NTP Developmental and Reproductive Toxicity Technical Report on the Modified One-Generation Study of 2-Hydroxy-4-methoxybenzophenone (CASRN 131-57-7) Administered in Feed to Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats with Prenatal and Reproductive Performance Assessments in F_1 Offspring

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Foreword

The National Toxicology Program (NTP), established in 1978, is an interagency program within the Public Health Service of the U.S. Department of Health and Human Services. Its activities are executed through a partnership of the National Institute for Occupational Safety and Health (part of the Centers for Disease Control and Prevention), the Food and Drug Administration (primarily at the National Center for Toxicological Research), and the National Institute of Environmental Health Sciences (part of the National Institutes of Health), where the program is administratively located. NTP offers a unique venue for the testing, research, and analysis of agents of concern to identify toxic and biological effects, provide information that strengthens the science base, and inform decisions by health regulatory and research agencies to safeguard public health. NTP also works to develop and apply new and improved methods and approaches that advance toxicology and better assess health effects from environmental exposures.

The NTP Technical Report series for developmental and reproductive toxicity (DART) studies began in 2019. The studies described in this NTP Technical Report series (i.e., the NTP DART Report series) are designed and conducted to characterize and evaluate the developmental or reproductive toxicity of selected substances in laboratory animals. Substances (e.g., chemicals, physical agents, and mixtures) selected for NTP reproductive and developmental studies are chosen primarily on the basis of human exposure, level of commercial production, and chemical structure. The interpretive conclusions presented in NTP DART Reports are based only on the results of these NTP studies, and extrapolation of these results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection for study per se is not an indicator of a substance's developmental or reproductive toxicity potential.

NTP conducts its studies in compliance with its laboratory health and safety guidelines and the Food and Drug Administration Good Laboratory Practice Regulations and meets or exceeds all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Studies are subjected to retrospective quality assurance audits before they are presented for public review. Draft reports undergo external peer review before they are finalized and published.

The NTP DART Reports are available free of charge on the NTP website and cataloged in PubMed, a free resource developed and maintained by the National Library of Medicine (part of the National Institutes of Health). Data for these studies are included in NTP's Chemical Effects in Biological Systems database.

For questions about the reports and studies, please email NTP or call 984-287-3211.

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Explanation of Levels of Evidence for Developmental and Reproductive Toxicity

The National Toxicology Program (NTP) describes the results of individual studies of chemical agents and other test articles and notes the strength of the evidence for conclusions regarding each study. Generally, each study is confined to a single laboratory animal species, although in some instances, multiple species may be investigated under the purview of a single study report. Negative results, in which the study animals do not exhibit evidence of developmental toxicity, do not necessarily imply that a test article is not a developmental toxicant, but only that the test article is not a developmental toxicant under the specific conditions of the study. Positive results demonstrating that a test article causes developmental toxicity in laboratory animals under the conditions of the study are assumed to be relevant to humans, unless data are available that demonstrate otherwise. In addition, such positive effects should be assumed to be primary effects, unless there is clear evidence that they are secondary consequences of excessive maternal toxicity. Given that developmental events are intertwined in the reproductive process, effects on developmental toxicity may be detected in reproductive studies. Evaluation of such developmental effects should be based on the NTP Criteria for Levels of Evidence for Developmental Toxicity.

It is critical to recognize that the "levels of evidence" statements described herein describe only developmental **hazard**. The actual determination of **risk** to humans requires exposure data that are not considered in these summary statements.

Five categories of evidence of reproductive toxicity are used to summarize the strength of the evidence observed in each experiment: two categories for positive results **(clear evidence** and **some evidence)**; one category for uncertain findings **(equivocal evidence)**; one category for no observable effects **(no evidence)**; and one category for experiments that cannot be evaluated because of major design or performance flaws **(inadequate study)**. Application of these criteria requires professional judgment by individuals with ample experience with and understanding of the animal models and study designs employed. For each study, conclusion statements are made using one of the following five categories to describe the findings; if warranted, these conclusion statements should be made separately for males and females. These categories refer to the strength of the evidence of the experimental results and not to potency or mechanism.

Levels of Evidence for Evaluating Reproductive Toxicity

- **Clear evidence** of reproductive toxicity is demonstrated by a dose-related effect on fertility or fecundity, or by changes in multiple interrelated reproductive parameters of sufficient magnitude that by weight of evidence implies a compromise in reproductive function.
- **Some evidence** of reproductive toxicity is demonstrated by effects on reproductive parameters, the net impact of which is judged by weight of evidence to have potential to compromise reproductive function. Relative to clear evidence of reproductive toxicity, such effects would be characterized by greater uncertainties or weaker relationships with regard to dose, severity, magnitude, incidence, persistence, or decreased concordance among affected endpoints.

- **Equivocal evidence** of reproductive toxicity is demonstrated by marginal or discordant effects on reproductive parameters that may or may not be related to the test article.
- **No evidence** of reproductive toxicity is demonstrated by data from a study with appropriate experimental design and conduct that are interpreted as showing no biologically relevant effects on reproductive parameters that are related to the test article.
- **Inadequate study** of reproductive toxicity is demonstrated by a study that, because of major design or performance flaws, cannot be used to determine the occurrence of reproductive toxicity.

Levels of Evidence for Evaluating Developmental System Toxicity

- **Clear evidence** of developmental toxicity is demonstrated by data that indicate a dose-related effect on one or more of its four elements (embryo-fetal death, structural malformations, growth retardation, or functional deficits) that is not secondary to overt maternal toxicity.
- **Some evidence** of developmental toxicity is demonstrated by dose-related effects on one or more of its four elements (embryo-fetal death, structural malformations, growth retardation, or functional deficits), but are greater uncertainties or weaker relationships with regard to dose, severity, magnitude, incidence, persistence, or decreased concordance among affected endpoints occur.
- **Equivocal evidence** of developmental toxicity is demonstrated by marginal or discordant effects on developmental parameters that may or may not be related to the test article.
- **No evidence** of developmental toxicity is demonstrated by data from a study with appropriate experimental design and conduct that are interpreted as showing no biologically relevant effects on developmental parameters that are related to the test article.
- **Inadequate study** of developmental toxicity is demonstrated by a study that, because of major design or performance flaws, cannot be used to determine the occurrence of developmental toxicity.

When a conclusion statement for a particular study is selected, consideration must be given to key factors that would support the selection of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of developmental and reproductive toxicity studies in laboratory animals, particularly with respect to interrelationships between endpoints or malformation, impact of the change on reproductive function and/or developmental outcomes, relative sensitivity of endpoints, normal background incidence, and specificity of the effect. For those evaluations that may be on the borderline between two adjacent levels, some factors to consider in selecting the level of evidence of reproductive toxicity are given below:

- Increases in severity and/or prevalence (more individuals and/or more affected litters) as a function of dose generally strengthen the level of evidence, keeping in mind that the specific manifestation may be different with increasing dose. For example,

histological changes at a lower dose level may reflect reductions in fertility at higher dose levels.

- In general, the more animals affected, the stronger the evidence; however, effects on a small number of animals across multiple related endpoints should not be discounted, even in the absence of statistical significance for the individual endpoint(s). In addition, effects with low background incidence when interpreted in the context of historical controls may be biologically important.
- Effects seen in many litters may provide stronger evidence than effects confined to one or a few litters, even if the incidence within those litters is high.
- Because of the complex relationship between maternal physiology and development, evidence for developmental toxicity may be greater for a selective effect on the embryo-fetus or pup.
- Concordant effects (syndromic) may strengthen the evidence of developmental toxicity. Single endpoint changes by themselves may be weaker indicators of effect than concordant effects on multiple endpoints related by a common process or mechanism.
- In order to be assigned a level of "clear evidence" the endpoint(s) evaluated should normally show a statistical increase in the deficit, or syndrome, on a litter basis.
- Consistency of effects across generations may strengthen the level of evidence. However, special care should be taken for decrements in reproductive parameters noted in the F_1 generation that were not seen in the F_0 generation, which may suggest developmental as well as reproductive toxicity. Alternatively, if effects are observed in the F_1 generation but not in the F_2 generation (or the effects occur at a lesser frequency in the F_2 generation), this may be due to the nature of the effect resulting in selection for resistance to the effect (i.e., if the effect is incompatible with successful reproduction, then the affected individuals will not produce offspring).
- Transient changes (e.g., pup weight decrements) by themselves are weaker indicators of effect than persistent changes.
- Single end point changes by themselves are weaker indicators of effect than concordant effects on multiple, interrelated end points.
- Marked changes in multiple reproductive tract endpoints without effects on integrated reproductive function (i.e., fertility and fecundity) may be sufficient to reach a conclusion of clear evidence of reproductive toxicity.
- Insights from supportive studies (e.g., toxicokinetics, ADME [absorption, distribution, metabolism, and excretion], computational models, structure-activity relationships) and reproductive findings from other in vivo animal studies (NTP or otherwise) should be drawn upon when interpreting the biological plausibility of an effect.
- New assays or techniques need to be appropriately characterized to build confidence in their utility: their usefulness as indicators of effect is increased if they can be associated with changes in traditional endpoints.

For more information visit: http://ntp.niehs.nih.gov/go/10003.

Peer Review

The National Toxicology Program (NTP) convened a virtual external ad hoc panel to peer review the draft *NTP Developmental and Reproductive Toxicity Technical Report on the Modified One-Generation Study of 2-Hydroxy-4-methoxybenzophenone (CASRN 131-57-7) Administered in Feed to Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats with Prenatal and Reproductive Performance Assessments in F_1 Offspring* on October 14, 2021. NTP announced the peer-review meeting in the Federal Register (86 FR 42869. August 5, 2021). The public could view the proceedings online and opportunities were provided for submission of written and oral public comments. The selection of panel members and conduct of the peer review were in accordance with federal policies and regulations. The panel was charged to:

(1) Review and evaluate the scientific and technical elements of each study and its presentation.

(2) Determine whether each study's experimental design, conduct, and findings support NTP's conclusions under the conditions of each study.

NTP carefully considered the panel's recommendations in finalizing the report. The peer-review report is provided in Appendix D. Other meeting materials are available on the NTP website (https://ntp.niehs.nih.gov/go/meeting).

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Abstract

2-Hydroxy-4-methoxybenzophenone (2H4MBP), also known as oxybenzone and benzophenone-3, is approved by the U.S. Food and Drug Administration for use in sunscreens and other personal care products in concentrations of <6%, either alone or in combination formulations, and as an indirect food additive in acrylic and modified acrylic plastics that come into contact with food. Mechanistic screening studies have shown that 2H4MBP and its metabolites are capable of activating the estrogen receptor and antagonizing the androgen receptor to varying degrees. The objective of the present study was to characterize the potential for 2H4MBP to adversely affect any phase of development, maturation, and ability to reproduce in Sprague Dawley (Hsd:Sprague Dawley® SD®) rats administered 2H4MBP in 5K96 feed, a diet low in phytoestrogens, using the National Toxicology Program (NTP) modified one-generation (MOG) study design. Exposure concentrations were based on a dose range-finding study that demonstrated 25,000 ppm 2H4MBP did not induce excessive maternal toxicity or affect parturition, litter size, or pup viability. Exposure concentrations of 3,000, 10,000, and 30,000 ppm were selected; ethinyl estradiol (EE), a synthetic form of estrogen, was included at 0.05 ppm as a positive reference control. 2H4MBP intake by F_0 females in the 3,000, 10,000, 25,000, and 50,000 ppm 2H4MBP groups, based on feed consumption and dietary concentrations from gestation day (GD) 6 through GD 21, was approximately 215, 695, 2,086, and 6,426 mg 2H4MBP/kg body weight/day (mg/kg/day), respectively; from lactation day (LD) 1 through LD 14, 2H4MBP intake was approximately 577, 1,858, 4,460, and 12,029 mg/kg/day, respectively.

Modified One-Generation Study

F_0 exposure began on GD 6 and was continual. At weaning on postnatal day (PND) 28, F_1 offspring were assigned to either reproductive performance (2/sex/litter), prenatal (1/sex/litter), or biological sampling (1/sex/litter) cohorts. Upon sexual maturity, F_1 mating and pregnancy indices were evaluated. In the prenatal cohort, F_2 prenatal development (litter size, fetal weight, and morphology) was assessed on GD 21. In the reproductive performance cohort, littering indices, F_2 viability, and growth were assessed until PND 28. The likelihood of identifying potential 2H4MBP-induced adverse effects (similarity and magnitude thereof) at any phase of growth or development was increased by examining related endpoints in multiple pups within a litter throughout life, across cohorts, and across generations.

2H4MBP exposure at the tested concentrations did not induce any effects on mating or pregnancy indices. In the prenatal cohort, exposure to 30,000 ppm was associated with significantly decreased mean numbers of corpora lutea and F_2 implants and a slightly lower number of live fetuses on GD 21 than in the control group. In the reproductive performance cohort, total F_2 mean litter size on PND 0 was also significantly decreased compared to the control group. 2H4MBP exposure might have affected litter size, although the effect was small in magnitude. Collectively, given the minimal apparent response that may or may not be a direct effect of 2H4MBP, this was considered equivocal evidence of an adverse effect on reproductive performance. EE exposure did not affect F_1 live litter size on PND 0, but significantly decreased mean number of corpora lutea and total F_2 implants were observed.

2H4MBP was associated with lower F_1 and F_2 preweaning and F_1 postweaning mean body weights. At 30,000 ppm 2H4MBP, preweaning F_1 mean body weights of both males and females were progressively lower over time, relative to their respective control groups. The response was

lessened in F_2 males and even more so in F_2 females. Significantly decreased F_1 postweaning mean body weights were not associated with concurrent lower feed consumption. The effects on body weights associated with exposure to 2H4MBP were considered some evidence of developmental toxicity. 2H4MBP intake by F_0 females in the 3,000, 10,000, and 30,000 ppm 2H4MBP groups, based on feed consumption and dietary concentrations from GD 6 through GD 21 was approximately 205, 697, and 2,644 mg/kg/day, respectively; from LD 1 through LD 13, 2H4MBP intake was approximately 484, 1,591, and 5,120 mg/kg/day, respectively. 2H4MBP intake by the F_1 generation postweaning (PND 28 through PND 91) in the 3,000, 10,000, and 30,000 ppm groups was approximately 267, 948, and 3,003 mg/kg/day (males) and 287, 983, and 3,493 mg/kg/day (females), respectively. 2H4MBP intake by the adult F_1 females in the 3,000, 10,000, and 30,000 ppm groups was approximately 240, 825, and 2,760 mg/kg/day (GD 0 through GD 21) and 426, 1,621, and 5,944 mg/kg/day (LD 1 through LD 13), respectively.

Diaphragmatic hernias were observed at a low incidence in 2H4MBP-exposed animals in both the F_1 and F_2 generations but were not observed in any control animals. Most of the diaphragmatic hernias were associated histologically with hepatodiaphragmatic hernias. Low incidences of diaphragmatic and hepatodiaphragmatic hernias have been reported in control groups in other NTP MOG studies. Therefore, it is unclear whether the occurrences of diaphragmatic and hepatodiaphragmatic hernias in both the F_1 and F_2 generations were related to 2H4MBP exposure.

2H4MBP did not alter estrogen or androgen-mediated developmental markers, and no gross lesions were observed at adult necropsy consistent with perturbation of normal estrogen receptor- or androgen-receptor-mediated development. Expected estrogenic responses were observed in the EE group. In the 30,000 ppm group, adult weights of male androgen-dependent reproductive tissues were slightly lower than those of the control males, likely secondary to the apparent growth retardation, and occurred in the absence of histopathological findings. Sperm and spermatid counts were not affected by 2H4MBP exposure. The ability of F_1 males in either cohort to successfully mate, resulting in pregnancy, also was not affected. Unlike findings reported for in vitro cell models, 2H4MBP had no apparent effect on estrogen receptor- or androgen-receptor-dependent processes, nor did it affect mating or pregnancy indices.

2H4MBP exposure in F_1 rats was associated with higher kidney weights, renal tubule epithelial regeneration, interstitial chronic active inflammation, renal tubule and pelvic concretions, renal tubule dilation, papillary necrosis, urothelial hyperplasia, and urothelial ulcers. F_1 females also displayed renal tubule epithelial degeneration, pelvic dilation, chronic progressive nephropathy, and mineralization. 2H4MBP-exposed F_1 males and females displayed higher liver weights relative to their respective control groups. The absolute weight of the adrenal glands was significantly decreased in the 30,000 ppm female group relative to the control group in the reproductive performance cohort. Several other decreases in organ weights were not associated with histological correlates and were considered related to changes in body weights.

F_2 fetal findings of hydronephrosis of the kidney and enlarged liver were observed in the 30,000 ppm group. F_2 offspring in the 30,000 ppm group exhibited dilation of the renal pelvis. The observed fetal, PND 28, and adult necropsy findings were consistent with previously reported studies that identified the kidney and liver as target tissues of 2H4MBP-mediated toxicity.

Conclusions

Under the conditions of this modified one-generation (MOG) study, there was *equivocal evidence of reproductive toxicity* of 2-hydroxy-4-methoxybenzophenone (2H4MBP) in Hsd:Sprague Dawley® SD® rats based on a decrease in F_2 litter size in both the prenatal and reproductive performance cohorts.

Under the conditions of this MOG study, there was *some evidence of developmental toxicity* of 2H4MBP in Hsd:Sprague Dawley® SD® rats based on the observed postnatal growth retardation. The relationship of the increased occurrence of diaphragmatic and hepatodiaphragmatic hernias in F_1 adults and F_2 pups to 2H4MBP exposure is unclear.

Exposure to 2H4MBP was not associated with signals consistent with alterations in estrogenic, androgenic, or antiandrogenic action. Exposure to 2H4MBP was associated with lower F_1 and F_2 mean body weights; this effect on body weight contributed to the apparent 2H4MBP-related decreases in male reproductive organ weights. Mating and littering were not significantly affected by 2H4MBP exposure. Exposure to 2H4MBP was associated with nonneoplastic kidney lesions in the F_0, F_1, and F_2 generations. Expected estrogenic responses were observed in the EE group.

Synonyms: Benzophenone-3; (2-hydroxy-4-methoxyphenyl)-phenylmethanoneoxybenzone; oxybenzone

Summary of Exposure-related Findings in Rats in the Modified One-Generation Study of 2-Hydroxy-4-methoxybenzophenone

	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm
F_0 Generation					
Maternal Parameters					
Number mated	25	25	25	25	25
Number pregnant (%)	22 (88.0)	21 (84.0)	22 (88.0)	20 (80.0)	20 (80.0)
Number not pregnant (%)	3 (12.0)	4 (16.0)	3 (12.0)	5 (20.0)	5 (20.0)
Number littered (%)	22 (100.0)	21 (100.0)	22 (100.0)	20 (100.0)	18 (90.0)
Clinical Observations	None	None	None	None	None
Mean Body Weight and Feed Consumption[a,b]					
Body weight: GD 21	375.2 ± 4.5**	366.6 ± 5.6	357.2 ± 4.7**	338.5 ± 3.9**	328.2 ± 5.1**
Body weight gain: GD 6–21	132.3 ± 3.0**	127.1 ± 3.4	118.1 ± 3.2**	99.3 ± 2.5**	86.4 ± 3.8**
Feed consumption: GD 6–21	20.0 ± 0.3*	19.6 ± 0.4	19.7 ± 0.5	23.9 ± 1.0*	20.3 ± 1.5
Body weight: LD 28	286.3 ± 3.1**	282.1 ± 3.7	277.1 ± 3.0	257.4 ± 4.0**	249.3 ± 4.0**
Body weight gain: LD 1–28	18.0 ± 3.3	22.0 ± 2.4	22.6 ± 2.8	12.7 ± 3.2	23.8 ± 1.9
Feed consumption: LD 1–13	45.3 ± 0.9*	45.8 ± 1.0	43.8 ± 0.9	43.6 ± 1.9	41.3 ± 1.7*
Necropsy Observations	None	None	None	None	None
F_1 Generation (Preweaning)[b]					
Clinical Observations	None	None	None	None	None
Live Litter Size					
PND 0	12.4 ± 0.6	12.5 ± 0.7	12.8 ± 0.5	11.7 ± 0.4	12.3 ± 0.6
PND 4 (prestandardization)	12.2 ± 0.5	13.0 ± 0.5	12.5 ± 0.5	11.7 ± 0.4	11.4 ± 0.9
PND 4 (poststandardization)	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1	8.0 ± 0.0	7.9 ± 0.1
PND 28	7.8 ± 0.1	7.9 ± 0.1	7.7 ± 0.1	7.8 ± 0.1	7.4 ± 0.2**
Male Pup Mean Body Weight					
PND 1	7.26 ± 0.10**	7.17 ± 0.10	6.89 ± 0.11*	6.88 ± 0.10*	6.34 ± 0.19**
PND 28	89.91 ± 1.08**	86.26 ± 1.53	81.11 ± 1.21**	67.93 ± 2.16**	80.46 ± 1.15**
Female Pup Mean Body Weight					
PND 1	6.88 ± 0.11*	6.87 ± 0.10	6.61 ± 0.11	6.63 ± 0.09	6.23 ± 0.12**
PND 28	80.35 ± 1.19**	78.14 ± 1.62	73.04 ± 1.12**	60.70 ± 1.53**	74.64 ± 1.11**
F_1 Generation (Postweaning)					
Mean Body Weight and Feed Consumption[a,b]					
Male body weight: PND 28	87.6 ± 1.1**	84.7 ± 1.5	79.5 ± 1.2**	65.7 ± 2.3**	78.2 ± 1.2**
Male body weight: PND 91	393.0 ± 5.0**	387.6 ± 4.3	372.5 ± 5.2*	330.4 ± 6.8**	322.8 ± 4.5**
Male feed consumption: PND 28–91	24.1 ± 0.4	23.9 ± 0.4	24.3 ± 0.3	23.0 ± 0.5	20.8 ± 0.3**

