., mg (20)	257.9 ± 15.0	282.8 ± 14.7	240.5 ± 15.0	246.1 ± 12.6	$p = 0.360$
Absolute Right Testis Wt., g (20)	1.42 ± 0.03	1.40 ± 0.03	1.34 ± 0.04	1.43 ± 0.03	$p = 0.837$
Relative Rt. Epididymal Wt.	0.79 ± 0.02	0.78 ± 0.02	0.74 ± 0.02	0.78 ± 0.02	$p = 0.760$
Female Terminal Body Wt., g (20)	228.6 ± 5.5	222.0 ± 5.3	206.5 ± 3.5*	203.0 ± 3.9*	$p < 0.001$
Relative Kidneys Wt.	9.68 ± 0.15	9.66 ± 0.17	9.39 ± 0.28	9.76 ± 0.17	$p = 0.840$
Female Renal Tubule Dilatation/Cysts (10)	0/0	3/2	10/9	7/6	
Male Renal Tubule Dilatation/Cyst/Mineralization/Hydronephrosis (10)	1/1/0/0	5/4/4/0	10/10/9/3	8/8/6/4	

Note. Mean ± SEM (*n*). Relative organ weights: (g organ wt/kg body wt) ± SEM.
[a] Trend = linear regression.
* $p < 0.05$ compared to controls.

21. AGD index on pnd 1 was reduced in 2000NP males (from a control value of 0.47 to *0.42), whereas at pnd 21, the AGD index was increased by *9% and *6% at 650NP and 2000NP, respectively. There was the same effect in females at pnd 21 for AGD index (not shown). Pup growth or survival to weaning was unaffected by NP exposure.

At pnd 21, male body weights were reduced by *13% at 650NP; no other changes were noted. Females at 650NP were *14% lighter than their controls; there was a trend towards heavier reproductive tract at 650NP and 2000NP that failed to reach significance (Table 5). No gross abnormalities of male reproductive tract structure were noted.

Vaginal opening was accelerated at 650NP and 2000NP; day of testis descent was delayed by *≈ 3 days at 650NP only (Table 5). Prepuce separation was unaltered.

Due to logistical constraints, the study was terminated when the F_3 animals were 55–58 days of age. At this age, males at 2000NP were *7% lighter than their controls; absolute epididymal weights were reduced at 650NP and 2000NP by *13% and *9%, respectively. There were no reductions in adjusted male organ weights.

Female body weights were reduced at 650NP and 2000NP by *10% and *11%, respectively.

No microscopic lesions were noted in stomachs, duodena, left testis or epididymis, ovaries, or uteri. In dosed rats, the incidence in males of renal tubular dilatation, cysts, and mineralization, and in females of renal tubular dilatation and cysts, was greater than those of the respective controls (Table 5). All renal effects were minimal to mild.

DISCUSSION

Previous studies have shown that NP was able to stimulate some responses similar to those of 17β estradiol, though the magnitude of the response was less, and the dose required for activation was vastly more. Those studies primarily used transfected cells or estrogen-responsive cells *in vitro* (Shelby *et al.*, 1996; Soto *et al.*, 1991), as well as short term *in vivo* dosing and evaluation (Shelby *et al.*, 1996). Male trout hepatocytes produce vitellogenin (an egg protein) after exposure to an estrogenic stimulus (Pelissero *et al.*, 1993), a response that is also stimulated by exposure to NP both *in vitro (*White *et al.*, 1994) and *in vivo (*Lech *et al.*, 1996). However, this latter study measured mRNA levels only. Indeed, the estrogenicity of NP has been so frequently reported that it has become a standard "weak estrogen" in setting up newer assays (e.g., Coldham *et al.*, 1997; Gaido *et al.*, 1997; Harris *et al.*, 1997). Although it has been recently shown that exposure to NP inhibits normal testis growth in trout (Jobling *et al.*, 1996), several questions were unaddressed until the present study. Would these *in vitro* and piscine activities translate into meaningful estrogenicity in mammals *in vivo*, and at what levels? Would exposure to this weak estrogenic compound over several generations affect sperm count, as proposed by Sharpe and Skakkebaek (1993), in a consistent and interpretable way? Would females also suffer detectable reproductive alterations?