	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm
Female body weight: PND 28	78.0 ± 1.0**	75.6 ± 1.6	71.5 ± 1.3**	58.7 ± 1.6**	72.3 ± 1.1**
Female body weight: PND 91	246.6 ± 3.5**	242.8 ± 3.2	236.9 ± 3.2	211.9 ± 2.7**	204.3 ± 3.0**
Female feed consumption: PND 28–91	17.4 ± 0.3	17.2 ± 0.3	17.2 ± 0.3	18.3 ± 0.3	16.7 ± 0.5
F_1 and F_2 Generations					
Endocrine Endpoints, Developmental Landmarks, and Pubertal Endpoints[b]					
Vaginal opening (F_1)					
Mean day of vaginal opening (litter mean)	35.3 ± 0.2**	35.4 ± 0.4	35.9 ± 0.3	38.1 ± 0.4**	24.3 ± 0.3**
Adjusted mean day of vaginal opening (litter mean)[c]	35.9 ± 0.2*	35.8 ± 0.3	35.9 ± 0.3	37.0 ± 0.3	24.3 ± 0.2**
Body weight at acquisition[a]	115.7 ± 1.9**	114.3 ± 1.6	111.5 ± 1.6	109.0 ± 1.9*	59.0 ± 1.5**
Balanopreputial separation (F_1)					
Mean day of balanopreputial separation (litter mean)	43.7 ± 0.3**	44.0 ± 0.4	44.9 ± 0.3*	47.1 ± 0.4**	45.8 ± 0.3**
Adjusted mean day of balanopreputial separation (litter mean)[c]	44.7 ± 0.3	44.7 ± 0.3	44.8 ± 0.3	45.4 ± 0.3	44.8 ± 0.3
Body weight at acquisition[a]	204.4 ± 2.9**	203.3 ± 2.9	196.4 ± 2.2	192.1 ± 2.8**	184.7 ± 2.2**
Prenatal Cohort					
Mating and Fertility Performance					
Number of mating pairs	22	20	22	20	15
Number mated	19	19	21	19	15
Mated females/paired (%)	86.4	95.0	95.5	95.0	100.0
Precoital interval (days)[b]	4.3 ± 0.7	5.3 ± 1.0	4.1 ± 0.8	3.9 ± 0.6	3.4 ± 0.5
Number not pregnant	4	2	2	1	0
Mean Body Weight and Feed Consumption[a,b]					
Body weight gain: GD 6–21	138.9 ± 4.2**	136.4 ± 3.0	117.9 ± 6.3*	103.6 ± 7.4**	108.4 ± 4.4**
Feed consumption: GD 0–21	23.5 ± 0.4	22.7 ± 0.6	23.2 ± 0.7	24.1 ± 0.9	23.1 ± 1.4
Uterine Content Data[b]					
Mean number of corpora lutea/female	18.56 ± 0.77**	17.56 ± 0.77	17.40 ± 0.89	14.89 ± 0.87**	13.53 ± 0.47**
Implantations/female	15.61 ± 0.65**	14.94 ± 0.67	13.28 ± 1.17	12.94 ± 0.88*	12.13 ± 0.79**
Live fetuses/litter	14.94 ± 0.82	14.63 ± 0.59	12.67 ± 1.17	13.24 ± 0.57	11.60 ± 0.76**
Fetal Findings					
External findings	None	None	None	None	None

	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm
Visceral findings[d]					
Enlarged liver – [M]					
Fetuses	0 (0.0)	1 (0.43)	2 (0.88)	7 (3.11)	0 (0.0)
Litters	0 (0.00)	1 (6.25)	1 (5.56)	2 (11.76)	0 (0.00)
Distended ureter, bilateral – [V]					
Fetuses	4 (1.49)	11 (4.7)	15 (6.58)[#]	10 (4.44)	12 (6.9)[#]
Litters	3 (16.67)	6 (37.50)	8 (44.44)	5 (29.41)	7 (46.67)
Distended ureter – [V]					
Fetuses	13 (4.83)	25 (10.68)	29 (12.72)	19 (8.44)	22 (12.64)
Litters	8 (44.44)	10 (62.50)	9 (50.00)	6 (35.29)	7 (46.67)
Skeletal findings	None	None	None	None	None
Reproductive Performance Cohort					
Mating and Fertility Performance					
Number of mating pairs	41	40	40	40	30
Number mated	40	37	35	35	29
Mated females/paired (%)	97.6	92.5	87.5	87.5	96.7
Precoital interval[b]	4.7 ± 0.6	4.8 ± 0.5	5.1 ± 0.7	4.2 ± 0.8	4.0 ± 0.6
Number not pregnant	6	3	7	8	2
Mean Body Weight and Feed Consumption[a,b]					
Body weight gain: GD 6–21	141.6 ± 3.7**	136.2 ± 3.3	123.3 ± 3.7**	101.1 ± 4.8**	112.9 ± 3.3**
Feed consumption: GD 0–21	27.8 ± 0.8	26.6 ± 0.7	26.1 ± 0.8	25.4 ± 0.6	22.5 ± 0.9**
Body weight: LD 28	317.8 ± 5.1**	316.4 ± 4.0	300.9 ± 3.9*	260.9 ± 4.0**	255.9 ± 4.7**
Body weight gain: LD 1–28	8.6 ± 2.9	7.0 ± 2.7	12.6 ± 3.2	12.8 ± 4.0	12.3 ± 2.5
Feed consumption: LD 1–13	44.8 ± 1.1*	45.9 ± 1.3	48.6 ± 1.7	50.4 ± 2.1	45.6 ± 1.6
Live Litter Size[b]					
PND 0	13.6 ± 0.5*	12.9 ± 0.6	12.4 ± 0.9	12.0 ± 0.4*	11.3 ± 0.5**
PND 4 (prestandardization)	13.1 ± 0.4*	12.6 ± 0.6	11.9 ± 0.8	11.5 ± 0.4	10.8 ± 0.5**
PND 4 (poststandardization)	7.8 ± 0.2	7.6 ± 0.2	7.6 ± 0.3	7.9 ± 0.1	7.6 ± 0.2
PND 28	5.7 ± 0.4	5.9 ± 0.3	5.7 ± 0.3	5.9 ± 0.3	6.7 ± 0.3*
Male Pup Mean Body Weight[b]					
PND 1	6.95 ± 0.12	7.17 ± 0.14	7.06 ± 0.14	6.75 ± 0.09	6.53 ± 0.10**
PND 28	72.28 ± 1.90**	80.42 ± 2.01**	75.41 ± 1.76	61.82 ± 2.46**	76.78 ± 1.19
Female Pup Mean Body Weight[b]					
PND 1	6.67 ± 0.13**	6.90 ± 0.12	6.52 ± 0.13	6.37 ± 0.09	6.22 ± 0.10**
PND 28	69.12 ± 1.70**	70.49 ± 1.96	66.19 ± 1.70	54.49 ± 2.09**	71.12 ± 1.03

	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm
Adult Necropsies					
Gross Necropsy Findings					
Prenatal Cohort					
Male	Kidney: dilation, unilateral [0, 0, 2 (2), 0, 0]; enlarged, unilateral [0, 0, 0, 1 (1), 0]; enlarged, bilateral [0, 0, 0, 5 (5), 0]; discolored, dark, unilateral [0, 0, 0, 0, 0]; discolored, dark, bilateral [0, 0, 0, 4 (4), 0]; discolored, dark, unilateral or bilateral [0, 0, 0, 4 (4), 0]; discolored, pale, unilateral [0, 0, 0, 4 (4), 0]; discolored, pale, bilateral [0, 0, 0, 0, 0]; discolored, pale, unilateral or bilateral [0, 0, 0, 4 (4), 0]; discolored, mottled, unilateral [0, 0, 0, 0, 0]; discolored, mottled, bilateral [0, 0, 0, 1 (1), 0]; discolored, mottled, unilateral or bilateral [0, 0, 0, 1 (1), 0] Urinary bladder: discoloration, brown [0, 0, 0, 9 (9), 0] Diaphragm: hernia [0, 0, 0, 0, 0]				
Reproductive Performance Cohort					
Male	Kidney: dilation, unilateral [1 (1), 0, 0, 1 (1), 0]; enlarged, unilateral [0, 0, 0, 0, 0]; enlarged, bilateral [0, 0, 1 (1), 1 (1), 0]; discolored, dark, unilateral [0, 0, 0, 4 (4), 0]; discolored, dark, bilateral [0, 0, 0, 15 (12), 0]; discolored, dark, unilateral or bilateral [0, 0, 0, 19 (14), 0]; discolored, pale, unilateral [0, 0, 0, 4 (4), 0]; discolored, pale, bilateral [0, 0, 0, 1 (1), 0]; discolored, pale, unilateral or bilateral [0, 0, 0, 5 (5), 0] Urinary bladder: discoloration, brown [0, 0, 0, 16 (14), 0] Diaphragm: hernia [0, 0, 0, 1 (1), 1 (1)]				
Female	Kidney: dilation, unilateral [0, 1 (1), 0, 2 (2), 0]; enlarged, unilateral [0, 0, 0, 1 (1), 0]; discolored, dark, unilateral [0, 0, 0, 1 (1), 0]; discolored, dark, bilateral [0, 0, 0, 1 (1), 0]; discolored, dark, unilateral or bilateral [0, 0, 0, 2 (2), 0]; discolored, pale, unilateral [0, 0, 0, 4 (3), 0]; discolored, pale, bilateral [0, 0, 0, 3 (3), 0]; discolored, pale, unilateral or bilateral [0, 0, 0, 7 (6), 0]; discolored, mottled, unilateral [0, 0, 0, 0, 0]; discolored, mottled, bilateral [0, 2 (2), 0, 0, 0]; discolored, mottled, unilateral or bilateral [0, 2 (2), 0, 0, 0] Diaphragm: hernia [0, 2 (2), 1 (1), 3 (3), 0]				
Organ Weights					
Prenatal Cohort					
Male	–	↑Kidney, liver weights	↑Kidney, liver weights	↑Kidney, liver weights ↓Testis, epididymis weights	↑ Kidney, liver weights
Female	–	↑Liver weights	↑Liver weights ↓Ovary weights	↑Liver weights ↓Ovary weights	↑Liver weights ↓Ovary weights

	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm
Reproductive Performance Cohort					
Male	–	↑Kidney, liver weights	↑Kidney, liver weights	↑Kidney, liver weights ↓Testis, epididymis, ventral prostate weights	↑Kidney, liver weights
Female	–	↑Kidney, liver weights	↑Kidney, liver weights ↓Ovary weight	↑Kidney, liver weights ↓ Ovary, adrenal gland weights	↑Liver weight ↓Ovary weight
Nonneoplastic Lesions					
Reproductive Performance Cohort[e]					
Male	Kidney: renal tubule, epithelium, regeneration [0, 0, 0, 33 (17)]; interstitium, inflammation, chronic active [0, 0, 0, 22 (14)]; renal tubule, concretion [0, 0, 0, 35 (19)]; pelvis, concretion [0, 0, 0, 17 (13)]; renal tubule, dilation [0, 0, 0, 37 (20)]; urothelium, hyperplasia, total [0, 1 (1), 0, 18 (15)]; urothelium, ulcer [0, 0, 0, 12 (9)]; papilla, necrosis [0, 0, 0, 10 (10)] Diaphragm: hepatodiaphragmatic hernia [0, 0, 1 (1), 1 (1)]				
Female	Kidney: renal tubule, epithelium, regeneration [0, 0, 3 (3), 13 (12)]; interstitium, inflammation, chronic active [0, 0, 0, 8 (8)]; renal tubule, concretion [0, 0, 0, 13 (12)]; pelvis, concretion [0, 0, 0, 9 (5)]; renal tubule, dilation [0, 0, 0, 28 (19)]; urothelium, hyperplasia, diffuse [0, 0, 0, 15 (12)]; urothelium, ulcer [0, 0, 0, 6 (6)]; papilla, necrosis [0, 0, 0, 4 (3)]; renal tubule, epithelium, degeneration [0, 0, 0, 21 (14)]; pelvis, dilation, total [0, 1 (1), 0, 5 (5)]; chronic progressive nephropathy [18 (14), 35 (19), 29 (19), 22 (17)]; mineralization [9 (8), 28 (17), 24 (18), 10 (8)] Diaphragm: hepatodiaphragmatic hernia [0, 2 (2), 1 (1), 4 (3)]				

Level of Evidence of Reproductive Toxicity: Equivocal evidence

Level of Evidence of Developmental Toxicity: Some evidence

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; ** at $p \leq 0.01$.
#Statistically significant at $p \leq 0.05$ in litter-based analysis of fetuses.
EE = ethinyl estradiol; GD = gestation day; LD = lactation day; PND = postnatal day; [M] = malformation; [V] = variation.
[a]Body weight results given in grams. Feed consumption results given in grams/animal/day.
[b]Data are presented as mean ± standard error.
[c]Adjusted based on body weight at weaning.
[d]Upper row denotes number of affected fetuses (%) and lower row the number of affected litters (%).
[e]Nonneoplastic lesions were not evaluated in the EE group.

Overview

The National Toxicology Program (NTP) has assessed the potential adverse effects of sunscreens using in vitro and in vivo model systems; the data presented herein are part of that larger effort. The scope of 2-hydroxy-4-methoxybenzophenone (2H4MBP) studies includes the assessment of potential endocrine activity in the U.S. Environmental Protection Agency Endocrine Disruptor Screening Program Phase 1 studies (estrogen- and androgen-receptor binding and activation, Hershberger and uterotrophic assays, aromatase inhibition, and steroid synthesis inhibition) and characterization of the potential effects of continuous 2H4MBP exposure over multiple generations using the NTP modified one-generation study design. In this study, exposure to 2H4MBP in feed began on gestation day (GD) 6. At weaning, 1 and 2 pups/sex/litter were allocated to prenatal and reproductive performance cohorts, respectively; an additional 1 pup/sex/litter was allocated to the biological sampling cohort. In addition to an assessment of reproductive performance, F_2 fetal outcomes (GD 21 fetal examinations) were assessed in the prenatal cohort and the potential effects on parturition and early growth of the F_2 generation were assessed in the reproductive performance cohort. Internal dose metrics were also assessed. Apical indicators sensitive to endocrine modulation were measured. The U.S. Food and Drug Administration's National Center for Toxicological Research (NCTR), in partnership under an Interagency Agreement, has also examined the effects of maternal and lactational exposure to 2H4MBP on development and reproductive organs in male and female rat offspring and on transcriptional changes in the testes and prostates of young rats. NCTR is also conducting fertility, embryo-fetal, and pre- and postnatal rat studies to characterize the potential effects of 2H4MBP exposure. This report complements the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) S5r2 guideline studies on 2H4MBP conducted by NCTR and allows for the comparison of study designs and outcomes. NTP previously conducted rat and mouse 2- and 13-week toxicity studies by dermal and oral routes of exposure and assessed the genotoxic potential of 2H4MBP. Potential effects of 2H4MBP exposure on mouse reproduction were assessed using the Reproductive Assessment by Continuous Breeding protocol. NTP has also conducted 2-year toxicology and carcinogenesis studies in rats (including perinatal exposure) and mice using dietary exposure.

Introduction

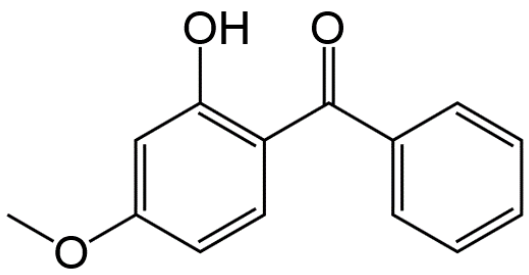


Figure 1. 2-Hydroxy-4-methoxybenzophenone (CASRN 131-57-7; Chemical Formula: $C_{14}H_{12}O_3$; Molecular Weight: 228.25)

Synonyms: Benzophenone-3; (2-hydroxy-4-methoxyphenyl)-phenylmethanoneoxybenzone; oxybenzone.

Chemical and Physical Properties

2-Hydroxy-4-methoxybenzophenone (2H4MBP) is an off-white to light-yellow powder with a melting point of 62°C to 65°C. 2H4MBP is relatively insoluble in water (69 mg/kg at 25°C) and is readily soluble in most organic solvents. 2H4MBP absorbs ultraviolet (UV) A (320–400 nm) and UVB (290–320 nm) light and is photostable.[1]

Production, Use, and Human Exposure

2H4MBP is synthesized by condensation of benzoic acid with resorcinol monomethyl ether in the presence of heat, zinc chloride, and polyphosphoric acid or by the Friedel-Crafts reaction of benzoyl chloride with 3-hydroxyanisole.[2]

2H4MBP is commonly used in sunscreens and other personal care products at concentrations of up to 6% to protect the user from solar erythema. According to the Environmental Working Group's Guide to Sunscreens database,[3] 2H4MBP is found in more than 1,000 products, including beach, sport, and baby sunscreens (619), moisturizers with SPF (150), and lip balms (109). 2H4MBP is also used as a photostablizer for synthetic resins and polymers to prevent UV degradation.[4; 5] Exposure can occur when present in acrylic and modified acrylic plastics that come into contact with food.[6]

2H4MBP and its metabolites are typically excreted in urine. A study using National Health and Nutrition Examination Survey (NHANES) cycle data from 2004 to 2012 demonstrated that more than 96% of the 10,232 samples (representing all populations) contained measurable urinary concentrations of 2H4MBP. Creatinine-adjusted urinary least square geometric mean concentrations ranged from 9 to 17 ng/mL in males, and from 18 to 45 ng/mL in females. Children and adolescent concentrations ranged from 17 to 27 ng/mL and from 13 to 24 ng/mL, respectively.[7; 8] Higher urinary concentrations of 2H4MBP were observed in non-Hispanic whites (28 ng/mL) than in Mexican Americans (17 ng/mL) or non-Hispanic blacks (13 ng/mL) and have been attributed to increased sunscreen use.[9] Higher urinary concentrations in females have been ascribed to the use of personal care products (e.g., lip balms, cosmetics) that often contain 2H4MBP.[9]

Regulatory Status

2H4MBP is approved by the U.S. Food and Drug Administration (FDA) for use as a sunscreen when present up to 6%, either alone or in combination formulations and as an indirect food additive present in acrylic and modified acrylic plastics that come into contact with food.[6; 10] Section 8(a) of the Toxic Substances Control Act requires manufacturers of 2H4MBP to report preliminary assessment information concerned with production, exposure, and use to the U.S. Environmental Protection Agency (EPA). The FDA has drafted a proposed rule, "Sunscreen Drug Products for Over-the-Counter Human Use."[10]

Absorption, Distribution, Metabolism, and Excretion

Experimental Animals

2H4MBP was well absorbed (≥63.9%) following a single oral gavage administration of [^{14}C]2H4MBP (3.01–2,570 mg/kg body weight) in male Fischer 344 (F344)/N rats, with the administered dose excreted primarily via urine (63.9% to 72.9%) and feces (19.3% to 41.7%) by 72 hours postadministration. The radioactivity remaining in tissues 72 hours after administration was low (approximately 0.1%) in all dose groups.[11] Following dermal application of 51.6, 204, and 800 μg [^{14}C]2H4MBP (in ethanol) to male rats, the dose was excreted mainly via urine (32.4%, 39.2%, and 13.2%, respectively) and feces (16.9%, 22.2%, and 9.15%, respectively) by 72 hours postapplication. The dose excreted in urine and feces suggests that the applied dose absorbed was 49.3%, 61.4%, and 22.4%, respectively, for 51.6, 204, and 800 μg [^{14}C]2H4MBP. When the dose (50 μg) was applied dermally in a lotion vehicle, the dose absorbed (51.8%) was similar to that in ethanol with 33.9% and 17.9% of the dose recovered in urine and feces, respectively.[11]

Absorption, distribution, metabolism, and excretion (ADME) were also investigated in male and female Sprague Dawley rats and B6C3F1/N mice following gavage administration of [^{14}C]2H4MBP.[12] Following a single gavage administration (10, 100, or 500 mg/kg [^{14}C]2H4MBP) in rats, most of the administered dose was excreted in urine (53% to 58%) and feces (25% to 42%) by 72 hours postadministration with no observable sex difference in excretion. The radioactivity in urine suggests that ≥53% of the administered dose was absorbed. Following a single 100 mg/kg gavage dose in male mice, urinary (≥34%) and fecal (≥24%) excretion was similar to that of rats. Mice excreted a higher percentage (5% to 15%) of the administered dose as exhaled CO_2, however, compared to rats (approximately 1%). The retention of dose in tissues was low at 72 hours (<1%) in all gavage groups.