In evaluating data from the current study, we place more weight on effects that were seen consistently across similarly treated generations. Interpretation of effects that manifest in

just one of several similarly treated generations must necessarily be done cautiously.

There were significant female effects in this study: vaginal opening was accelerated at 650NP and 2000NP in all three generations exposed developmentally. Estrous cycle length was increased in F_1 and F_2 adults at 2000NP; it was not assessed in the F_3 animals. The pnd 21 weight of uterus/vagina was increased, but only in the F_1 animals. All three changes are markers of an estrogenic response (rev. in Reel *et al*., 1996). In humans, late onset of menarche reduces the risk of breast cancer later in life (rev. in Harris *et al*., 1992). If the reverse is true, that early estrogenization predisposes to increased cancer risk (Key and Pike, 1988), then these indications of early estrogenization may not be just changes, but may be adverse. Further studies with NP are planned that will evaluate effectively the same exposure scenario used in the present study and incorporate a lifetime cancer subset of animals (Delclos and Newbold, 1997) to explore this possibility directly.

The increase in estrous cycle length in F_1 and F_2 animals, although small, has been seen before with other estromimetics (e.g., Chapin *et al*., 1997a) and with estrogen itself (Biegel *et al*., 1998). This change in cycle is considered both hormone responsive (rev. in Cooper *et al*., 1993) and adverse, because of the association between lengthened cycle and reduced fecundity in larger populations of animals (Chapin *et al*., 1997b).

The follicle count data are perplexing. On one hand, they are sufficiently 'noisy' (there is a great deal of interanimal variation, which raises the standard deviations and reduces statistical power) to prevent the attainment of statistical significance, even with a $\approx$ 50% reduction in the mean number of growing and large follicles. This is consistent with other reports (e.g., Bolon *et al*., 1997; Plowchalk *et al*., 1993). On the other hand, this 50% reduction, together with an increase in the apparent pool of small/resting follicles, is also consistent with a reduction in recruitment of follicles into the pool of growing follicles for subsequent ovulation. However, if the effect of NP was to reduce recruitment, then one would have expected reduced litter size, which was clearly unaffected in the F_2 litters. For follicle counts, we can only conclude that the method is currently too noisy to be of significant use. Further work is ongoing in this area (Bolon *et al*., 1997).

There was no consistent effect on fertility. Although the $\approx$ 1 pup per litter decrease in the F_0 mating was statistically significant, the two pup per litter decrease in the F_1 mating was not, and the mean number of pups per litter was equal in the F_2 mating. Overall, based on these variances and numbers of pairs, this study had a 90% chance of seeing 2.7 fewer pups per litter. There may be a consistent effect of NP on rat fertility, but we conclude that it must be smaller than $\approx$ 2 pups out of a possible 15, a relatively small effect.

Nonetheless, females did show a consistent acceleration of vaginal opening in all generations in which it was assessed. In contrast to the consistent vaginal effect, there was no consistent detectable effect on the males. Based on previous reviews showing that prenatal estrogen exposure alters male reproductive development and suggesting that low-level estrogenic signals from environmental chemicals might be responsible for a significant proportion of male reproductive problems (Sharpe, 1993; Sharpe and Skakkebaek, 1993), we were quite prepared to find effects on spermatogenesis and reproductive organ structure, and greater effects in developmentally exposed animals (i.e., in F_1, F_2, and F_3 males) than in the adult-only exposed group (F_0 animals). The F_0 animals showed, indeed, no trends or change in any reproductive organ weight or in any sperm parameter. The same is true of the F_1 animals, the first generation exposed both developmentally and through to mating (and beyond): no changes or trends were seen in any sperm measure or in any reproductive organ weight. The organ weights from the F_3 males must be considered cautiously because spermatogenesis, although qualitatively complete, is not nearly to the quantitative levels found in adults (Blazak *et al*.,1985), and so these organ weights must still be considered peripubertal.