ADME of 2H4MBP was investigated in Sprague Dawley rats and B6C3F1/N mice at 72 hours following dermal application of 0.1 or 10 mg/kg [^{14}C]2H4MBP formulated in several vehicles.[12] In male rats, the highest absorption was observed following application in light paraffin oil (80%). Absorption following application in ethanol, ethanol:coconut oil (1:1), or coconut oil alone was comparable to paraffin oil (64% to 73%). In contrast, the absorption of 2H4MBP from the lotion vehicle (olive oil:emulsifying wax:water [15:15:70 v:v:v]) in male (10 mg/kg, 46%) and female (15 mg/kg, 29%) rats was lower relative to other vehicles. Both male and female mice absorbed approximately 60%–69% of the 10 mg/kg dose in ethanol or acetone and 37%–46% of the 10 mg/kg dose when formulated in the lotion vehicle. There was no dose-related effect on absorption (0.1 versus 10 mg/kg) in either male rats or mice.[12]

Kinetics of disposition of 2H4MBP have been investigated in rats in limited studies. Following a single gavage dose of 100 mg/kg 2H4MBP in male Sprague Dawley rats, the time (T_{max}) to reach the maximum plasma concentration, C_{max} (21.21 µg/mL) was 3 hours; the elimination of 2H4MBP in plasma was biphasic with alpha and beta half-lives of 0.88 and 15.9 hours, respectively. Of the tissues examined, the liver had the highest concentration of 2H4MBP and conjugated 2H4MBP at 6 hours.[13] In another study, following a 100 mg/kg gavage dose in male Sprague Dawley rats, similar plasma T_{max} (2.72 hours) and C_{max} (21.21 µg/mL) were observed, with an elimination half-life of 4.58 hours.[14] Following a single gavage dose of 10 mg/kg in male and female Sprague Dawley rats, plasma T_{max} and C_{max} were 6.0 hours and 8.5 ng/mL, respectively, for males and 2.3 hours and 2.9 ng/mL, respectively, for females. The plasma elimination half-life was 6.4 hours for males and 18.5 hours for females. The bioavailability of 2H4MBP in male and female rats was <1%, demonstrating extensive first-pass metabolism of 2H4MBP following gavage administration.[12]

Consistent with low bioavailablity, 2H4MBP is metabolized via numerous pathways in rodents, including demethylation, oxidation, glucuronidation, and sulfation. Products identified in bile and/or urine of rodents following administration of 2H4MBP were 2H4MBP, 2,4-dihydroxybenzophenone (DHB), 2,3,4-trihydroxybenzophenone (THB), 2,5-dihydroxy-4-methoxybenzophenone (D2H4MBP), and their corresponding glucuronide and sulfate conjugates (Figure 2).[11-13; 15] Similar metabolites were also observed in vitro following incubation of 2H4MBP with microsomes.[16; 17] 2H4MBP and DHB have been quantified in serum from pregnant rats.[18]

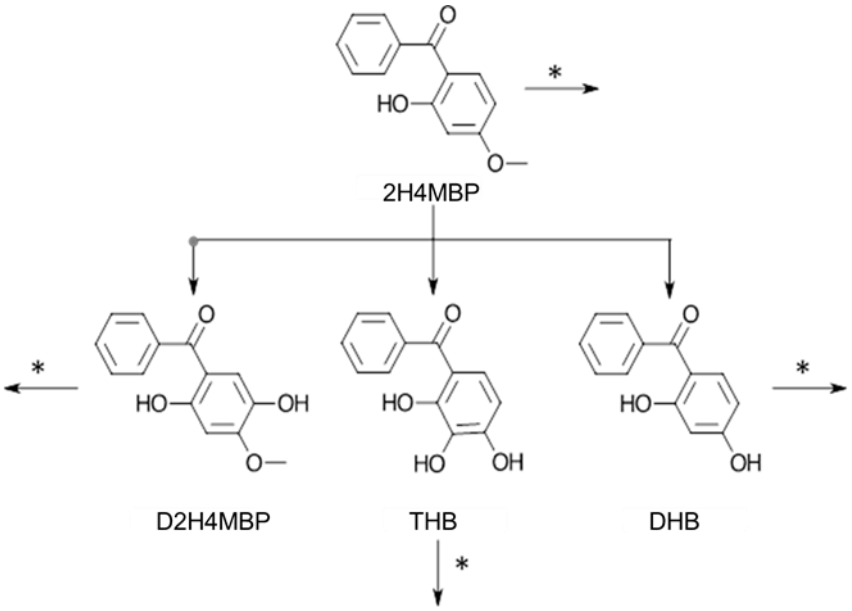


Figure 2. Metabolism of 2-Hydroxy-4-methoxybenzophenone in Rodents

2H4MBP = 2-hydroxy-4-methoxybenzophenone; D2H4MBP = 2,5-dihydroxy-4-methoxybenzophenone;
THB = trihydroxybenzophenone; DHB = dihydroxybenzophenone.
*Indicates glucuronide and sulfate conjugates.

Humans

ADME data on 2H4MBP in humans are limited. Human studies with sunscreens have demonstrated that 2H4MBP is readily absorbed from the skin.[19] A study that used excised human epidermis in Franz diffusion cells showed that approximately 10% of the dermally applied dose of 2H4MBP is absorbed.[20] When applied dermally, 2H4MBP and the metabolites DHB and 2,2'-dihydroxy-4-methoxybenzophenone can be detected in serum and are excreted in urine.[21; 22] A study examining the absorption of 2H4MBP and subsequent irradiation with UVA and UVB rays demonstrated that participants excreted 1.2%–8.7% (mean 3.7%) of the total applied dose in urine. 2H4MBP was detected in urine 3–5 days after application. UV irradiation did not affect the amount of 2H4MBP excreted.[23] Frequency of sunscreen use is also related to urinary 2H4MBP concentrations, with frequent users having much higher urinary concentrations.[24] 2H4MBP has been detected in maternal urine[25] and breast milk.[26; 27] Human geometric mean maximum plasma concentrations of 2H4MBP have been shown to be approximately 200 ng/mL when topically applied. This concentration exceeds the FDA guidance of 0.5 ng/mL that would necessitate the conduct of additional nonclinical toxicity studies.[28]

Developmental and Reproductive Toxicity

Models of Endocrine Activity

2H4MBP has been reported to bind to and activate estrogen receptor (ER) alpha (ERα) with a median effective concentration (EC_{50}) ranging from approximately 3 to 20 μM.[29-32] 2H4MBP can also activate estrogen receptor beta (ERβ),[31; 33] and reports indicate that 2H4MBP can act as ERα, ERβ, and progesterone receptor antagonists.[31-33] In NTP-sponsored ER binding and activation studies[34] conducted under OPPTS[a] 890.1250[35] and OPPTS 890.1300,[36] maximal mean specific binding was >75%, which categorizes 2H4MBP as "not interactive"; however, 2H4MBP was able to induce a luciferase response, albeit weak (>10%; logEC_{50}s of −3.2 and −4.0 M). 2H4MBP acts as an estrogen in stimulating MCF7 cell proliferation (EC_{50} of 3.7×10^{-6} M). 2H4MBP has been shown to induce a uterotrophic response (median effective dose [ED_{50}] of 1,000–1,500 mg/kg per day) in immature rats,[37] but 2H4MBP did not cause a uterotrophic response in ovariectomized rats when tested ≤1 g/kg in an NTP study.[34] 2H4MBP was evaluated in quantitative (dose-response) high-throughput screening assays by NTP in the Toxicology in the 21st Century (Tox21) program, and activity was observed in assays measuring stimulation of ER, progesterone receptor, constitutive androstane receptor, pregnane X receptor, retinoic acid receptor, and estrogen-related receptor signaling pathways. In addition, 2H4MBP was shown to inhibit androgen-receptor signaling (https://pubchem.ncbi.nlm.nih.gov/compound/4632#section=BioAssay-Results&fullscreen=true).

2H4MBP exposure in male rainbow trout and Japanese medaka has been shown to induce vitellogenin production (an estrogenic response), decrease the number of eggs produced, and reduce egg viability and hatching.[38] 2H4MBP has also been shown to increase plasma

[a]Guidelines issued before April 22, 2010, refer to "OPPTS" because the office name changed from "Office of Prevention, Pesticides and Toxic Substances" to "Office of Chemical Safety and Pollution Prevention" (or "OCSPP").

concentrations of testosterone in male adult Japanese medaka and to decrease the estradiol-to-testosterone ratio in both male and female fish with concomitant downregulation of gonadal steroidogenic genes (*star*, *cyp11a*, *cyp17*, *hsd3b*, *hsd17b3*, and *cyp19a*).[39]

Experimental Animals

The effects of 2H4MBP exposure on sperm density and vaginal cytology have been reported.[40] Rats and mice received 0, 3,125, 12,500, or 50,000 ppm in the diet for 90 days. Male rats exposed to 50,000 ppm weighed 30% less than control animals, displayed lower epididymis and caudal epididymis weights (17% and 22%, respectively), and lower sperm density (27%). Females displayed a slight increase in estrous cycle length (>1 day) in the 12,500 and 50,000 ppm groups. Male mice in the 50,000 ppm group displayed a 27% decrease in sperm density and weighed 16% less than control animals. Female mice in the 50,000 ppm group displayed a slight increase in estrous cycle length relative to control animals (>0.5 days). NTP conducted a Reproductive Assessment by Continuous Breeding (RACB) study in mice at exposure concentrations of 12,500, 25,000, or 50,000 ppm in the diet.[41] 2H4MBP exposure had no effect on F_0 fertility, but the number of live pups per litter was significantly reduced in the 25,000 and 50,000 ppm groups, which was associated with lower parental mean body weights. There were no changes in sperm density or estrous cyclicity; however, the cumulative days to litter were increased in the 50,000 ppm group. 2H4MBP had minimal effects on fertility in the F_1 generation, but pup weights were significantly decreased relative to the control group. Collectively, the studies indicated that 2H4MBP caused systemic toxicity but had minimal effects on fertility and reproduction at the exposure concentrations used. Another study examined the effects of 0, 10, 20, 100, or 400 mg/kg of 2H4MBP dermally applied to mice 5 days per week for 13 weeks. No effects on mean body weight, organ weights, sperm density, or testicular histopathology were attributed to 2H4MBP exposure.[42]

The effects of maternal and lactational exposure to 2H4MBP on F_1 development and reproductive organs have been assessed.[18] Rats received 0, 1,000, 3,000, 10,000, 25,000, or 50,000 ppm 2H4MBP in the diet from gestation day (GD) 6 until weaning on postnatal day (PND) 23. Exposure to 2H4MBP was associated with increased liver and kidney weights in dams. Clinical pathology findings in dams assessed on GDs 10, 15, and 20 and lactation day 23 included elevation of glucose, alanine aminotransferase, alkaline phosphatase, cholesterol, and total bile acids, as well as depression of aspartate aminotransferase, blood urea nitrogen, and creatinine. These findings occurred primarily in the higher dosed groups and often at all time points. Alanine aminotransferase and cholesterol were elevated in the male and female offspring at the 25,000 and 50,000 ppm exposure concentrations. No significant differences were observed in littering parameters. Male and female pups in the 25,000 and 50,000 ppm groups displayed lower body weights than control pups. Male anogenital distance, adjusted for body weight at PND 23, was significantly decreased in the 50,000 ppm group relative to the control group. At necropsy on PND 23, relative female liver weights were higher than those of the control group at exposure concentrations ≥10,000 ppm. In the 50,000 ppm group, spermatocyte development was impaired and ovarian follicular development was delayed.

Endocrine Disruptor Screening Panel Studies

The potential for 2H4MBP to bind to the ER was assessed in accordance with EPA guideline OPPTS 890.1250.[35] In each of three independent experiments, the maximal mean specific

binding was >75% at every soluble 2H4MBP concentration assessed, thereby categorizing 2H4MBP as "not interactive." When the specific binding was averaged using the scoring system as described in the OPPTS guideline, 2H4MBP was classified as "not interactive" with a median inhibitory concentration (IC_{50}) of approximately 2.3×10^{-4} to 14.8×10^{-4} M. In the ER transcriptional activation assay, conducted in accordance with EPA guideline OPPTS 890.1300,[35] 2H4MBP at 10^{-5} M induced relative luciferase activity of 14.9% and 20.9% in each respective run. 2H4MBP was considered a "positive" agent, per OPPTS 890.1300, because it exceeded 10% of the response of the positive control. 2H4MBP was assessed in a uterotrophic assay in accordance with OPPTS 890.1600,[35] and 2H4MBP did not significantly alter uterine wet or blotted weights.[34]

The potential for 2H4MBP to bind to the rat androgen receptor was assessed in accordance with OPPTS 890.1150.[35] 2H4MBP tested up to 10^{-4} M did not displace more than 50% of the [^{3}H]-R1881, a synthetic androgen-receptor agonist, categorizing 2H4MBP as "equivocal." The potential for 2H4MBP to induce androgenic agonist and antagonist transactivation activity was assessed in MDA-kb2 reporter cells that had been stably transfected with a mouse mammary tumor virus luciferase-neo reporter construct containing the androgen response element. In all independent runs of the agonist transcriptional activation assay, 2H4MBP did not increase in luciferase activity at any of the viable soluble concentrations tested. In two of three runs, the decrease in dihydrotestosterone-induced luciferase activity resulting from 2H4MBP exposure was approximately 25% at the highest feasible dose of $10^{-4.5}$ M, with the first run exhibiting a luciferase activity of 72.2% of maximal. The potential for 2H4MBP to have an androgenic or antiandrogenic response was assessed in a Hershberger bioassay conducted in accordance with OPPTS 890.1400.[35] In the absence of androgenic action, 2H4MBP up to 1,000 mg/kg did not have any effect on androgen-dependent organ weights, demonstrating that 2H4MBP does not exhibit any in vivo androgenic activity in this model system. Rats co-administered 1,000 mg/kg of 2H4MBP and testosterone propionate displayed significantly decreased day 10 mean body weight and body weight gain (7% and 28%, respectively) relative to the control group. The mean weights of the glans penis and ventral prostate were also significantly decreased (6% and 20%, respectively). The weight of the seminal vesicles was also significantly decreased; however, when concurrent body weight is used as a covariate, the magnitude of the response is lower and no longer attains statistical significance. The fact that these organ weight changes only occurred in the presence of lower body weights at the highest dose assessed suggests that they could be secondary to effects on body weight.[34]

The potential for 2H4MBP to act as an inhibitor of aromatase activity was assessed using human CYP19 (aromatase) and P450 reductase Supersomes™ 2H4MBP in accordance with OPPTS 890.1200.[35] 2H4MBP was classified as equivocal, as it produced a mean aromatase activity level of 51% (±13% SD) of control activity at the highest soluble test concentration of 10^{-4} M.[34]

Humans

Maternal 2H4MBP exposure, determined primarily via third trimester urinary concentrations, was associated with lower birth weight of girls and higher birth weight of boys.[43] In another study, maternal gestational urinary 2H4MBP concentrations were positively associated with weight and head circumference at birth in male newborns.[44] Maternal exposure to 2H4MBP has been postulated to be involved in the development of Hirschsprung's disease. One hypothesis is

that this complex congenital disease is caused by gene–environment interactions that can lead to intestinal obstruction and chronic constipation in the offspring.[45] Pregnant women who had higher 2H4MBP concentrations in urine exhibited higher odds (2.4 to 2.6:1) of having a child with Hirschsprung's disease.[25] In the 293T and SH-SY5Y cell migration model of Hirschsprung's disease, 2H4MBP suppressed migration and altered the levels of key migratory proteins at both the ribonucleic acid and transcribed protein levels in the absence of cytotoxicity.[25; 45]

A study looking at the potential effect of 2H4MBP dermal application and serum hormone changes in young men and postmenopausal women concluded that the amount of 2H4MBP absorbed did not alter the endogenous reproductive hormone homeostasis.[19]

General Toxicity

Experimental Animals

The acute rat dermal median lethal dose (LD_{50}) has been reported to be >16 g/kg. Concomitant local skin reactions consisting of mild to moderate erythema were observed in the absence of significant pathological findings.[5] The acute rat oral LD_{50} for 2H4MBP has been reported to be >12.8 g/kg.[46] These authors also reported that administration of 0.5% or 1% 2H4MBP in rat diet for 12 weeks was associated with growth depression. Upon examination at week 6, female rats exposed to 0.5% or 1% displayed a leukocytosis with an increase in the lymphocyte count and a decrease in the neutrophil count, as well as a decrease in hemoglobin concentration compared to control females. At week 12, exposed rats displayed anemia and lymphocytosis with a reduction in granulocytes. The relative weights of the pituitary gland, thymus, heart, adrenal gland, lung, and spleen were also lower in both sexes. The 0.5% females showed higher relative thyroid weight than the control group, as well as the first stages of kidney degeneration. Degenerative nephrosis was diagnosed both macro- and microscopically in the kidneys of both sexes at 1%.

NTP has reported the findings of three studies conducted in F344 rats exposed to: (1) 0, 3,125, 6,250, 12,500, 25,000, or 50,000 ppm 2H4MBP in feed for 2 or 13 weeks; (2) 0, 1.25, 2.5, 5, 10, or 20 mg/kg 5 days per week for 2 weeks dermally in acetone or lotion; and (3) 12.5, 25, 50, 100, or 200 mg/kg in acetone or lotion 5 days per week for 13 weeks.[40] After dietary administration for 2 weeks, 6,250 ppm 2H4MBP and higher concentrations were associated with higher liver weights and marked hepatocyte cytoplasmic vacuolization. As was observed in the 2-week study, kidney and liver weights were higher in the 2H4MBP-exposed rats in the 13-week study at exposure concentrations of 3,125 ppm and higher (liver) or 25,000 ppm and higher (kidney). Histopathological kidney findings included dilated tubules and tubular epithelial cell regeneration. These findings were observed primarily in high-dosed rats. In the 13-week feed study, 2H4MBP administration was associated with lower body weight gains of 50,000 ppm male and female rats. Additionally, in the 13-week feed study, kidney lesions progressed to include papillary degeneration or necrosis and inflammation. Although cytoplasmic vacuolization was not observed in the liver, liver enzymes remained elevated at 13 weeks. In the 2-week dermal study, small and variable increases in liver and kidney weights were observed in exposed groups, with statistically significant differences observed primarily in the higher dose groups. In the 13-week dermal study, female rats in the higher dose groups displayed higher kidney weights than the control group. No other findings were attributed to 2H4MBP exposure. A 4-week dermal study in rats using 100 mg/kg 2H4MBP in petroleum jelly twice a day did not

affect body weight; liver, kidney, or testes weights; or histopathology.[15] 2H4MBP exposure did lower rat blood glutathione-*S*-transferase levels.

Humans

The literature contains no studies on the general toxicity of 2H4MBP in humans.

Immunotoxicity

Experimental Animals

A study conducted for irritation per the Draize method concluded that an occlusive patch containing 0.5 mL or 0.5 mg at 2H4MBP concentrations from 4% to 100% was nonirritating to intact and abraded albino rabbit skin.[5] 2H4MBP at 100% up to 100 mg was found not to be irritating to the rabbit eye using the modified FSLA or Draize methods. A sunscreen containing 6% 2H4MBP was found not to be photosensitizing in albino rabbits and was negative for sensitization potential in the Klingman Maximization Procedure[5] and local lymph node assay.[47]

Humans

Some reports have indicated that 2H4MBP might induce allergenic and sensitization responses.[5] In a sunscreen sensitization study, researchers detected allergy and/or photoallergy in 3.7% of the human subjects, which was attributed to application of moisturizing creams that contained 2H4MBP.[48] A subsequent study sponsored by Schering-Plough HealthCare Products reported the results of the meta-analysis of 64 unpublished studies conducted at 10 independent clinical laboratories representing the results of 19,570 individuals subjected to human repeat insult patch tests and photoallergy studies between 1992 and 2006.[49] These studies were aggregated and analyzed to evaluate the irritancy and sensitization potential of sunscreen products containing 2H4MBP concentrations between 1% and 6%. Forty-eight dermal responses were considered suggestive of sensitization or irritation with a mean rate of response of 0.26%. The authors concluded that sunscreen products formulated with 1% to 6% 2H4MBP do not possess a significant sensitization or irritation potential for the general public. 2H4MBP was also negative in an in vitro phototoxicity assay using SkinEthic™, a human epidermis model.[50]

Study Rationale

2H4MBP was nominated to NTP by the National Cancer Institute because of high exposure via use of 2H4MBP-containing sunscreen products and lack of chronic toxicity and carcinogenicity data. 2H4MBP was also nominated by a private individual to ascertain genotoxic potential. Furthermore, there are concerns about the endocrine activity of 2H4MBP. Under the purview of the Sunscreen Innovation Act of 2014, FDA is in the process of reviewing toxicity data on specific commonly used sunscreens to ascertain whether the available data support a positive GRASE (generally recognized as safe and effective)[51] designation. FDA is also in the process of finalizing and making effective the Sunscreen Monograph, which will update conditions under which over-the-counter sunscreen products can be marketed in the United States. FDA had expressed concern about the potential long-term adverse effects,[52] or effects not otherwise readily detected from human use, and specifically identified reproductive toxicity and carcinogenicity as concerns. This concern was elevated due to data in the published literature suggesting potential for endocrine activity.

To understand the potential effects on reproduction and development, NTP conducted this study with continual 2H4MBP exposure in a sensitive animal model to address the potential for 2H4MBP to (1) exhibit endocrine activity, (2) affect the ability of offspring to reproduce, and (3) induce adverse fetal effects. In addition, this study allowed for quantification of 2H4MBP in the blood at different ages for comparison to human blood concentrations. This report complements ICH[b] S5r2 guideline studies (fertility and early embryonic development, embryo-fetal development, and pre- and postnatal developmental studies in rats) on 2H4MBP[53] conducted by FDA's National Center for Toxicological Research, an interagency NTP partner, and allows for the comparison of study designs and outcomes. Potential endocrine activity that could result in neoplastic/tumorigenic responses was assessed in the concurrently conducted mouse and rat 2-year toxicology and carcinogenesis studies. The 2-year rat study also included perinatal exposure.[34]

As disposition is similar following oral and dermal exposure, 2H4MBP exposure via the diet was selected for this study, rather than topical application, to sustain internal exposure. It was also recognized that if applied topically, internal dose would be influenced by intra- and inter-animal grooming behavior. To minimize the potential endocrine activity of phytoestrogens that are often present in rodent diets, a diet low in phytoestrogens was used. Ethinyl estradiol, a synthetic form of estrogen, was selected as a positive control to provide context for any potential estrogen-like findings in 2H4MBP-exposed rats, if present.

[b]ICH = International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use.

Materials and Methods

Overview of Pre- and Postnatal Dose Range-finding and Modified One-Generation Study Designs

Modified one-generation (MOG) studies are composed of two interrelated parts: (1) a dose range-finding study (Figure 3) and (2) a MOG study (Figure 4, Table 1). If the acceptable range of exposure concentrations required to avoid excessive general and perinatal toxicity is unknown, a pre- and postnatal dose range-finding study is conducted. Nulliparous females are mated at the animal vendor and sent to the testing laboratory. Dosing typically begins at implantation (gestation day [GD] 6) through weaning on lactation day (LD) 28. Offspring are exposed in utero, during lactation, and through consumption of dosed feed.

In MOG studies, time-mated females are administered the test article from GD 6 through weaning (evidence of mating = GD 0). The subsequent F_1 litters are standardized to a specified litter size (n = 8 or 10), with equal representation of both sexes. These offspring are continuously exposed to the test article via the same route of exposure and dose concentration as their dams. Multiple endpoints indicative of potential endocrine alteration (e.g., anogenital distance [AGD], nipple retention in males, pubertal markers) are measured (Table 1). Randomly selected F_1 animals are taken to adulthood for gross and histopathological examinations and can be allocated at weaning (postnatal day [PND] 28) to various cohorts. Histopathological examination of multiple animals per litter increases the power of statistical tests to detect adverse effects.[54]

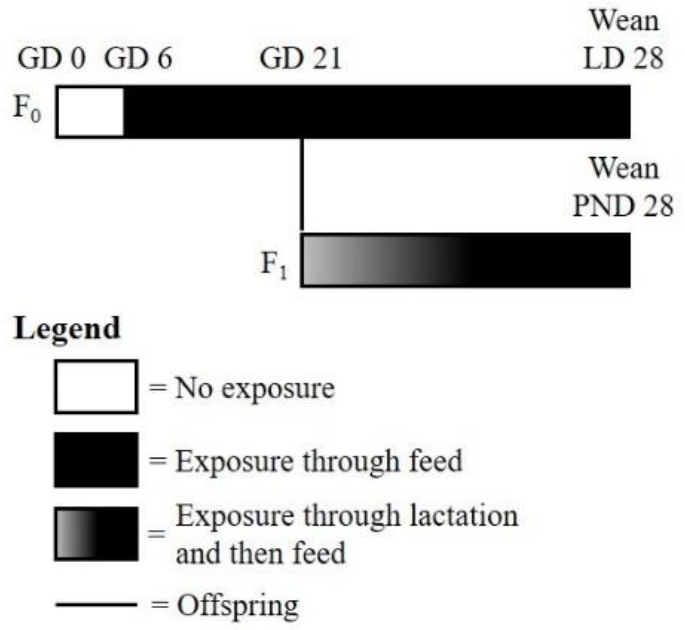


Figure 3. Design of a Dose Range-finding Study

F_0 dams are exposed to the test article from gestation day (GD) 6 through weaning on lactation day (LD) 28 and evaluated for maternal toxicity. F_1 offspring are exposed in utero through postnatal day (PND) 28 and evaluated for signs of in utero and postnatal toxicity.

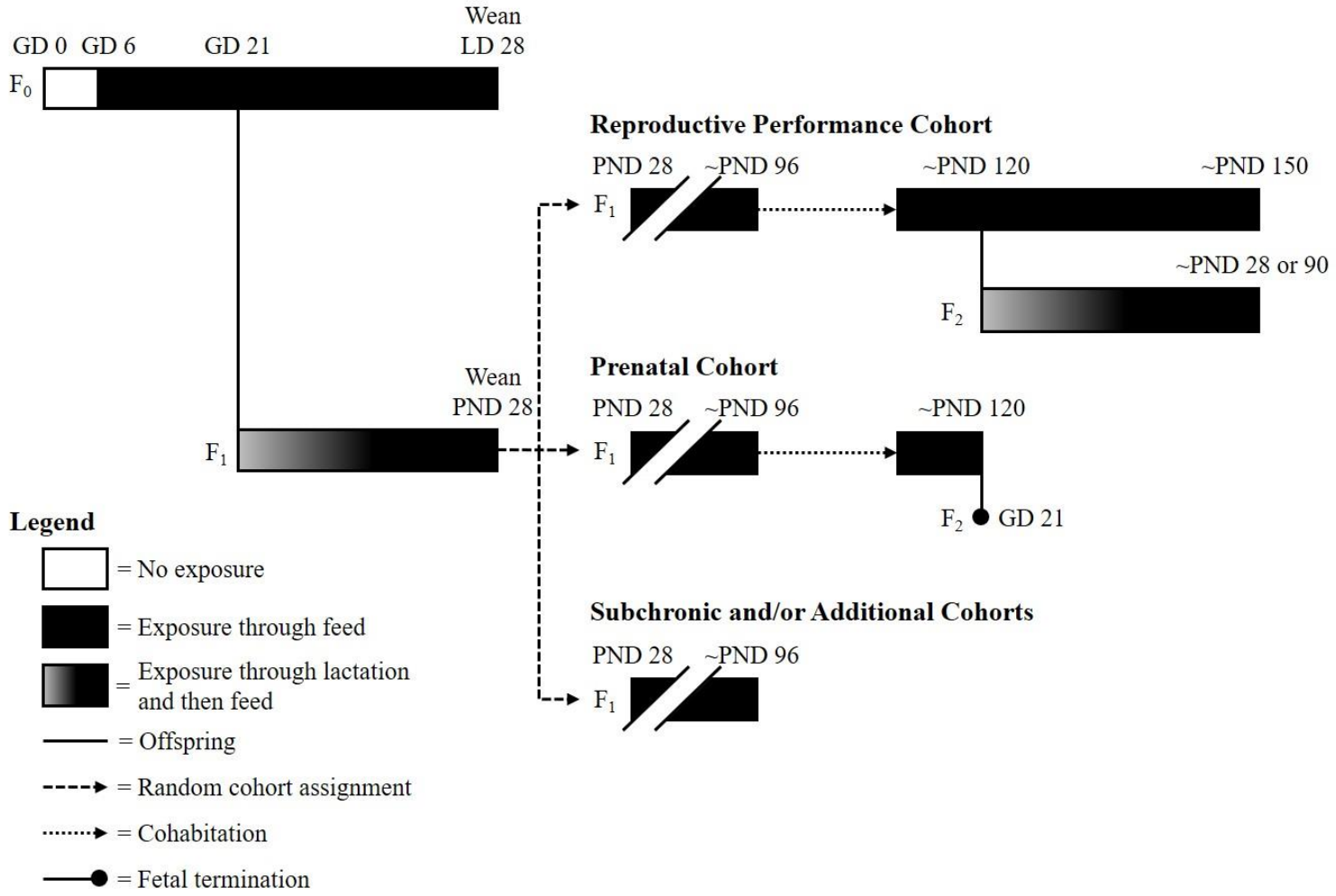


Figure 4. Design of a Modified One-Generation Rat Study

F_0 dams are exposed to the test article from gestation day (GD) 6 through weaning on lactation day (LD) 28 and evaluated for maternal toxicity. F_1 offspring are exposed in utero and during lactation through postnatal (PND) 28 and evaluated for signs of toxicity. After weaning, F_1 offspring are allocated into cohorts for prenatal, reproductive performance, or additional assessments (e.g., subchronic or biological sampling cohorts) and exposure to test article continues until necropsy. F_2 offspring are exposed in utero and during lactation and postweaning until necropsy (reproductive performance cohort).

The ability of F_1 animals to mate and produce viable offspring is evaluated in the reproductive performance cohort. The potential for the test article to induce fetal defects is assessed in the prenatal cohort: F_2 fetuses are examined on GD 21, which includes examination of external morphology, fetal viscera, head (soft tissue and skeletal components), and skeleton (osseous and cartilaginous defects). Abnormalities are categorized as either malformations, which are permanent structural changes that could adversely affect survival, development, or function; or variations, which are a divergence beyond the usual range of structural constitution that might not adversely affect survival or health,[55] consistent with descriptions by Makris et al.[56] Endpoints common to most cohorts are described in Table 1.

Table 1. Key Modified One-Generation Study Design Endpoints

Cohort	Key Endpoints
F_0 Dams	Maternal toxicity endpoints (body weight, feed consumption, clinical observations)
F_1 Generation[a]	Clinical observations
	Body weights
	Feed consumption
	Necropsy
	Pup survival
	Anogenital distance, nipple/areola retention, testis descent, vaginal cytology
Reproductive Performance Cohort	F_1 reproductive performance
	F_1 andrology and sperm parameters
	F_1 histopathology
	F_2 litter size, viability, and growth
	F_2 necropsy
Prenatal Cohort	F_1 reproductive performance
	F_2 fetal external, visceral, skeletal, and head soft tissue examinations
	F_2 necropsy
Subchronic Cohort	F_1 hematology
	F_1 clinical chemistry
	F_1 histopathology

[a]Additional cohorts (e.g., biological sampling cohort) and associated endpoints may be included in the study design.

Subchronic toxicity, including effects on clinical chemistry and hematology, are assessed in a 3-month cohort. Other cohorts can also be added (e.g., for internal dose estimation, neurobehavioral, toxicokinetic, and/or immunotoxicity assessments) to identify potential hazards across multiple functional outcomes. If necessary, more than one animal per sex can be selected from each litter and assigned to a cohort (e.g., reproductive performance). The F_1 litter remains the statistical unit but examining multiple animals per litter increases the likelihood of detecting adverse responses and collectively makes the most use of the animals produced.

In the studies reported here, F_0 females were administered the test article in feed beginning on GD 6. F_1 and F_2 offspring were exposed in utero, during lactation, and through consumption of dosed feed.

Procurement and Characterization

2-Hydroxy-4-methoxybenzophenone

2-Hydroxy-4-methoxybenzophenone (2H4MBP) was obtained from Ivy Fine Chemicals (Cherry Hill, NJ) in a single lot (20100801), which was used in the dose range-finding and MOG studies. Identity and purity analyses were conducted under the analytical chemistry laboratory and study laboratory at Battelle (Columbus, OH) (Appendix A). Reports on analyses performed in support of the 2H4MBP studies are on file at the National Institute of Environmental Health Sciences (NIEHS).

Lot 20100801 of the chemical, a light-yellow powder, was identified as 2H4MBP by infrared (IR) and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The IR spectrum was in good agreement with a reference spectrum (BP #824 from the Sadtler Basic Monomers and Polymers Library [Bio-Rad Laboratories, Hercules, CA]) and the structure of 2H4MBP. ^{1}H and ^{13}C NMR spectra were consistent with computer-predicted spectra and the structure of the test article.

The purity of 2H4MBP lot 20100801 was determined using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, as well as gas chromatography (GC) with flame ionization detection (FID). Lot 20100801 was screened for common residual volatile solvents using GC with electron capture detection (ECD) and FID. Differential scanning calorimetry (DSC) was also used to determine the purity of 2H4MBP. Karl Fisher titration of 2H4MBP lot 20100801 was conducted to estimate moisture content.

Purity assessment by HPLC/UV and GC/FID found one major peak with no reportable impurities ≥0.1%. Purity by DSC was 99.9%. Karl Fischer analysis indicated that no quantifiable water was present in 2H4MBP lot 20100801. No significant halogenated or nonhalogenated volatile impurities were found in the lot. The overall purity of 2H4MBP lot 20100801 was determined to be >99%.

To ensure stability, the bulk 2H4MBP was stored at room temperature (approximately 25°C) in sealed amber glass containers. Periodic analysis of the lot by the study laboratory using HPLC/UV showed no degradation of the bulk 2H4MBP chemical.

Ethinyl Estradiol

Ethinyl estradiol (EE) was obtained in a single lot (090M1241V) from Sigma-Aldrich (St. Louis, MO) via Government Scientific Source, Inc. (Reston, VA). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at Battelle (Columbus, OH) (Appendix A).

EE lot 090M1241V was a white powder. The lot identity was confirmed using IR spectroscopy and ^{1}H and ^{13}C NMR spectroscopy; all spectra were consistent with the structure of EE and matched available reference and predicted spectra. Elemental analysis indicated that the sample

was approximately 80.4% carbon, 11.5% oxygen, 7.9% hydrogen, and >0.5% nitrogen, which is consistent with theoretical values.

HPLC/UV showed a major peak with 99.8% and one minor peak with 0.23% of the total peak area, and analysis for volatiles using headspace GC/FID found the sample contained approximately 0.023% acetone. DSC yielded a purity of 99.7% and a melting point of 184°C. Karl Fischer analysis indicated that the water content of lot 090M1241V was approximately 0.4%. These data indicated the EE purity of lot 090M1241V to be ≥99.7%, consistent with the manufacturer-reported purity of 99%.

To ensure stability, the EE positive control was stored in sealed glass containers at room temperature. Prior to the study and at study termination, lot 090M1241V was analyzed using HPLC/UV to ensure chemical stability.

Preparation and Analysis of Dose Formulations

2-Hydroxy-4-methoxybenzophenone

Dosed feed formulations were prepared monthly (dose range-finding study) or eight times (MOG study) (Table A-2) using irradiated low-phytoestrogen feed (5K96 Casein diet). Formulations were stored at approximately 5°C for up to 42 days in amber glass bottles. The homogeneity of 2H4MBP formulations in 5K96 feed was confirmed before conducting the studies. The analytical chemistry laboratory at Battelle (Columbus, OH) conducted all dose formulation analyses throughout the study.

Stability studies conducted on a 1,000 ppm formulation when sealed and stored in amber plastic bags for 42 days at 5°C or −20°C showed that the formulation was within 10% of the day 0 value. An animal room simulation of a 1,000 ppm formulation stored in open glass containers at room temperature, with and without rodent urine and feces, showed that 2H4MBP over 7 days was within 10% of the day 0 concentration. The preadministration dosed feed formulations were analyzed three times over the course of the study (Table A-3, Table A-4) using HPLC/UV. All preadministration samples were within 10% of the target concentration. For one set of dosed feed formulations, postadministration samples were collected from the animal room approximately 1 month after preparation. These formulations were also within 10% of the target concentrations.

Ethinyl Estradiol

Dosed feed formulations were prepared eight times (Table A-2) using 5K96 feed. Formulations were stored at −20°C for ≤57 days in sealed amber plastic bags. The homogeneity of 0.05 ppm EE formulations in 5K96 feed was confirmed before conducting the studies.

Stability studies conducted on the 0.05 ppm formulation, when stored in sealed amber plastic bags at −20°C, approximately 5°C, or room temperature for 57 days, showed that the formulation was within 10% of the day 0 value. An animal room simulation of the 0.05 ppm formulation in open glass containers, with and without rodent urine and feces, showed that EE over 8 days was within 10% of the day 0 value.

The preadministration dosed feed formulations were analyzed four times over the course of the study (Table A-4) using HPLC/UV. All preadministration samples were within 10% of the target concentration with the exception of two formulations, one of which was 11% below and the other 12% above. Postadministration samples were collected from the animal room at the end of the exposure period and sent to Battelle (Columbus, OH) for analysis. The concentrations of the animal room samples were within 10% of the preadministration analyses and, therefore, demonstrated acceptable stability during the study.

Animal Source

Female Sprague Dawley (Hsd:Sprague Dawley® SD®) rats were obtained from Envigo (formerly Harlan Laboratories, Inc., Dublin, VA) for use in the dose range-finding and MOG studies. Sexually mature (12 to 13 weeks old) females were time-mated overnight at the vendor and were received on GD 1 or GD 2 (13 to 14 weeks old) for both the dose range-finding and MOG studies. GD 0 was defined as the day positive evidence of mating was observed.

Animal Health Surveillance

In accordance with the National Toxicology Program (NTP) Sentinel Animal Program (Appendix C), 20 nonmated female rats were designated for disease monitoring after arrival; samples were collected for serological analyses, and the rats were euthanized, necropsied, and examined for the presence of disease or parasites. All test results were negative.

Animal Welfare

Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. All animal studies were conducted in a facility accredited by AAALAC International. Studies were approved by the RTI International Animal Care and Use Committee and conducted in accordance with all relevant National Institutes of Health and NTP animal care and use policies and applicable federal, state, and local regulations and guidelines.

Experimental Design

Dose Range-finding Study

Time-mated female rats were received on GD 1 or GD 2, randomized based on GD 3 body weight, and placed on a 5K96 Casein diet containing 0, 3,000, 10,000, 25,000, or 50,000 ppm 2H4MBP from GD 6 through LD 28. Feed and water were available ad libitum; information on feed composition and contaminants is provided in Appendix B. Dose selection was based in part on Fischer 344/N rat studies reported in Toxicity Report 21.[40]

Eight time-mated rats were allocated to each exposure group. Six additional time-mated female rats were allocated to the control, 3,000, and 50,000 ppm groups for collection of tissues for bioanalytical method development. Viability, clinical observations, body weights, pup counts (litters were not standardized), and feed consumption were recorded to help determine the maximum exposure concentration that could be tolerated by the dams while not affecting the number of pups, so the MOG study could be populated with a sufficient number of offspring. Maternal plasma, amniotic fluid, and fetuses were collected from three separately allocated dams on GD 18. On LD 4 and PND 4, maternal plasma and pups (three per sex), respectively, were

collected from two to three dams per group. On LD 28, a piece of the left lateral lobe of the liver, left and right kidneys, left and right ovaries, and uterus were collected from five dams per group. In addition, left and right testes, left and right epididymides, and the brain were collected from 10 male pups per group on PND 28. All other dams and pups were euthanized without further examination on LD 28 and PND 28, respectively. Females that did not litter were euthanized approximately 5 days after expected littering, received a gross necropsy, and had their pregnancy status determined. If present, the numbers of implantation sites were recorded. F_1 pups that were removed for health reasons or morbidity received a gross necropsy. Further details of animal maintenance and study design are given in Table 2.

Modified One-Generation Study with Prenatal and Reproductive Performance Cohorts

Time-mated F_0 female rats, 25 per group, were received on GDs 1 or 2, randomized based on GD 3 body weight, and placed on a 5K96 Casein diet containing 0, 3,000, 10,000, or 30,000 ppm 2H4MBP or 0.05 ppm EE ad libitum on GD 6. The exposure concentration of 30,000 ppm was expected to result in minimal maternal toxicity and to ensure that the model system was appropriately challenged, increasing the likelihood of identifying any toxicological signal in the offspring. The F_1 and F_2 generations were exposed to 2H4MBP or EE via the mother during gestation and lactation, and directly via 5K96 feed at the same exposure concentration as their respective dams. Viability, clinical observations, body weights, pup counts, and feed consumption were recorded. F_1 and F_2 litters were standardized to 8 pups (4/sex/litter, when possible) on PND 4. At weaning on PND 28, offspring were randomly assigned to reproductive performance (2/sex/litter), prenatal development (1/sex/litter), or biological sample collection (1/sex/litter) cohorts. Information on feed composition and contaminants is provided in Appendix B. Additional details of animal maintenance and study design are given in Table 2 and Table 3.

Endocrine-sensitive and Pubertal Endpoints

AGD and corresponding body weight (for covariate analyses) were recorded for each F_1 and F_2 pup on PND 1. AGD was measured using a stereomicroscope with a calibrated ocular reticle. The distance between the midpoint of the anal opening to the caudal edge of the genital papilla was recorded and converted to millimeters (mm). F_1 and F_2 male pups were evaluated for retention of areolae/nipples on PND 13 and observed for testicular descent over 25 (F_1) or 28 (F_2) days beginning on PND 14. Acquisition of balanopreputial separation (BPS), defined as complete retraction of the prepuce from the glans penis, was evaluated in all F_1 males over 59 days beginning on PND 35, and body weight was recorded upon BPS acquisition. External genitalia were examined for malformations and undescended testes (cryptorchidism). The acquisition of vaginal opening (VO) was evaluated in F_1 females over 48 days beginning on PND 23, and the corresponding body weight recorded upon VO acquisition.

Vaginal Cytology

Beginning on PND 75, vaginal lavages were collected from the F_1 females in the prenatal and reproductive performance cohorts for 16 consecutive days for evaluation of estrous cyclicity and confirmation of mating. Vaginal vaults were moistened with saline, if necessary, and samples of vaginal fluid and cells were spotted onto a slide and stained with toluidine blue. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were

determined and used to ascertain estrous cycle stages (diestrus, proestrus, estrus, and metestrus).[57]

F_1 Cohabitation and Assessment of Mating

Sexually mature F_1 animals in the prenatal (14–15 weeks; 1 male and 1 female/litter) and reproductive performance (17–18 weeks; 2 males and 2 females/litter) cohorts were randomly assigned a mating partner, avoiding sibling pairings, and paired in a 1:1 ratio for up to 15 days. Mating was confirmed by daily examination for the presence of a vaginal copulation plug or sperm in a vaginal lavage. The day of confirmed mating was considered GD 0. Females that did not exhibit evidence of mating or did not deliver a litter were necropsied 25 days after the cohabitation period ended. The uterus was examined grossly and stained with ammonium sulfide to identify potential implantation sites. The number of corpora lutea on the ovary were enumerated, and gross lesions were examined for histopathological changes.

Prenatal Cohort

On GD 21, fetuses were removed from the uterus, individually weighed (live fetuses only), and examined externally for alterations, including inspection of the oral cavity for cleft palate. Placental morphology was also evaluated. Live fetuses were subsequently euthanized by oral administration of sodium pentobarbital. Females with no evidence of mating were necropsied and examined for gross lesions, which were retained and examined histologically. Fetal sex was confirmed by inspection of gonads in situ. All fetuses were examined for soft tissue alterations under a stereomicroscope.[58; 59] The heads were removed from approximately half of the fetuses in each litter, fixed in Bouin's solution, and subsequently examined by freehand sectioning.[60] This technique precludes skeletal evaluations of the skull; therefore, remaining heads and all fetuses were eviscerated, fixed in ethanol, macerated in potassium hydroxide, stained with Alcian blue and Alizarin red, and examined for subsequent cartilage and osseous alterations.[61; 62] External, visceral, and skeletal fetal findings were recorded as developmental variations or malformations. After positive evidence of mating, male sires were necropsied, selected organs were weighed, and gross lesions were collected for potential histological examination.