In light of the above, the male sperm count effects seen in the F_2 adults are perplexing. On one hand, although F_2 cauda epididymal and testis weights are entirely unchanged by NP exposure, there is a clear monotonic downward trend in whole epididymal weight and sperm density (number of sperm per milligram of cauda epididymis), although whole epididymis weight was never significantly lower than control. Both of these endpoints changed after pre-natal exposure to estradiol (Cook *et al*., 1998). Both of these changes must be considered adverse, given their implicit and explicit relationship to fertility (Chapin *et al*., 1997b). Additionally, both spermatids per milligram testis and total spermatids per testis are reduced at 2000NP. The reduced epididymal sperm count and testicular spermatid counts are internally consistent and suggest reduced spermatogenesis. The clear dose relationship of these changes lends weight to their being taken as treatment related. There appears to be an increase in sperm abnormalities (see discussion below, however). Thus, there is some internal consistency within the set of data.

On the other hand, (a) testis weight and cauda epididymis weight were unchanged, (b) there were no microscopically-detectable structural lesions or changes in high-resolution sections of testis or epididymis, and (c) these effects were seen only in this generation, whereas the preceding F_1 animals were treated identically and lacked any hint of such effects. It has long been believed that a Sertoli cell will support a species-dependent number of developing germ cells, and that this number is quite constant across individuals within a species (rev. in Sharpe, 1994). If, as postulated (Sharpe and Skakkebaek, 1993) the effect of a prenatal estrogenic stimulus would be to prematurely halt Sertoli cell division, the resultant adult testis would have qualitatively normal spermatogenesis in a smaller (lighter) testis that had fewer Sertoli cells and therefore reduced absolute sperm output, but equal spermatogenic efficiency; that is, sperm per milligram would be the same across

treatment groups, whereas sperm per total testis would be reduced. In the present study, both spermatid endpoints were reduced at 2000NP in the F_2 animals, implying a lesion in spermatogenesis. However, no lesions were visible to a staging-competent pathologist reading these GMA-embedded, PAS-H-stained sections, and mean testis weight was clearly the same across dose levels (Table 3). These are puzzling internal inconsistencies that are not easily dismissed.

An additional difficulty is that the epididymal sperm density effects only manifest in the second developmentally exposed generation. This might be explained by some degree of bioconcentration across generations. There are no mammalian data that might support or refute this suggestion. Data from aquatic species show that algae and fish do bioaccumulate NP from the water (Ahel *et al*. in Nimrod and Benson, 1996; Ekelund *et al*. in Jobling *et al.,* 1996) up to ≈ 300-fold. Compared to bioconcentration factors for *o,p*-DDT of 25,000–100,000 (rev. in Nimmo, 1985), this seems relatively modest. Additionally, NP lacks the tendency to accumulate in lipid-rich environments (see Nimrod and Benson, 1996). Nonetheless, if the F_2 animals were developmentally exposed to levels of NP that were higher than those for the developing F_1 rats, this might explain our seeing sperm changes only in the F_2 animals. However, the changes in the F_2 rats are still internally inconsistent, so bioaccumulation cannot explain all of what was observed.

Alternatively, we assume that the reproductive system of the F_2 rats would develop identically to the F_1 animals. It is always possible that these assumptions are wrong, and that these data tell us something new about the reproductive system and its development under mild but constant estromimetic signals. NP is one of the compounds being tested in a five-generation study performed by the National Institute of Environmental Health Sciences and NCTR (Delclos and Newbold, 1997), so that a replicate of this general study design is planned, which may shed some light on the replicability of these effects.

Finally, testis weight was not consistently reduced across generations: it was slightly reduced in the F_1 animals, but not the F_2 or F_3 animals. The cautious interpretation of these data would have to be that there was no effect on Sertoli cell number. These data are consistent with those presented recently by Cook *et al.* (1998), who reported that prenatal and developmental exposure to 17β-estradiol did not change Sertoli cell number.