Reproductive Performance Cohort

Fertility and fecundity were assessed in two males and two females from each F_1 litter and all exposure groups. Pup viability was assessed daily during lactation. F_2 offspring were standardized to a litter size of 8 pups (4/sex/litter, when possible) on PND 4. F_1 males were euthanized at approximately 22 weeks of age after assessment of fertility, fecundity, and F_2 generation pup survival. The F_1 females and the F_2 offspring were euthanized on PND 28, when the F_1 females were 18–24 weeks of age. F_2 offspring were given a gross necropsy. F_1 sires were necropsied after mating, selected organs were weighed, and gross lesions were collected for potential histological examination. Given the absence of functional changes, a crossover mating to determine affected sex was deemed unnecessary.

Immediately after euthanasia, the left testis and epididymis were removed, trimmed, and weighed. The cauda epididymis was then weighed, and samples were collected for determining cauda epididymal sperm motility, number, and density via automated sperm analyzer (Hamilton Thorne, Inc., Beverly, MA). The sampled left cauda epididymis and the intact corpus and caput were frozen at −80°C for subsequent determination of epididymal sperm concentration from the left cauda epididymis. The left testis was frozen at −80°C for subsequent determination of

homogenization-resistant spermatid head counts for calculations of daily sperm production and efficiency of daily sperm production.[63] The right testis and epididymis were examined histologically. Gross lesions took precedence over sperm parameter assessments (i.e., if the left testis was grossly abnormal, it and the left epididymis would be examined histologically, and the right testis and epididymis, if grossly normal, would be subjected to sperm assessments).

Biological Sampling Cohort

On PND 28 and PND 56 (5/sex/time point/exposure group), kidneys, epididymides, testes, ovaries, and liver were collected and frozen for potential future analyses. Plasma samples were also collected from these rats on PNDs 28 and 56 (5/sex/time point/exposure group) and analyzed for 2H4MBP and metabolites.[64]

Necropsy and Histopathology

Complete necropsies were performed on adult F_1 males and F_1 females in the reproductive performance cohort; unscheduled deaths, F_0 females, F_1 males, and F_1 females in the prenatal cohort; F_1 females in the reproductive performance cohort that either had no evidence of mating or did not produce a litter; and F_2 offspring. All gross lesions were examined histologically. In addition, several protocol-required tissues were examined microscopically from the adult F_1 males and females in the reproductive performance cohorts.

The initial histological examination was performed by an experienced, board-certified veterinary pathologist. The slides, individual animal data records, and pathology tables were subsequently evaluated by an independent quality assessment (QA) laboratory. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. A QA pathologist evaluated selected slides from the various cohorts. Kidney histopathology was reviewed from all males and females in the F_1 reproductive performance cohort and from animals in other cohorts in which the kidney had gross lesions. The urinary bladder, thyroid gland, liver, testis, epididymis, and ovaries were reviewed from all animals in the F_1 reproductive performance cohort for which the tissue had been previously examined by the study laboratory pathologist.

The QA report and the reviewed slides were submitted to the NTP pathologist, who reviewed and addressed any inconsistencies in the diagnoses made by the laboratory and QA pathologist. The QA pathologist, who served as the coordinator of the Pathology Working Group (PWG), presented representative histopathology slides containing examples of lesions related to test article administration, examples of disagreements in diagnoses between the laboratory and QA pathologist, or lesions of general interest to the PWG for review. The PWG consisted of the NTP pathologist and other pathologists experienced in rodent toxicological pathology. When the PWG consensus differed from the opinion of the laboratory pathologist, the diagnosis was changed. Final diagnoses for reviewed lesions represent a consensus between the laboratory pathologist, QA pathologist, and the PWG. Details of these review procedures have been described, in part, by Maronpot and Boorman[65] and Boorman et al.[66]

Table 2. Experimental Design and Materials and Methods in the Dose Range-finding and Modified One-Generation Studies of 2-Hydroxy-4-methoxybenzophenone (Preweaning)

Dose Range-finding Study	Modified One-Generation Study
Study Laboratory	
RTI International (Research Triangle Park, NC)	Same as dose range-finding study
Strain and Species	
Sprague Dawley (Hsd:Sprague Dawley® SD®) rats	Same as dose range-finding study
Animal Source	
Envigo (formerly Harlan Laboratories, Inc., Dublin, VA)	Same as dose range-finding study
Day of Arrival	
July 19, 2011 (GD 1 or GD 2)	February 14 or 16, 2012 (GD 1 or GD 2)
Average Age on Arrival	
~14 weeks	13–14 weeks
Weight Range at Randomization	
179.1–236.1 g on GD 3	186.4–258.8 g on GD 3
Date of First Exposure	
GD 6 (July 23, 2011)	F_0 females: GD 6 (February 18–21, 2012)
	F_1 rats (all cohorts): lifetime exposure
	F_2 rats: lifetime exposure
Duration of Exposure	
GD 6 through LD 28	F_0 females: GD 6 through LD 28
	F_1 rats (biosampling cohort): lifetime exposure through PND 56
	F_1 rats (prenatal cohort): lifetime exposure through PND 111–113 (males) or through PND 109–132 (females)
	F_1 rats (reproductive performance cohort): lifetime exposure through PND 153–155 (males) or through PND 127–168 (females)
	F_2 rats (reproductive performance cohort): in utero through PND 28
Date of Last Exposure	
LD 28 (September 7, 2011)	F_0 females: LD 28 (April 3–6, 2012)
	F_1 rats (biosampling cohort): PND 56 (May 2, 2012)
	F_1 rats (prenatal cohort): PND 111–113 (through June 28, 2012) (males) or PND 116–132 (through July 15, 2012) (females)
	F_1 rats (reproductive performance cohort): PND 153–155 (through August 10, 2012) (males) or PND 127–168 (through August 21, 2012) (females)

Dose Range-finding Study	Modified One-Generation Study
	F_2 rats (reproductive performance cohort): PND 28 (through August 21, 2012)
Necropsy Dates	
Gross necropsies were conducted on F_0 females that did not deliver a litter or were euthanized early and F_1 offspring that were euthanized moribund or found dead.	F_0 females: LD 28 (April 6, 2012)
	F_1 rats (biosampling cohort): not performed
	F_1 rats (prenatal cohort): June 26–28, 2012 (males) or July 2–15, 2012 (females)
	F_1 rats (reproductive performance cohort): August 6–10, 2012 (males) or August 7–21, 2012 (females)
	F_2 rats (reproductive performance cohort): August 7–21, 2012
Average Age at Necropsy	
Not performed	F_0 females: ~21 weeks
	F_1 rats (biosampling cohort): not performed
	F_1 rats (prenatal cohort): 111–113 days (males) or 109–132 days (females)
	F_1 rats (reproductive performance cohort): 153–155 days (males) or 127–168 days (females)
	F_2 rats: 28 days
Size of F_0 Study Groups	
8–14 time-mated females	25 time-mated females
Method of Randomization and Identification	
Time-mated animals were individually identified by ink tail marking and assigned to exposure group by stratified randomization of GD 3 body weights using Provantis® (Instem, Stone, United Kingdom) electronic data collection system.	Same as dose range-finding study, except F_1 and F_2 pups were identified by ink paw marking, and postweaning F_1 males and F_1 females were identified by ink tail marking.
Animals per Cage	
1 (with litter)	F_0 females: 1 (with litter)
	F_1 rats (biosampling cohort): ≤2 (males or females) until approximate termination
	F_1 rats (prenatal cohort): ≤2 (males or females) until approximate PND 91
	F_1 rats (reproductive performance cohort): ≤2 (males or females) until PND 91, then housed individually except during cohabitation or when housed with their litters

Dose Range-finding Study	Modified One-Generation Study
Diet	
Irradiated certified Advanced Protocol Verified Casein Diet 1 IF 5K96 (PMI Nutrition International, Richmond, IN), available ad libitum	Irradiated certified Advanced Protocol Verified Casein Diet 1 IF 5K96 (PMI Nutrition International, St. Louis, MO), available ad libitum
Water	
Tap water (Durham, NC) via automatic watering system (Avidity Science, formerly Edstrom Industries, Inc., Waterford, WI), available ad libitum	Same as dose range-finding study
Cages	
Solid-bottom polycarbonate cages (Lab Products, Inc., Seaford, DE), rotated once weekly and changed at least once/week	Same as dose range-finding study
Bedding	
Certified irradiated Sani-Chips® hardwood cage bedding (P.J. Murphy Forest Products Corp., Montville, NJ)	Same as dose range-finding study
Cage Filters	
Filter paper (Granville Milling Co., Creedmoor, NC), changed weekly	Same as dose range-finding study
Racks	
Stainless steel (Ancare, Bellmore, NY), changed and rotated every 2 weeks during the study	Stainless steel (Lab Products, Inc., Seaford, DE), changed and rotated every 2 weeks during the study
Animal Room Environment	
Temperature: 71.05°F to 72.8°F Relative humidity: 39.98% to 55.91% Room fluorescent light: 12 hours/day Room air changes: 10/hour	Temperature: 72°F ± 3°F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: 10/hour
Exposure Concentrations	
0, 3,000, 10,000, 25,000, or 50,000 ppm 2H4MBP in feed, available ad libitum	0, 3,000, 10,000, or 30,000 ppm 2H4MBP in feed, available ad libitum; 0.05 ppm EE in feed, available ad libitum
Type and Frequency of Observation of F_0 and F_1 Dams	
Viability was assessed at least twice daily, and clinical observations were recorded at least once daily. Female body weights were recorded daily during gestation (GD 3–21) and during lactation on LDs 1, 4, 7, 14, 21, 25, and 28. Feed consumption was recorded at 3-day intervals from GD 3 through GD 21 and for LDs 1–4, 4–7, 7–14, 14–21, 21–25, and 25–28.	Viability was assessed at least twice daily, and clinical observations were recorded at least once daily. Female body weights were recorded daily during gestation (GD 3–21) and during lactation on LDs 1, 4, 7, 10, 13, 16, 19, 21, 25, and 28. Feed consumption was recorded at 3-day intervals from GD 3 through GD 21 and LD 1 through LD 28.

Dose Range-finding Study	Modified One-Generation Study
Type and Frequency of Observation of F_1 and F_2 Pups	
Viability was assessed at least twice daily, and clinical observations were recorded at least once daily. The number of live and dead pups in each litter was counted daily. Individual pups were sexed and weighed on PNDs 1, 4, 7, 14, 21, 25, and 28. Litters were not standardized on PND 4, and all offspring (unless euthanized and biological samples collected for subsequent analytical method development) were retained until PND 28 to assess litter size, sex distribution, pup body weights, and survival during lactation.	Viability was assessed at least twice daily, and clinical observations were recorded at least once daily. The number of live and dead pups in each litter was counted daily. Individual pups were sexed and weighed on PNDs 1, 4, 7, 10, 13, 16, 19, 21, 25, and 28. Litters were standardized to a litter size of 8 pups (4/sex/litter, when possible) on PND 4. Endocrine F_1/F_2 endpoints: AGD and corresponding pup weight on PND 1; areolae/nipple retention on PND 13; testicular descent beginning on PND 14
Primary Method of Euthanasia	
100% carbon dioxide (F_0 females and PND 28 pups); intraperitoneal injection of a solution containing sodium pentobarbital or decapitation (GD 21 fetuses; PND 4 pups)	100% carbon dioxide (adults and PND 28 pups) or administration of a solution containing sodium pentobarbital (PND 4 pups [intraperitoneal injection]; GD 21 fetuses [oral])
Necropsy and Postmortem Evaluation	
F_0 dams were euthanized on LD 28 without necropsy. Females that did not litter were euthanized ~5 days after expected littering, received a gross necropsy, and had their pregnancy status determined. If present, the numbers of implantation sites and corpora lutea were recorded. F_1 pups that were removed for health reasons or died received a gross necropsy.	F_0 dams were euthanized on LD 28, received a gross necropsy, and had their number of implantation sites recorded. Females that did not litter were euthanized 3 days after expected littering, received a gross necropsy, and had their pregnancy status determined. If present, the number of implantation sites and corpora lutea were recorded. Histopathological analysis of gross lesions was performed if collected.
Internal Dose Assessment/Additional Tissue Collection	
On GD 18, maternal plasma, amniotic fluid, and fetuses were collected from 3 pregnant dams/exposure group from the 0, 3,000, and 50,000 ppm groups. On LD 4, maternal plasma was collected from 2 or 3 dams/exposure group from the 0, 3,000, and 50,000 ppm groups. On PND 4, pups (3/sex) were collected from 2 or 3 dams/exposure group from the 0, 3,000, and 50,000 ppm groups. On LD 28, a piece of the left lateral lobe of the liver, left and right kidneys, left and right ovaries, and uterus were collected from 5 dams/exposure group. In addition, left and right testes, left and right epididymides, and the brain were collected from 10 male pups/exposure group on PND 28. Sample collection preceded the analytical method protocol development and method validation. Following the analysis and evaluation of a sample subset, the analysis of the full sample set was not pursued due to potential instability of analytes during long-term storage.	On PNDs 28 and 56 (5/sex/time point/exposure group), kidneys, epididymides, testes, ovaries, and liver were collected from rats in the biological sampling cohort and frozen for potential future analyses. Plasma samples were also collected from these rats on PNDs 28 and 56 (5/sex/time point/exposure group) and analyzed for 2H4MBP and metabolites.[64]

GD = gestation day; LD = lactation day; PND = postnatal day; 2H4MBP = 2-hydroxy-4-methoxybenzophenone; EE = ethinyl estradiol; AGD = anogenital distance.

Table 3. Experimental Design and Materials and Methods in the Modified One-Generation Study of 2-Hydroxy-4-methoxybenzophenone (Postweaning)

Modified One-Generation Study
F_1 Postweaning Assessments
All Cohorts: Viability was assessed at least twice daily, and clinical observations recorded at least once daily. F_1 male body weights and feed consumption were recorded once weekly. F_1 female body weights and feed consumption were recorded at least once weekly during the premating interval. Vaginal opening (and concomitant body weight) was evaluated beginning on PND 23, balanopreputial separation (and concomitant body weight) was evaluated beginning on PND 35. **Prenatal and Reproductive Performance Cohorts**: After collection of vaginal lavage samples for 16 days, F_1 nonsibling mating pairs (1 male and 1 female/litter or 2 males and 2 females/litter) from the same exposure group were cohabitated until evidence of mating or for ≤15 days. F_1 dams were observed for the same gestational endpoints as the F_0 dams. **Reproductive Performance Cohort:** F_1 dams and F_2 pups were evaluated for the same lactational endpoints as the F_0 dams and F_1 pups. A crossover mating would have been considered if an effect on fertility was observed.
F_1 Necropsy and Postmortem Evaluation
Prenatal Cohort: F_1 dams were euthanized on GD 21. Necropsies were performed on all females. Terminal body weights and adrenal glands (paired), liver, ovaries (left and right), and gravid uterus weights were recorded. The number of corpora lutea on each ovary was recorded. The number and location of all fetuses and resorptions (early or late) and the total number of implantation sites were recorded. If there were no macroscopic evidence of pregnancy, the uterus was stained to visualize potential evidence of implantation sites. Live fetuses were counted, sexed, weighed, and examined for external morphological abnormalities, including examination of the oral cavity for cleft palate. Placental morphology was also evaluated. Live fetuses were euthanized and then examined for visceral morphological abnormalities by fresh dissection. The sex of each fetus was confirmed by internal examination. The heads from approximately one-half of the fetuses in each litter were fixed, sectioned, and examined. All fetuses were eviscerated, fixed, stained, and examined for skeletal developmental variations, malformations, or other morphological findings. After positive evidence of mating, male sires were weighed, euthanized, and necropsied, and the following organ weights recorded: adrenal glands (paired), testes (left and right), epididymides (left and right), kidneys, liver, dorsolateral and ventral prostate gland, seminal vesicles with coagulating glands, thyroid gland (fixed), LABC muscle, Cowper's glands (paired), and preputial glands. Histopathology of gross lesions was assessed. **Reproductive Performance Cohort:** F_1 dams were euthanized on LD 28, and sires were euthanized within approximately 1 week of their mating partner. Terminal body weights and the following organ weights were recorded: adrenal glands (paired), liver, kidneys (left and right), ovaries (left and right), uterus, cervix, vagina, testes (left and right), epididymides (left and right), cauda epididymis, dorsolateral and ventral prostate gland, seminal vesicles with coagulating glands, thyroid gland (fixed), LABC muscle, Cowper's glands (paired), and preputial glands. Histopathology was performed on the following organs (predominantly reproductive tissues): adrenal glands, liver, kidneys, pituitary gland, thyroid gland, ovaries, testes, epididymides, dorsolateral and ventral prostate gland, seminal vesicles, coagulating glands, LABC muscle, Cowper's glands, preputial glands, and gross lesions. Cauda epididymal sperm motility, cauda epididymal sperm concentration, and testicular sperm head counts were also assessed. **Biological Sampling Cohort:** At weaning, F_1 rats were randomly allocated for collection of biological samples. Rats were subjected to a gross necropsy, and the following tissues were collected on PNDs 28 and 56 (5/sex/time point/exposure group): plasma, kidneys, epididymides, testes, ovaries, and liver. Tissues were frozen at −70°C until analysis. Results of the plasma analyses had been reported previously.[64]

PND = postnatal day; GD = gestation day; LABC = levator ani/bulbocavernosus; LD = lactation day..

Statistical Methods

Statistical methods were chosen based on distributional assumptions as well as on the need to incorporate within-litter correlation among animals. Unless specifically mentioned, all endpoints were tested for a trend across exposure groups, followed by pairwise tests for each exposed group against the negative control group. Significance of all trend and pairwise tests is reported at both 0.05 and 0.01 levels.

In the main study, the positive control (EE) was analyzed only by a single pairwise comparison to the negative control. The positive control analysis was kept separate from that of the other exposed groups and was excluded from all trend tests.

Analysis of Fetal Malformations and Variations

Incidences of malformations and variations in the fetuses were summarized as number of litters affected and as number of fetuses affected. Trend and pairwise analyses of the fetal malformations and variations was conducted using a Cochran-Armitage test with a Rao-Scott adjustment, as described below.

The tendency of fetuses from the same litter to respond more similarly than fetuses from different litters has been referred to as the "litter effect"[67] and reflects littermates' similarities in genetics and in utero experiences. Failure to account for correlation within litters leads to underestimates of variance in statistical tests, resulting in higher probabilities of Type I errors ("false positives"). Therefore, the Cochran-Armitage test was modified to accommodate litter effects using the Rao-Scott approach.[68] The Rao-Scott approach accounts for litter effects by estimating the ratio of the variance in the presence of litter effects to the variance in the absence of litter effects. This ratio is then used to adjust the sample size downward to yield the estimated variance in the presence of litter effects. The Rao-Scott approach was implemented in the Cochran-Armitage test as recommended by Fung et al.,[69] formula $\bar{T}_{RS2}$.

Analysis of Incidences of Gross Pathology and Morphology Findings

For the F_0 dams, incidences of gross findings and histopathology were summarized as number of animals affected. Because some of these animals did not survive until the removal day for their cohort, analysis of the histopathological findings was conducted using the Poly-3 test, as described below.

The Poly-k test[70-72] was used to assess neoplasm and nonneoplastic lesion prevalence. This test is a survival-adjusted quantal-response procedure that modifies the Cochran-Armitage trend test to account for survival differences. Following Bailer and Portier,[70] a value of k = 3 was used in the analysis of site-specific lesions. Variation introduced by the use of risk weights, which reflect differential mortality, was accommodated by adjusting the variance of the Poly-3 statistic as recommended by Bieler and Williams.[73] Poly-3 tests used the continuity correction described by Nam.[74]

For the F_1 and F_2 animals, incidences of gross findings and histopathology were summarized as number of litters affected and number of animals affected. To account for within-litter correlation, the Rao-Scott adjustment (as described earlier) was applied to the Cochran-Armitage

test in the analysis of this data. For histopathological data in F_1 cohorts in which survival issues could apply, the Poly-3 correction was also applied.

All p values calculated for gross pathological and histopathological data are one-sided and include a continuity correction.

Analysis of Continuous Endpoints

Before statistical analysis, extreme values identified by the outlier test of Dixon and Massey[75] for small samples ($n < 20$) and Tukey's outer fences method[76] for large samples ($n \geq 20$) were examined by NTP personnel, and implausible values were eliminated from the analysis.

In some instances, no considerations for litter effects were necessary in the analysis of the continuous data. This was the case for the F_0 generation and for the F_1 prenatal cohort for which there was only one animal per litter. In these instances, organ and body weight measurements, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett[77] and Williams.[78; 79]

When litter effects were present, organ and body weight endpoints were analyzed using linear mixed models, with litters as a random effect. To adjust for multiple comparisons, a Dunnett-Hsu adjustment was used.[80] Pup and fetal weights were adjusted for litter size by covariate analysis (see below) before analysis. AGD was adjusted for the body weight of the pup taken on the day of AGD measurement. The adjusted AGDs were analyzed as normal variates with litter effects using a linear mixed model.

Feed consumption, litter sizes, pup survival, implantations, number of resorptions, uterine content endpoints, spermatid, and epididymal spermatozoal measurements typically have skewed distributions. When litter effects were not present, these endpoints were analyzed using the nonparametric multiple comparison methods of Shirley[81] (as modified by Williams[82] and Dunn[83]). For these endpoints, the Jonckheere test[84] was used to assess the significance of the exposure concentration-related trends and to determine, at the 0.01 level of significance, whether a trend-sensitive test (the Williams or Shirley test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic exposure concentration-related trend (the Dunnett or Dunn test).

When litter effects were present for non-normally distributed continuous endpoints, the trend across exposure groups was analyzed by a permutation test based on the Jonckheere trend test implemented by randomly permuting whole litters across exposure groups and bootstrapping within the litters (see, for example, Davison and Hinckley[85]). Pairwise comparisons were made using a modified Wilcoxon test that incorporated litter effects.[86] The Hommel procedure was used to adjust for multiple comparisons.[87]

Analysis of Gestational and Fertility Indices

When litter effects were not present, Cochran-Armitage trend tests were used to test the significance of trends in gestational and fertility indices across exposure groups. Fisher's exact test was used to conduct pairwise comparisons of each exposed group with the control group. P values for these analyses are two-sided.

When litter effects were present, as with the F_1 reproductive performance cohort, the gestational and fertility indices were tested using the Rao-Scott adjustment to the Cochran-Armitage test. This practice was used for both the trend and pairwise tests.

Body Weight Adjustments

Because body weights typically decrease with increasing litter size, adjusting body weight for litter size in the analysis of fetal and pup weights can provide additional precision to detect test article effects.[88] Body weight adjustments are appropriate when the litter effect, as evidenced by decreasing weights with increasing litter size, is relatively constant across exposure concentrations. Adjusted fetal weights were calculated by fitting a linear model to littermean fetal weights as a function of litter size and exposure concentration, and the estimated coefficient of litter size was then used to adjust each litter mean fetal weight based on the difference between its litter size and the mean litter size. Preweaning pup body weights were adjusted for live litter size as follows. A linear model was fit to body weights as a function of exposure concentration and litter size. The estimated coefficient of litter size was then used to adjust each pup body weight based on the difference between its litter size and the mean litter size. Prestandardization PND 4 body weights were adjusted for PND 1 litter size, and body weights measured between PND 4 poststandardization and PND 21 were adjusted for PND 4 poststandardization litter size. After adjustment, mean body weights were analyzed with a linear mixed model with a random litter effect.

Analysis of Time-to-Event Data

Time-to-event endpoints, such as day of attainment of testicular descent, BPS, and VO, have four features that require careful model selection: (1) they might display non-normality; (2) litter-based correlation might be present; (3) values might be censored, meaning attainment is not observed before the end of the observation period; and (4) growth retardation, reflected in the weaning weight, is an important covariate in the case of BPS and VO given the relationship between normal day of expected attainment and body weight.

For this study, attainment times were approximately normally distributed and attainment was observed in all but three animals (from the same litter, BPS only). Under these circumstances, a mixed model approach is appropriate. The mixed model used here was fit to attainment day as a function of exposure concentration, as well as a function of both exposure concentration and weaning weight (for BPS and VO) with a random litter effect.

To calculate mean attainment values adjusted for weaning weight, a linear model was fit to attainment day as a function of exposure concentration and weaning weight. The estimated coefficient of weaning weight was then used to adjust each attainment day based on the difference between the measured weaning weight and the mean weaning weight.

Cumulative response percentage, obtained using the methods of Kaplan-Meier,[89] was plotted against time to attainment for unadjusted attainment times as well as attainment times adjusted for weaning weight. For litter-based plots, the litter median was used as time to attainment if >50% of the pups for that litter attained. Otherwise, litters with ≤50% of the pups attaining had time to attainment set to the final day of observation. These litters are included in the denominator of Kaplan-Meier calculations but not the numerator.

Analysis of Vaginal Cytology Data

Vaginal cytology data consist of daily observations of estrous cycle stages over a 16-day period. Differences from the control group for cycle length and number of cycles were analyzed using a Datta-Satten modified Wilcoxon test with a Hommel adjustment for multiple comparisons.

To identify disruptions in estrous cyclicity, a continuous-time Markov chain model (multi-state model) was fit using a maximum likelihood approach,[90] producing estimates of stage lengths for each exposure concentration group. Confidence intervals for these estimates were obtained based on bootstrap sampling of the individual animal cycle sequences. Stage lengths that were significantly different than the control group were identified using permutation testing with a Hommel adjustment.

Historical Control Data

The concurrent control group is the most valid comparison to the exposed groups and is the only control group analyzed statistically in NTP developmental and reproductive toxicity studies. However, historical control data are often helpful in interpreting potential exposure concentration-related effects, particularly for uncommon fetal findings that occur at a very low incidence. For meaningful comparisons, the conditions for studies in the historical control database must be generally similar. Factors that might affect the background incidences of fetal findings at a variety of sites are diet, strain/stock, route of exposure, study type, and/or laboratory that conducted the study. The NTP historical control database for fetal findings contains all fetal evaluations from teratology studies and/or modified one-generation studies for each laboratory. In general, the historical control database for a given study includes studies using the same route of administration and study design. However, historical control data for rats in this NTP Developmental and Reproductive Toxicity Technical Report contain data from feed and gavage (all routes) studies conducted at RTI International. The concurrent controls are included in the historical control data set. NTP historical controls are available online at https://ntp.niehs.nih.gov/data/controls/index.html.

Quality Assurance Methods

This study was conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations, Title 21 of the United States Code of Federal Regulations Part 58.[91] In addition, this study was audited retrospectively by an independent QA contractor. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Developmental and Reproductive Toxicity Report. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this report.

Results

Data Availability

The National Toxicology Program (NTP) evaluated all study data. Data relevant for evaluating toxicological findings are presented here. All study data are available in the NTP Chemical Effects in Biological Systems (CEBS) database: https://doi.org/10.22427/NTP-DATA-DART-05.[92]

Dose Range-finding Study

Maternal Findings

Viability and Clinical Observations

One F_0 rat in the 3,000 ppm group was euthanized on study day 5 before the start of dosed feed administration due to the presence of excessive red eye discharge (too early to determine pregnancy status) (Appendix E). No clinical observations were attributed to 2-hydroxy-4-methoxybenzophenone (2H4MBP) exposure in any group during gestation or lactation (Appendix E).

Body Weights and Feed Consumption

F_0 females exposed to 50,000 ppm 2H4MBP displayed lower body weights than the control group (Table 4; Figure 5). The mean body weight of dams in the 50,000 ppm group on gestation day (GD) 21 was significantly decreased by 11% compared to the control group, and the mean body weight gain of dams in the 50,000 ppm group over gestation (GD 6–21) was significantly decreased by 35%. This difference was attributed to a transient body weight loss over the GD 6–9 interval and lower body weight gains over most of the subsequent intervals and not attributed to smaller litters or lower fetal weights (Appendix E). F_0 females exposed to 10,000 or 25,000 ppm 2H4MBP displayed similar 20% significant decreases in body weight gain over the GD 6–21 interval, which were attributed to lower body weights during the early gestation period (Table 4).

Lactation mean body weights were significantly decreased in dams exposed to 50,000 ppm 2H4MBP relative to the control group (Table 4; Figure 5). This decrease was similar in magnitude to that observed at the end of gestation and likely related to the significantly decreased body weights observed during gestation.

In general, feed consumption during gestation in the 2H4MBP-exposed groups was higher than in the control group (Table 5). Feed consumption was significantly increased at several time intervals in the 25,000 and 50,000 ppm groups, and likely signifies poor palatability and subsequent feed wastage given the high concentration of 2H4MBP in the feed. 2H4MBP intake for F_0 females in the 3,000, 10,000, 25,000, and 50,000 ppm 2H4MBP groups, based on measured feed consumption and dietary concentrations for GD 6–21 interval, was approximately 215, 695, 2,086, and 6,426 mg 2H4MBP/kg body weight/day (mg/kg/day), respectively (Table 5).

2H4MBP exposure was not associated with lower feed consumption during lactation (Table 5). 2H4MBP intake for F_0 females in the 3,000, 10,000, 25,000, and 50,000 ppm 2H4MBP groups, based on feed consumption and dietary concentrations for lactation days (LDs) 1–14, was approximately 577, 1,858, 4,460, and 12,029 mg/kg/day, respectively (Table 5).

Table 4. Summary of Mean Body Weights and Body Weight Gains of F_0 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed during Gestation and Lactation (Dose Range-finding Study)

Parameter[a,b]	0 ppm	3,000 ppm	10,000 ppm	25,000 ppm	50,000 ppm
Gestation Day					
6	224.0 ± 4.3 (10)	223.2 ± 4.6 (11)	225.2 ± 2.8 (6)	226.9 ± 3.6 (5)	224.8 ± 5.0 (12)
9	236.6 ± 4.4* (10)	238.0 ± 4.6 (11)	233.7 ± 3.5 (6)	233.8 ± 3.0 (5)	224.0 ± 4.1 (12)
12	248.6 ± 5.4** (10)	245.9 ± 5.3 (11)	243.7 ± 3.7 (6)	241.6 ± 3.5 (5)	226.9 ± 5.5** (12)
15	264.9 ± 6.4* (10)	261.8 ± 5.1 (11)	258.1 ± 5.1 (6)	258.7 ± 3.8 (5)	247.9 ± 5.0 (12)
18	298.8 ± 7.5** (10)	295.0 ± 5.3 (11)	290.2 ± 7.1 (6)	288.7 ± 5.3 (5)	268.6 ± 5.9** (12)
21	343.3 ± 11.3** (7)	327.7 ± 7.3 (8)	321.7 ± 8.3 (6)	323.8 ± 6.2 (5)	305.4 ± 7.1** (9)
Gestation Weight Change					
Gestation Day Interval					
6–21	120.7 ± 6.1** (7)	106.1 ± 7.4 (8)	96.5 ± 5.9** (6)	96.9 ± 4.5** (5)	78.0 ± 2.5** (9)
6–9	12.7 ± 1.2** (10)	14.8 ± 0.7 (11)	8.5 ± 1.7* (6)	6.9 ± 1.4** (5)	−0.8 ± 1.3** (12)
9–12	11.9 ± 1.5** (10)	7.9 ± 1.4 (11)	9.9 ± 2.8 (6)	7.9 ±1.4 (5)	2.9 ± 2.3** (12)
12–15	16.3 ± 1.3* (10)	15.9 ± 1.4 (11)	14.5 ± 2.0 (6)	17.0 ± 1.9 (5)	21.1 ± 1.6 (12)
15–18	33.9 ± 2.9** (10)	33.3 ± 2.8 (11)	32.1 ± 3.8 (6)	30.0 ± 2.7 (5)	20.7 ± 1.3** (12)
18–21	40.6 ± 2.5* (7)	35.1 ± 2.9 (8)	31.6 ± 2.1* (6)	35.1 ± 2.2 (5)	33.4 ± 1.2 (9)
Lactation Day					
1	247.5 ± 7.7** (7)	241.8 ± 7.1 (7)	239.0 ± 5.0 (6)	232.2 ± 8.6 (5)	221.7 ± 5.0** (9)
4	261.7 ± 6.2** (7)	256.1 ± 6.5 (7)	251.4 ± 5.6 (6)	245.5 ± 6.7 (5)	225.1 ± 5.7** (9)
7	266.7 ± 9.9* (5)	262.8 ± 9.2 (5)	263.4 ± 5.0 (6)	246.2 ± 8.2 (5)	234.6 ± 5.6* (6)
14	277.8 ± 11.8** (5)	269.0 ± 10.6 (5)	280.3 ± 5.0 (6)	252.7 ± 7.5 (5)	222.8 ± 10.5** (6)
21	265.7 ± 8.1* (5)	269.4 ± 8.3 (5)	267.5 ± 3.5 (6)	252.3 ± 6.6 (5)	222.5 ± 15.2* (6)
Lactation Weight Change					
Lactation Day Interval					
1–21	18.7 ± 3.4 (5)	32.1 ± 9.4 (5)	28.5 ± 3.6 (6)	20.2 ± 3.6 (5)	3.8 ± 11.6 (6)
1–4	14.3 ± 3.1* (7)	14.2 ± 4.8 (7)	12.3 ± 2.3 (6)	13.3 ± 3.3 (5)	3.3 ± 2.7 (9)
4–7	5.1 ± 2.0 (5)	6.2 ± 4.3 (5)	12.0 ± 0.7 (6)	0.7 ± 6.1 (5)	13.7 ± 2.3 (6)
7–14	11.1 ± 3.5 (5)	6.2 ± 8.5 (5)	16.9 ± 2.3 (6)	6.5 ± 6.9 (5)	−11.8 ± 8.5 (6)
14–21	−12.1 ± 4.3 (5)	0.4 ± 8.0 (5)	−12.8 ± 3.3 (6)	−0.4 ± 2.9 (5)	−0.3 ± 9.5 (6)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Data are presented as mean ± standard error (n); body weight data are presented in grams. Changes in n are the result of animal removal (i.e., biological sampling, animal health concerns).
[b]Statistical analysis performed by the Jonckheere (trend) and Williams or Dunnett (pairwise) tests.

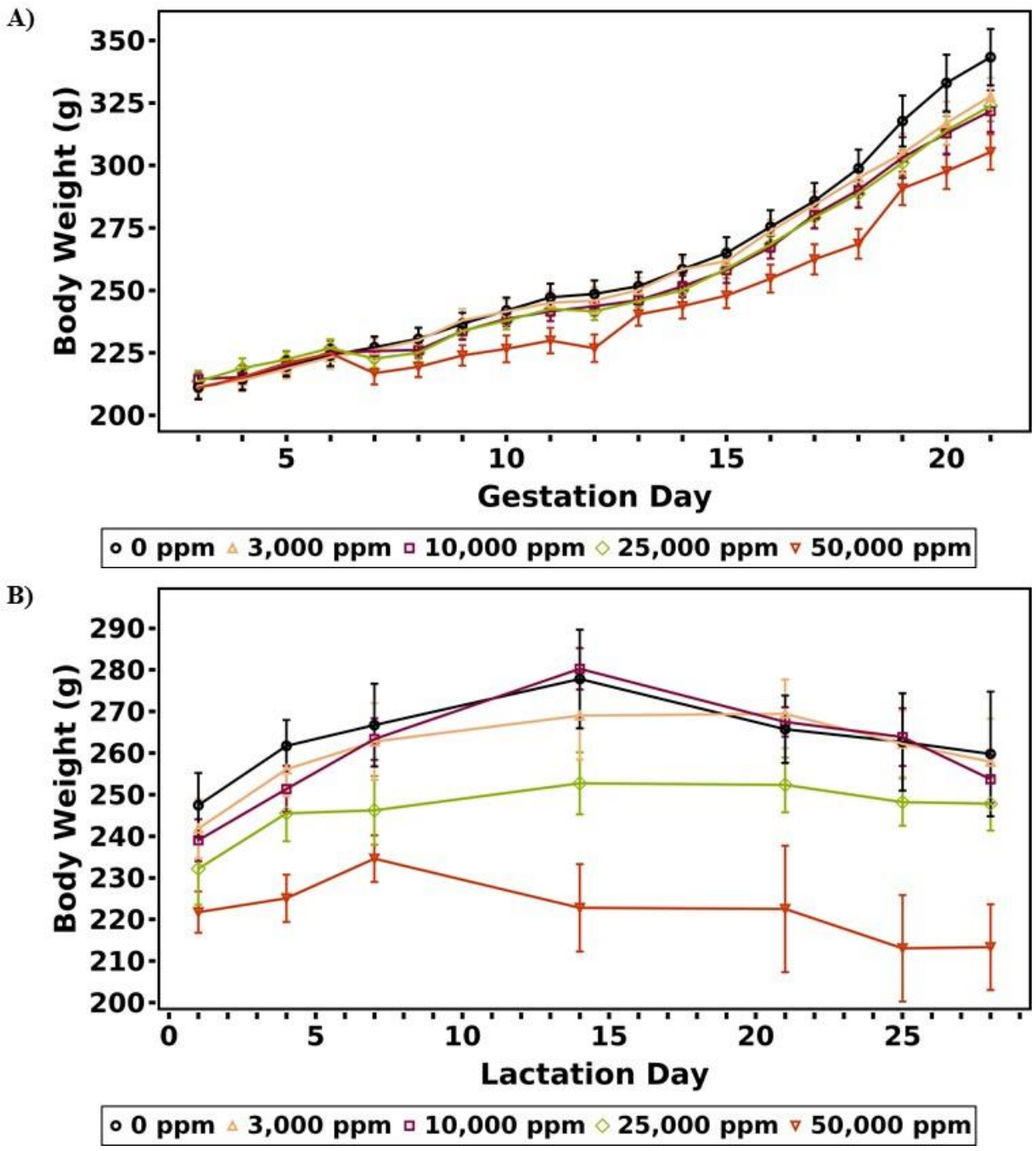


Figure 5. Growth Curves for F_0 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed during Gestation and Lactation (Dose Range-finding Study)

Growth curves shown for F_0 female rats during (A) gestation and (B) lactation. Information for statistical significance in maternal weights is provided in Table 4.

Table 5. Summary of Feed and Test Article Consumption of F_0 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed during Gestation and Lactation (Dose Range-finding Study)

Parameter[a,b]	0 ppm	3,000 ppm	10,000 ppm	25,000 ppm	50,000 ppm
Feed Consumption (g/animal/day)[c]					
Gestation Day Interval					
6–21	18.1 ± 0.7** (7)	18.7 ± 0.4 (7)	18.0 ± 0.8 (5)	21.8 ± 1.5* (4)	32.0 ± 1.5** (9)
6–9	16.3 ± 0.6** (10)	16.5 ± 0.4 (11)	14.6 ± 0.7 (5)	28.4 ± 4.7 (5)	39.3 ± 2.7** (12)
9–12	16.6 ± 0.8 (10)	17.4 ± 0.3 (11)	18.3 ± 1.5 (6)	18.3 ± 1.2 (5)	19.3 ± 2.0 (9)
12–15	16.8 ± 0.9** (10)	18.5 ± 0.4 (11)	17.9 ± 0.9 (6)	20.6 ± 1.1** (4)	42.4 ± 3.0** (11)
15–18	19.9 ± 0.7* (10)	21.2 ± 0.6 (11)	19.7 ± 1.0 (6)	21.2 ± 1.1 (5)	17.8 ± 0.9 (11)
18–21	20.1 ± 0.9** (7)	21.3 ± 0.8 (7)	18.8 ± 1.0 (6)	24.1 ± 2.3 (5)	34.7 ± 2.7** (9)
Lactation Day Interval					
1–14	47.5 ± 1.2 (5)	49.3 ± 2.1 (5)	47.9 ± 4.0 (6)	43.6 ± 3.4 (5)	53.6 ± 2.5 (6)
1–4	33.2 ± 1.4** (7)	34.2 ± 2.4 (7)	37.9 ± 7.2 (6)	43.3 ± 4.7 (5)	52.8 ± 3.7** (9)
4–7	42.1 ± 1.4 (5)	41.6 ± 2.6 (5)	44.6 ± 5.8 (6)	32.5 ± 3.0 (5)	35.1 ± 5.3 (4)
7–14	55.8 ± 1.5 (5)	58.1 ± 2.1 (5)	53.5 ± 2.8 (6)	48.5 ± 5.5 (5)	56.3 ± 3.6 (4)
Chemical Intake (mg/kg/day)[d,e]					
GD 6–21	0.0 ± 0.0 (7)	214.5 ± 4.7 (7)	695.2 ± 30.4 (5)	2,085.7 ± 161.2 (4)	6,426.4 ± 355.5 (9)
LD 1–14	0.0 ± 0.0 (5)	576.7 ± 18.4 (5)	1,858.3 ± 173.8 (6)	4,460.1 ± 310.8 (5)	12,028.5 ± 715.5 (6)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
GD = gestation day; LD = lactation day.
[a]Data are presented as mean ± standard error (n), where n = the number of dams. Feed consumption is not reported for nonpregnant animals during the gestation or lactation phase.
[b]Changes in n are the result of animal removal (i.e., biological sampling, animal health concerns). Additional animals removed as outliers include: GD 6–9 (one value in the 10,000 ppm group), GD 12–15 (one value in the 25,000 ppm group), GD 18–21 (one value in the 3,000 ppm group), and GD 6–21 (one value each in the 3,000, 10,000, and 25,000 ppm groups).
[c]Statistical analysis performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
[d]Chemical intake calculated as: ([exposure concentration × feed consumption]/[average body weight of day range]).
[e]No statistical analysis performed on the chemical intake data.

Maternal Reproductive Performance

Across all exposure groups, 13 out of 57 time-mated F_0 females were not pregnant: four in the control group; two each in the 3,000, 10,000, and 50,000 ppm groups; and three in the 25,000 ppm group (Table 6). There were no toxicologically relevant effects of 2H4MBP exposure on the proportion of dams that produced viable litters or on gestation length. There was no effect of 2H4MBP exposure on initial mean litter size or sex ratio.

Table 6. Summary of the Reproductive Performance of F_0 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed during Gestation (Dose Range-finding Study)

Parameter[a]	0 ppm	3,000 ppm	10,000 ppm	25,000 ppm	50,000 ppm
Time-mated Females (GD 6)	14[b]	13[b,c]	8	8	14[b]
Females Pregnant (%)	10 (71.4)	11 (78.6)	6 (75.0)	5 (62.5)	12 (85.7)
Females Not Pregnant (%)	4 (28.6)	2 (15.4)	2 (25.0)	3 (37.5)	2 (14.3)
Dams Not Delivering with Evidence of Pregnancy (%)	0 (0.0)[d]	1 (12.5)[d]	0 (0.0)	0 (0.0)	0 (0.0)[d]
Dams with Litters on PND 0 (%)[e]	7 (100.0)[d]	7 (87.5)[d]	6 (100.0)	5 (100.0)	9 (100.0)[d]
Gestation Length (days)[f,g,h]	22.1 ± 0.1 (7)	22.3± 0.2* (7)	22.0 ± 0.0* (6)	22.2 ± 0.2 (5)	22.1 ± 0.1 (9)
Live Litter Size on PND 0[f,h]	11.4 ± 0.7 (7)	10.9 ± 0.9 (7)	10.7 ± 1.4 (6)	11.8 ± 0.7 (5)	11.8 ± 0.7 (9)
PND 1 Pup Weight[h,i,j]	7.11 ± 0.09** 80 (7)	6.82 ± 0.19 74 (7)	6.39 ± 0.10* 64 (6)	6.58 ± 0.31 59 (5)	6.31 ± 0.14** 106 (9)
Percent Live Male Pups/Litter[f,h]	53.02 ± 5.86 (7)	47.64 ± 4.93 (7)	41.05 ± 4.66 (6)	47.84 ± 4.76 (5)	53.46 ± 3.80 (9)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
GD = gestation day; PND = postnatal day.
[a]Animals removed from the study between mating and littering were excluded from calculations of % littered females.
[b]Includes six time-mated (pregnant) rats used for biological sample collection for methods development.
[c]Excludes animal euthanized moribund on study day 5.
[d]Excludes three pregnant rats used for biological sample collection on GD 18.
[e]Percentage is the number of littered females/pregnant females. Statistical analysis performed by the Cochran-Armitage (trend) and Fisher's exact (pairwise) tests.
[f]Statistical analysis performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
[g]Gestation length calculated for time-mated females that delivered a litter.
[h]Data are displayed as mean ± standard error (n).
[i]n = the number of pups examined (number of litters).
[j]Statistical analysis performed using mixed effects models with litter as a random effect for both trend and pairwise tests, and a Dunnett-Hsu adjustment for multiple pairwise comparisons.