Taking all the above into consideration, our interpretation is that these F_2 sperm changes either (a) are "noise," or (b) are telling us something new about reproductive system development under a constant mild estrogenic stimulus. We feel that a clear conclusion cannot be drawn from these present data. The male F_2 sperm data are consistent enough to be compelling, but *not* consistent enough to be conclusive. Therefore, until these treatment effects are replicated, we feel it is unwarranted to conclude that this study shows an adverse effect of a mild estromimetic on male reproductive development.

The apparent increase in sperm morphologic abnormalities in F_2 adults is dose-related, and therefore must be seriously considered. However, all the values are within the range of the other control data from this study and this laboratory, are all very low in an absolute (and a relative) sense, and (more convincingly) the F_2 *control* value for abnormalities is unusually low: the 0.1% value contrasts with the 0.48 and 0.63% values from the F_1 and F_0 controls, respectively. Thus, we believe that the "increase" in morphologic abnormalities is an artifact, due to an unusually low control value, and we *do not* conclude that NP increases sperm morphologic abnormalities.

NP exposure consistently produced adult kidney effects. Weight increases were often seen (F_0 males, both sexes of F_1 animals, F_2 males). Renal tubular pathology (dilatation and cysts, mineralization) was seen in every generation/gender combination except for F_0 females. This is interpreted as being a slight acceleration of the tubular nephropathy normally seen in Sprague-Dawley rats, and tended to be more pronounced in males than in females, as is also the case with spontaneous nephropathy (Montgomery and Seely, 1990). Mineralization was seen in the low-dose males of all generations. Mineralization is a common, spontaneous, age-related renal change in female rats, and although the cause is unknown, imbalances in dietary or renal ion levels, protein, or lipid have all been suggested (rev.in Montgomery and Seely, 1990). In male rats, the background incidence of mineralization is low, and when increased, may reflect renal tubular damage. The presence of granular casts is another indication of tubular degeneration/necrosis. The increased incidence of these two lesions in males in this study suggests that NP may damage renal tubules. Although the mechanism for this is currently unclear, several observations may be relevant: NP is excreted by the kidneys (Knaak *et al*., 1966), so morphologic changes in an excretory organ might be expected. NP binds to and decreases the activity of testis membrane-associated calcium pumps (Michelangeli *et al*., 1996), which is consistent with the notion that nonionic surfactants can bind to and modify the activity of cell surface proteins (rev. in Cserhati, 1995). Finally, the kidney has estrogen receptors (rev. in Li and Li, 1996), which may be playing a role in this response (e.g., by modifying protein synthesis and/or expression patterns). Thus, one could hypothesize an alteration in tubular ion pump activity, which might explain the mineralization, tubular dilatation, and subsequent cyst formation by the cells lining those tubules. However, it's likely that multiple mechanisms are at work, for if ion pump effects alone were responsible, one might have expected lesions (and functional problems) in other membranes active in moving water or ions, such as the rete testis, gut epithelium, or choroid plexus. The rete and stomach lining were examined microscopically and found to be unaffected.

It is worth noting that the renal changes occurred consistently at *all* dose levels, whereas the next most sensitive and

consistent change (accelerated vaginal opening) was affected only at the middle and high doses.

It also should be pointed out that this design, by focusing on development and the early part of the reproductive process, omitted the process of reproductive aging, which might plausibly be altered by NP. Because premature aging-related infertility could reasonably be considered adverse, this important aspect of the reproductive life cycle should be examined for NP. A longer study could also have evaluated the occurrence of endocrine-related cancers (above), which were not a target of the current study. The NCTR/NIEHS study (Delclos and Newbold, 1997) will address this important issue.

Thus, the present data show that exposure to 4-nonylphenol for several generations of rats provided indications of estrogen activity in females (accelerated vaginal opening, estrous cycle disruption) and altered kidney structure and weight in both sexes, in the absence of consistent reproductive changes in males. These data show that relatively high doses of NP ($\approx$ 100–350 mg/kg/d) can plausibly be considered reproductively adverse, but that lower doses ($\approx$ 12–40 mg/kg/d) appear to pose a greater hazard to the kidneys than to the reproductive system of male or female rats. From these data, it would appear that 200 ppm NP is a no observed adverse effect level for reproductive effects.

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