F_1 Offspring Findings

Pup Viability and Body Weights

2H4MBP exposure was associated with a reduction in the mean number of live pups per litter in the 25,000 and 50,000 ppm groups (approximately 2–3 pups/litter from PND 0 through PND 28) (Table 7; Appendix E). Over the lactation period, there were 20 dead pups (from five litters) in the 25,000 ppm group and 16 dead pups (from five litters) in the 50,000 ppm group, compared to 3 dead pups (from two litters) in the control group. In the 25,000 ppm group, 12 of the 20 dead pups were from a single litter. In the 50,000 ppm group, 10 of the 16 dead pups were from a single litter (Appendix E). Male and female pup mean body weights of these exposed groups were significantly decreased (25%–50%) compared to those of control pups (Table 8; Figure 6, Figure 7). Adverse F_1 pup clinical observations in the 25,000 and 50,000 ppm groups were consistent with the effects of 2H4MBP exposure on pup survival (Appendix E). Findings included observations of pups found dead, cannibalized, missing, no milk band, bruised, stained fur, cold to touch, or emaciated. There were no notable gross findings in the limited number of F_1 offspring that received a necropsy. Necropsy findings for pups found dead on or after PND 1

were limited to the absence of milk/food in the stomach (Appendix E). Pups in the 10,000 ppm group displayed mean body weights that were lower (4%–16%) than those of the control group.

Table 7. Summary of F_1 Litter Size and Pup Survival Following Perinatal Exposure to 2-Hydroxy-4-methoxybenzophenone (Dose Range-finding Study)

Postnatal Day	0 ppm	3,000 ppm	10,000 ppm	25,000 ppm	50,000 ppm
No. of Live Pups (Litters)[a]					
0	80 (7)	76 (7)	64 (6)	59 (5)	106 (9)
Total Litter Size[b,c]					
0	11.7 ± 0.6 (7)	11.7 ± 1.1 (7)	11.0 ± 1.5 (6)	12.2 ± 0.5 (5)	12.0 ± 0.6 (9)
Live Litter Size[b,c]					
0	11.4 ± 0.7 (7)	10.9 ± 0.9 (7)	10.7 ± 1.4 (6)	11.8 ± 0.7 (5)	11.8 ± 0.7 (9)
1	11.4 ± 0.7 (7)	10.6 ± 0.9 (7)	10.7 ± 1.4 (6)	11.8 ± 0.7 (5)	11.8 ± 0.7 (9)
4	11.4 ± 0.7 (7)	10.6 ± 0.9 (7)	10.7 ± 1.4 (6)	10.6 ± 1.3 (5)	10.8 ± 0.7 (9)
7	11.2 ± 0.8 (5)	10.6 ± 1.2 (5)	10.7 ± 1.4 (6)	9.6 ± 2.0 (5)	9.7 ± 1.1 (6)
14	11.0 ± 0.8 (5)	10.0 ± 1.0 (5)	10.7 ± 1.4 (6)	10.3 ± 0.6 (4)	9.5 ± 1.1 (6)
21	11.0 ± 0.8 (5)	10.0 ± 1.0 (5)	10.7 ± 1.4 (6)	10.3 ± 0.6 (4)	9.5 ± 1.1 (6)
28	11.0 ± 0.8 (5)	10.0 ± 1.0 (5)	10.7 ± 1.4 (6)	10.3 ± 0.6 (4)	9.2 ± 1.0 (6)
No. of Dead Pups (Litters)[a]					
0	2 (1)	6 (3)	2 (2)	2 (1)	2 (2)
1–4	0 (0)	2 (2)	0 (0)	6 (1)	9 (2)
5–28	1 (1)	4 (3)	0 (0)	12 (4)	5 (4)
1–28	1 (1)	6 (4)	0 (0)	18 (4)	14 (4)
Dead per Litter[b,c]					
0	0.29 ± 0.29 (7)	0.86 ± 0.46 (7)	0.33 ± 0.21 (6)	0.40 ± 0.40 (5)	0.22 ± 0.15 (9)
1–4	0.00 ± 0.00 (7)	0.29 ± 0.18 (7)	0.00 ± 0.00 (6)	1.20 ± 1.20 (5)	1.00 ± 0.88 (9)
5–28	0.20 ± 0.20 (5)	0.80 ± 0.37 (5)	0.00 ± 0.00 (6)	2.40 ± 0.98 (5)	0.83 ± 0.31 (6)
1–28	0.20 ± 0.20 (5)	1.20 ± 0.49 (5)	0.00 ± 0.00 (6)	3.60 ± 2.14 (5)	2.33 ± 1.56 (6)
Survival Ratio[b,c]					
0	0.97 ± 0.03 (7)	0.94 ± 0.03 (7)	0.97 ± 0.02 (6)	0.97 ± 0.03 (5)	0.98 ± 0.01 (9)
1–4	1.00 ± 0.00 (7)	0.97 ± 0.02 (7)	1.00 ± 0.00 (6)	0.90 ± 0.10 (5)	0.93 ± 0.06 (9)
5–28	0.98 ± 0.02 (5)	0.94 ± 0.03 (5)	1.00 ± 0.00 (6)	0.70 ± 0.18 (5)	0.90 ± 0.04 (6)
1–28	0.98 ± 0.02 (5)	0.90 ± 0.04 (5)	1.00 ± 0.00 (6)	0.70 ± 0.18 (5)	0.83 ± 0.10 (6)

[a]n = the number of pups (number of litters).
[b]Data are displayed as mean ± standard error of the litter means (n), where n = number of litters.
[c]F_1 litter size and survival endpoints were analyzed using the Jonckheere (trend) and Shirley or Dunn (pairwise) tests. All calculations are based on the last litter observation of the day.

Table 8. Summary of F_1 Male and Female Pup Mean Body Weights Following Perinatal Exposure to 2-Hydroxy-4-methoxybenzophenone (Dose Range-finding Study)[a,b]

Postnatal Day[c]	0 ppm	3,000 ppm	10,000 ppm	25,000 ppm	50,000 ppm
Male					
1	7.32 ± 0.11** 42 (7)[d]	7.02 ± 0.21 36 (7)	6.55 ± 0.13* 26 (6)	6.70 ± 0.30 28 (5)	6.43 ± 0.17** 57 (9)
4	11.19 ± 0.22** 42 (7)	10.67 ± 0.42 36 (7)	9.40 ± 0.21** 26 (6)	8.39 ± 0.60** 24 (5)	8.52 ± 0.27** 52 (9)
7	15.60 ± 0.38** 29 (5)	14.87 ± 0.99 25 (5)	13.49 ± 0.37 26 (6)	11.50 ± 0.90** 22 (5)	11.46 ± 0.30** 29 (6)
14	30.11 ± 1.01** 28 (5)	30.79 ± 1.19 25 (5)	26.41 ± 0.61 26 (6)	25.51 ± 1.78* 17 (4)	21.76 ± 1.09** 29 (6)
21	46.44 ± 1.26** 28 (5)	47.97 ± 1.47 25 (5)	40.77 ± 1.24 26 (6)	36.77 ± 2.24** 17 (4)	27.90 ± 2.52** 29 (6)
28	81.97 ± 1.60** 28 (5)	82.02 ± 3.18 25 (5)	70.77 ± 1.73 26 (6)	63.24 ± 4.94** 17 (4)	40.22 ± 4.02** 29 (6)
Female					
1	6.83 ± 0.03** 38 (7)	6.67 ± 0.18 38 (7)	6.30 ± 0.09 38 (6)	6.44 ± 0.35 31 (5)	6.10 ± 0.11** 49 (9)
4	10.40 ± 0.15** 38 (7)	10.02 ± 0.39 38 (7)	9.11 ± 0.12* 38 (6)	8.38 ± 0.78** 29 (5)	8.25 ± 0.24** 45 (9)
7	14.66 ± 0.33** 27 (5)	14.73 ± 0.74 28 (5)	13.00 ± 0.21 38 (6)	12.02 ± 0.99* 26 (4)	11.27 ± 0.29** 29 (6)
14	27.07 ± 1.22** 27 (5)	29.49 ± 0.91 25 (5)	26.16 ± 0.23 38 (6)	24.64 ± 2.14 24 (4)	22.41 ± 1.54* 28 (6)
21	42.83 ± 1.07** 27 (5)	44.69 ± 1.99 25 (5)	39.35 ± 0.38 38 (6)	36.71 ± 2.71 24 (4)	28.02 ± 3.07** 28 (6)
28	74.01 ± 1.15** 27 (5)	74.13 ± 2.42 25 (5)	66.77 ± 1.00 38 (6)	58.12 ± 5.84* 24 (4)	37.12 ± 4.90** 26 (6)
Male and Female					
1	7.11 ± 0.09** 80 (7)	6.82 ± 0.19 74 (7)	6.39 ± 0.10* 64 (6)	6.58 ± 0.31 59 (5)	6.31 ± 0.14** 106 (9)
4	10.84 ± 0.18** 80 (7)	10.33 ± 0.38 74 (7)	9.19 ± 0.14** 64 (6)	8.41 ± 0.69** 53 (5)	8.43 ± 0.23** 97 (9)
7	15.16 ± 0.35** 56 (5)	14.69 ± 0.84 53 (5)	13.13 ± 0.23* 64 (6)	11.48 ± 0.92** 48 (5)	11.37 ± 0.29** 58 (60)
14	29.08 ± 0.90** 55 (5)	30.11 ± 0.99 50 (5)	26.15 ± 0.27 64 (6)	24.98 ± 1.94 41 (4)	22.09 ± 1.26** 57 (6)
21	44.85 ± 1.24** 55 (5)	46.32 ± 1.42 50 (5)	39.82 ± 0.66 64 (6)	36.67 ± 2.57* 41 (4)	28.03 ± 2.76** 57 (6)
28	78.55 ± 1.66** 55 (5)	77.73 ± 2.39 50 (5)	68.27 ± 1.14 64 (6)	60.37 ± 5.39** 41 (4)	38.80 ± 4.43** 55 (6)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.

[a]Statistical analysis performed using mixed effects models with litter as a random effect for both trend and pairwise tests, and a Dunnett-Hsu adjustment for multiple pairwise comparisons.
[b]Data are displayed as mean ± standard error of the litter means. Body weight data are presented in grams.
[c]As litters were not standardized, pup weights throughout the entire postnatal period were adjusted using the total live litter size on postnatal day 1.
[d]n = the number of pups examined (number of litters).

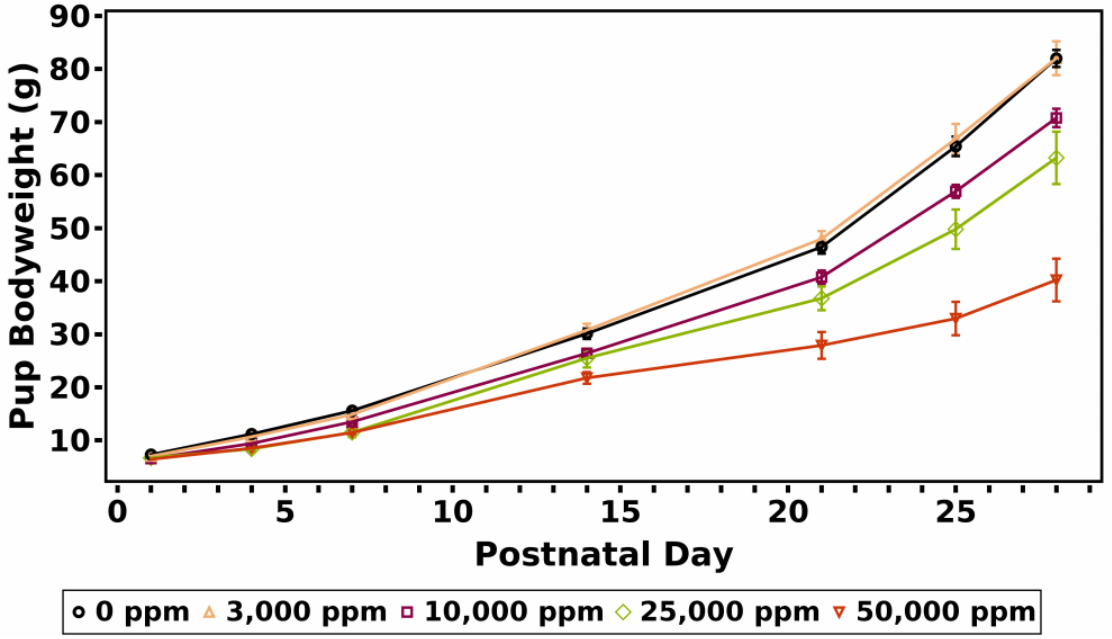


Figure 6. Lactation Growth Curves for F_1 Male Pups Following Perinatal Exposure to 2-Hydroxy-4-methoxybenzophenone (Dose Range-finding Study)

Information for statistical significance in male pup weights is provided in Table 8.

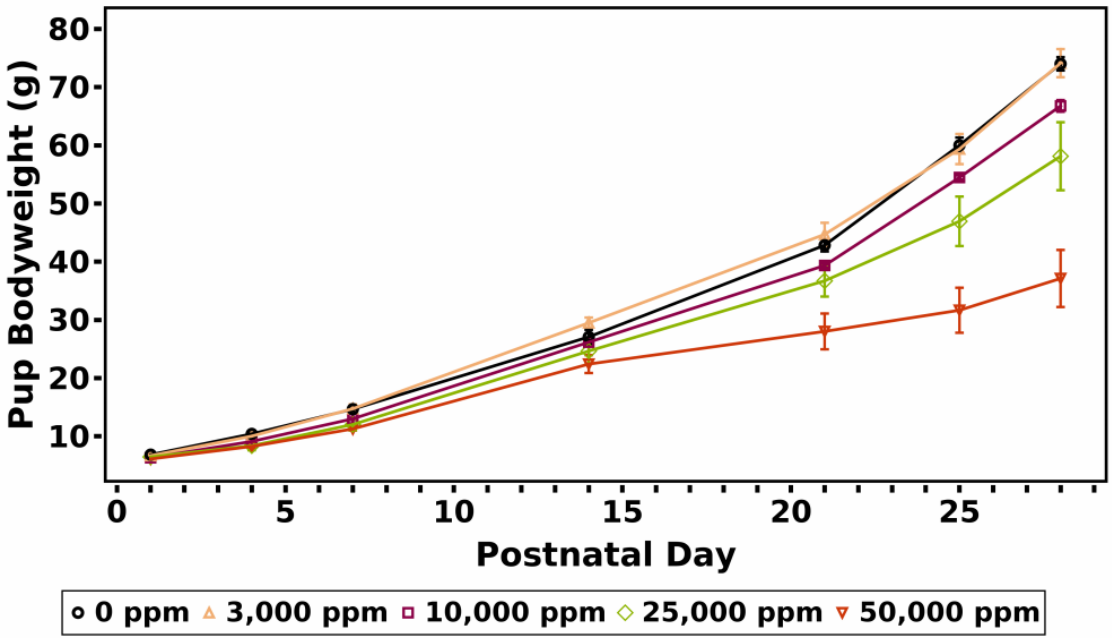


Figure 7. Lactation Growth Curves for F_1 Female Pups Following Perinatal Exposure to 2-Hydroxy-4-methoxybenzophenone (Dose Range-finding Study)

Information for statistical significance in female pup weights is provided in Table 8.

Exposure Concentration Selection Rationale for the Modified One-Generation Study of 2-Hydroxy-4-methoxybenzophenone

The selection of 30,000 ppm 2H4MBP as the high exposure concentration was based on the toxicity observed at 50,000 ppm and the marginal effect on pup survival at 25,000 ppm (most of the pup deaths at this exposure concentration were attributed to a single dam). Exposure concentration spacing (3,000, 10,000, 30,000 ppm) was selected to achieve a no-observed-adverse-effect level and to avoid excessive overlap of the ingested doses due to increased feed consumption during pregnancy. The selection of the 0.05 ppm ethinyl estradiol (EE) exposure concentration as a reference positive control was informed by the National Center for Toxicological Research studies,[93] which demonstrated that this exposure concentration accelerated time to vaginal opening (VO), delayed time to balanopreputial separation (BPS), caused transient alterations in estrous cyclicity, and induced male mammary gland hyperplasia.

Modified One-Generation Study

F_0 Generation: Maternal Findings

Maternal effects were evaluated from GD 6 through LD 28, as shown in Figure 8. Viability, clinical observations, gestation and lactation mean body weights, feed consumption, and reproductive performance results are presented below.

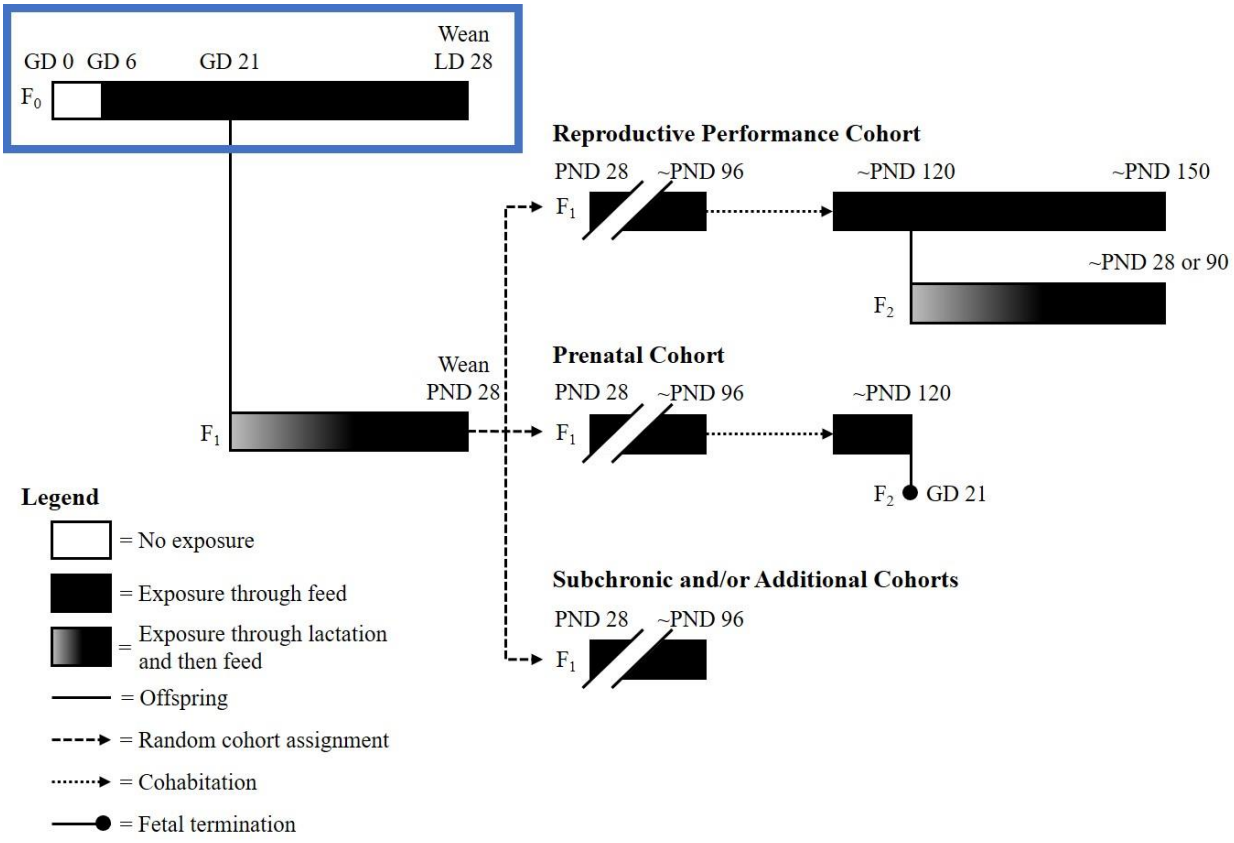


Figure 8. Design of the Modified One-Generation Study – F_0 Generation

GD = gestation day; LD = lactation day; PND = postnatal day.

F_0 Viability and Clinical Observations

2H4MBP exposure did not affect survival of the F_0 females (Appendix E). One female in the EE group was removed on GD 11 and was subsequently diagnosed with lymphoma. Given the singular incidence and early onset, this death was not considered related to EE exposure. No clinical observations were attributed to 2H4MBP exposure (Appendix E).

F_0 Gestation Body Weights and Feed Consumption

F_0 females exposed to 10,000 or 30,000 ppm 2H4MBP displayed lower gestation mean body weights and body weight gains (Table 9; Figure 9). On GD 21, female mean body weights were significantly decreased by 5% and 10% compared to those of control animals in the 10,000 and 30,000 ppm 2H4MBP groups, respectively. Body weight gains between GD 6 and GD 21 were significantly decreased by 11%, 25%, and 35% compared to those of the control group in the

10,000 and 30,000 ppm 2H4MBP and 0.05 ppm EE groups, respectively (Table 9). There was a transient loss in mean body weight (−1.0 g) between GD 6 and GD 9 in the 30,000 ppm 2H4MBP group compared to a gain of 13.7 g in the control group. This interval corresponds to the first interval the females were administered dosed feed and likely reflects lower palatability of the dosed feed; this is also consistent with what was observed in the dose range-finding study. Females in the 30,000 ppm groups also exhibited significantly decreased (approximately 12%) body weight gains in the GD 15–18 and GD 18–21 intervals (Table 9). Gestational mean body weights and weight gains in the EE group were less than those in the control group. Body weight gain in the EE group over the GD 6–21 interval was significantly decreased by approximately 35% compared to the control group (Table 9). There was no effect of 2H4MBP exposure on F_0 female mean body weights during gestation in the 3,000 ppm group. There was no reduction in litter size on PND 0 or pup mean body weight on PND 1 in the 2H4MBP-exposed groups (Appendix E), suggesting the lower relative maternal body weights were due to a maternal body weight effect of 2H4MBP rather than an effect on the collective weight of the uterine contents. Pup body weight on PND 1, but not litter size, was significantly decreased in the 0.05 ppm EE group (Appendix E) and likely contributed to the lower maternal body weight gain of that group compared to the control group.

Table 9. Summary of Mean Body Weights and Body Weight Gains of F_0 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed during Gestation

Parameter[a,b]	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[c]
Gestation Day					
6	242.9 ± 2.7 (22)	239.4 ± 3.2 (21)	239.0 ± 2.7 (22)	239.1 ± 2.7 (20)	241.4 ± 3.9 (20)
9	256.6 ± 2.9** (22)	251.4 ± 3.5 (21)	249.5 ± 2.9 (22)	238.1 ± 2.5** (20)	242.3 ± 3.6** (20)
12	272.4 ± 3.1** (22)	266.3 ± 3.5 (21)	262.1 ± 3.1* (22)	251.7 ± 2.7** (20)	251.5 ± 3.6** (19)
15	292.2 ± 3.0** (22)	285.1 ± 3.8 (21)	280.5 ± 3.2* (22)	268.8 ± 2.9** (20)	264.5 ± 3.6** (19)
18	331.4 ± 3.7** (22)	325.2 ± 4.5 (21)	317.6 ± 3.9* (22)	303.3 ± 3.4** (20)	297.4 ± 5.1** (19)
21	375.2 ± 4.5** (22)	366.6 ± 5.6 (21)	357.2 ± 4.7** (21)	338.5 ± 3.9** (20)	328.2 ± 5.1** (19)
Gestation Weight Change					
Gestation Day Interval					
6–21	132.3 ± 3.0** (22)	127.1 ± 3.4 (21)	118.1 ± 3.2** (22)	99.3 ± 2.5** (20)	86.4 ± 3.8** (19)
3–6	14.6 ± 1.4 (22)	12.7 ± 1.2 (21)	14.3 ± 1.1 (22)	12.5 ± 1.0 (20)	15.0 ± 1.6 (20)
6–9	13.7 ± 0.6** (22)	12.0 ± 0.9 (21)	10.5 ± 1.0* (22)	-1.0 ± 1.4** (20)	0.9 ± 1.1** (20)
9–12	15.8 ± 0.9* (22)	15.0 ± 0.9 (21)	12.7 ± 0.7* (22)	13.5 ± 0.9 (20)	9.3 ± 0.8** (19)
12–15	19.8 ± 0.8* (22)	18.8 ± 0.8 (21)	18.4 ± 0.8 (22)	17.2 ± 1.1 (20)	13.0 ± 0.9** (19)
15–18	39.2 ± 1.4** (22)	40.2 ± 1.5 (21)	37.0 ± 1.4 (22)	34.5 ± 1.3* (20)	32.9 ± 2.6* (19)
18–21	43.8 ± 1.7** (22)	41.3 ± 1.9 (21)	40.7 ± 1.4 (21)	35.1 ± 1.2** (20)	30.8 ± 1.7** (19)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
EE = ethinyl estradiol.
[a]Data are displayed as mean ± standard error (n); body weight data are presented in grams.
[b]Statistical analysis performed by the Jonckheere (trend) and Williams or Dunnett (pairwise) tests.
[c]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.

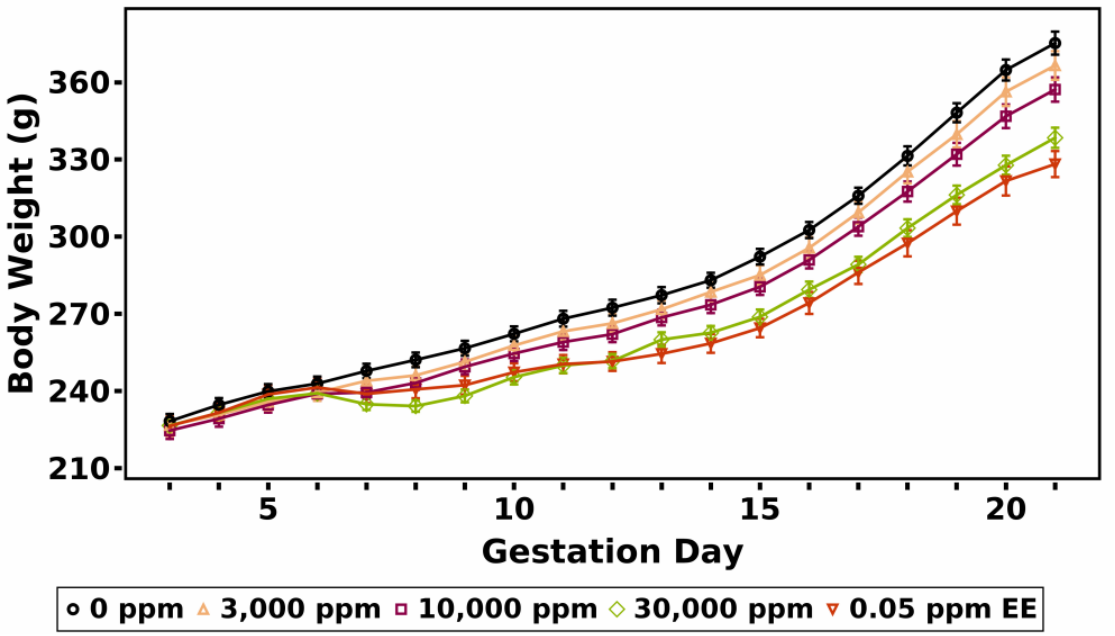


Figure 9. Growth Curves for F_0 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed during Gestation

EE = ethinyl estradiol. Information for statistical significance in maternal weights is provided in Table 9.

Despite sporadic differences, neither 2H4MBP nor EE exposure adversely affected feed consumption during gestation (Table 10). Observed higher feed consumption in the 30,000 ppm group likely represented feed wastage. 2H4MBP intake for F_0 females in the 3,000, 10,000, and 30,000 ppm groups, based on feed consumption and dietary concentrations over the GD 6–21 interval, was approximately 205, 697, and 2,644 mg/kg/day, respectively (Table 10). EE intake during gestation was approximately 0.004 mg/kg/day.

Table 10. Summary of Feed and Test Article Consumption of F_0 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed during Gestation

Gestation Day Interval[a]	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[b]
Feed Consumption (g/animal/day)[c]					
6–21	20.0 ± 0.3* (22)	19.6 ± 0.4 (21)	19.7 ± 0.5 (22)	23.9 ± 1.0* (20)	20.3 ± 1.5 (19)
3–6	17.5 ± 0.3 (22)	16.7 ± 0.4 (21)	16.8 ± 0.4 (22)	17.0 ± 0.5 (20)	17.4 ± 0.5 (20)
6–9	17.8 ± 0.3** (22)	17.6 ± 0.4 (21)	20.4 ± 1.5 (22)	30.8 ± 2.7** (20)	20.7 ± 2.5 (20)
9–12	18.7 ± 0.3* (22)	18.6 ± 0.4 (20)[d]	18.2 ± 0.6 (22)	17.1 ± 0.6 (20)	13.9 ± 0.5** (19)[e]
12–15	19.2 ± 0.4 (22)	19.4 ± 0.4 (21)	18.9 ± 0.6 (22)	27.2 ± 2.3* (20)	26.9 ± 3.3 (19)
15–18	22.6 ± 0.4** (22)	21.7 ± 0.4 (21)	21.0 ± 0.4** (22)	21.3 ± 0.3** (20)	18.9 ± 0.6** (16)[f]
18–21	21.8 ± 0.6 (22)	20.6 ± 0.5 (21)	20.0 ± 0.6 (22)	23.3 ± 1.8 (20)	19.7 ± 1.2** (19)
Chemical Intake (mg/kg/day)[g,h]					
GD 6–21	0.0 ± 0.0 (22)	204.5 ± 2.7 (21)	697.3 ± 15.4 (22)	2,644.4 ± 109.2 (20)	3.8 ± 0.2 (19)[i]

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
EE = ethinyl estradiol; GD = gestation day.
[a]Data are displayed as mean ± standard error (n), where n = the number of dams. Feed consumption is not reported for nonpregnant animals during the gestation phase.
[b]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[c]Statistical analysis performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
[d]Change in n is due to the exclusion of improbable data.
[e]Excludes one dam euthanized moribund on GD 11.
[f]Excludes feed consumption from cages where excess feed spillage was observed.
[g]Chemical intake calculated as: ([exposure concentration × feed consumption]/[average body weight of day range]).
[h]No statistical analysis performed on the chemical intake data.
[i]EE intake presented as µg/kg/day.

Maternal Reproductive Performance

Across all exposure groups, 20 of 125 time-mated rats were not pregnant: three each in the control and 10,000 ppm groups, four in the 3,000 ppm group, five in the 30,000 ppm group, and five in the EE group (Table 11; Appendix E). There was no effect of 2H4MBP exposure on the proportion of dams that produced viable litters or on gestation length. There was no effect of 2H4MBP exposure on initial mean litter size, PND 1 pup weight, or sex ratio. PND 1 pup weight in the EE group was significantly decreased by 13% compared to the control group (Table 11). Anogenital distance (AGD) measurements are presented in Appendix E.

Table 11. Summary of the Reproductive Performance of F_0 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed during Gestation

Parameter[a]	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[b]
Time-mated Females (GD 6)	25	25	25	25	25
Females Pregnant (%)	22 (88.0)	21 (84.0)	22 (88.0)	20 (80.0)	20 (80.0)
Females Not Pregnant (%)	3 (12.0)	4 (16.0)	3 (12.0)	5 (20.0)	5 (20.0)
Dams with Litters on PND 0 (%)[c]	22 (100.0)	21 (100.0)	22 (100.0)	20 (100.0)	18 (90.0)[d]
Gestation Length (days)[e,f,g]	22.3 ± 0.1 (22)	22.3 ± 0.1 (21)	22.2 ± 0.1 (22)	22.4 ± 0.1 (20)	22.4 ± 0.1 (18)
Live Litter Size on PND 0[e,g]	12.8 ± 0.6 (22)	13.0 ± 0.6 (21)	13.3 ± 0.6 (22)	12.4 ± 0.4 (20)	13.2 ± 0.6 (18)
PND 1 Pup Weight[g,h,i]	7.07 ± 0.10* 271 (22)	7.04 ± 0.09 260 (20)[j]	6.78 ± 0.10 276 (22)	6.78 ± 0.09 234 (20)	6.21 ± 0.18** 208 (18)
Percent Live Male Pups/Litter[e,g]	46.62 ± 2.90 (22)	52.08 ± 3.48 (21)	48.11 ± 4.01 (22)	52.56 ± 2.93 (20)	47.76 ± 3.60 (18)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
EE = ethinyl estradiol; GD = gestation day; PND = postnatal day.
[a]Animals removed from the study between mating and littering were excluded from calculations of % littered females.
[b]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[c]Percentage is the number of littered females/pregnant females. Statistical analysis performed by the Cochran-Armitage (trend) and Fisher's exact (pairwise) tests.
[d]Excludes one dam euthanized moribund on GD 11.
[e]Statistical analysis performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
[f]Gestation length was calculated for time-mated females that delivered a litter.
[g]Data are displayed as mean ± standard error (n).
[h]n = the number of pups examined (number of litters).
[i]Statistical analysis performed using mixed effects models with litter as a random effect for both trend and pairwise tests, and a Dunnett-Hsu adjustment for multiple pairwise comparisons.
[j]Excludes one litter in which the lone pup died on PND 1.

Lactation Body Weights and Feed Consumption

F_0 females in the 10,000 and 30,000 ppm 2H4MBP and 0.5 ppm EE groups displayed lower mean body weights during lactation compared to the control group (Figure 10; Table 12). The magnitude of response in female body weights at LD 1 and LD 28 was similar to that observed at the end of the gestation interval. There was no effect of 2H4MBP or EE exposure on body weight gain during the lactation interval. These observations collectively suggest that the lower lactation body weight was a consequence of exposure to 2H4MBP or EE during gestation and not a direct effect of exposure during lactation.

Feed consumption during lactation was similar among the groups. Dam 2H4MBP intake based on feed consumption and dietary concentrations during lactation from LD 1 through LD 13 (until the pups started consuming feed) for the 3,000, 10,000, and 30,000 ppm groups was approximately 484, 1,591, and 5,120 mg/kg/day, respectively (Table 12). EE intake during lactation was approximately 0.008 mg/kg/day.

Table 12. Summary of Mean Body Weights, Body Weight Gains, and Feed and Test Article Consumption of F_0 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed during Lactation[a]

Lactation Day	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[b]
Body Weight (g)[c]					
1	268.3 ± 3.7** (22)	260.5 ± 3.8 (21)	254.6 ± 3.7** (22)	244.6 ± 3.3** (20)	227.5 ± 3.5** (18)
28	286.3 ± 3.1** (22)	282.1 ± 3.7 (20)	277.1 ± 3.0 (22)	257.4 ± 4.0** (20)	249.3 ± 4.0** (15)[d]
Body Weight Gain (g)[c]					
1–28	18.0 ± 3.3 (22)	22.0 ± 2.4 (20)	22.6 ± 2.8 (22)	12.7 ± 3.2 (20)	23.8 ± 1.9 (15)
Feed Consumption[e]					
1–13 (g/animal/day)	45.3 ± 0.9* (22)	45.8 ± 1.0 (19)	43.8 ± 0.9 (22)	43.6 ± 1.9 (18)	41.3 ± 1.7* (15)
1–13 (g/kg/day)	157.9 ± 3.3 (22)	161.4 ± 3.0 (19)	159.1 ± 3.0 (22)	170.7 ± 7.2 (18)	168.9 ± 7.4 (15)
Chemical Intake (mg/kg/day)[f,g]					
1–13	0.0 ± 0.0 (22)	484.1 ± 8.9 (19)	1,590.7 ± 29.6 (22)	5,119.8 ± 216.3 (18)	8.4 ± 0.4 (15)[h]

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
EE = ethinyl estradiol.
[a]Data are displayed as mean ± standard error (n), where n = the number of dams.
[b]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[c]Statistical analysis performed by the Jonckheere (trend) and Williams or Dunnett (pairwise) tests.
[d]Excludes body weights of two dams on lactation day (LD) 4 and one dam on LD 7 scheduled for removal.
[e]Statistical analysis performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
[f]Chemical intake calculated as: ([exposure concentration × feed consumption]/[average body weight of day range]).
[g]No statistical analysis performed on the chemical intake data.
[h]EE intake presented as µg/kg/day.

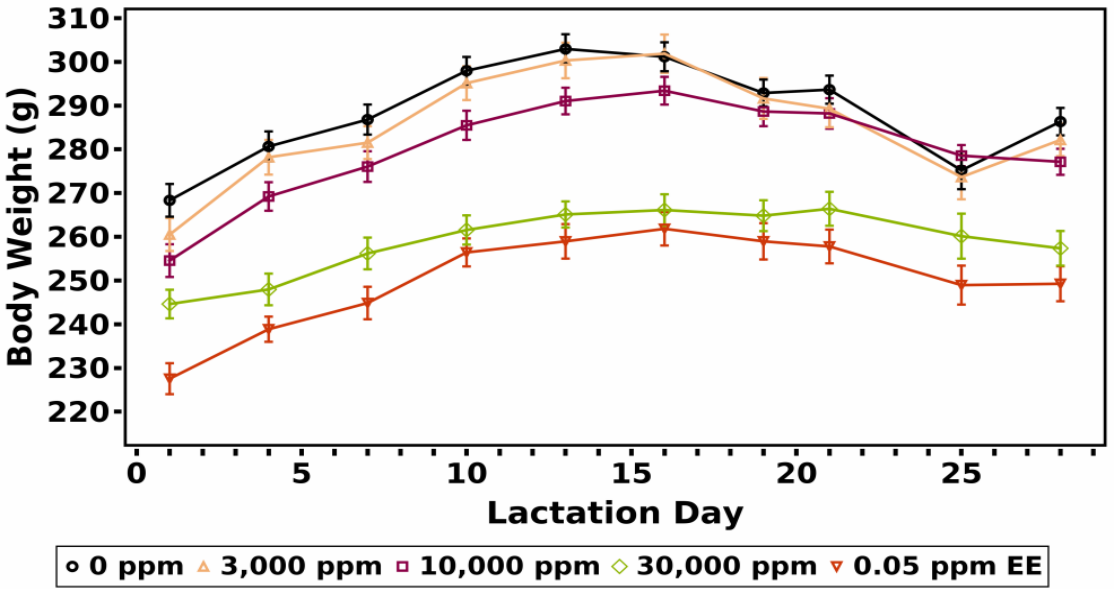


Figure 10. Growth Curves for F_0 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed during Lactation

EE = ethinyl estradiol. Information for statistical significance in maternal weights is provided in Table 12.

Collectively, these data indicate that 30,000 ppm 2H4MBP and 0.05 ppm EE challenged the dams (as demonstrated by significantly decreased GD 6–21 body weights), without adversely affecting F_1 litter size.

F_1 Generation: Preweaning

F_1 male and female rats were evaluated during the preweaning period from PND 0 through PND 28, as shown in Figure 11. Viability, clinical observations, and mean body weight results are presented below.

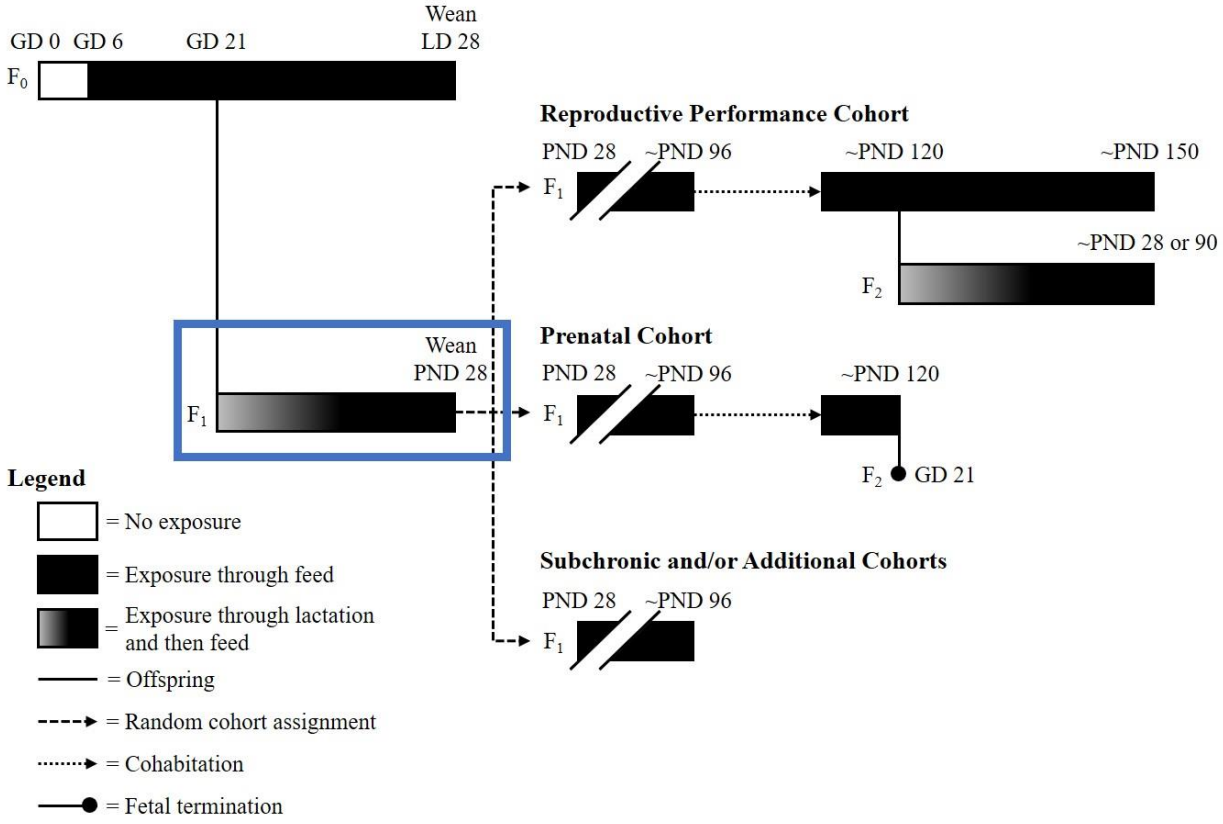


Figure 11. Design of the Modified One-Generation Study – F_1 Generation: Preweaning

GD = gestation day; LD = lactation day; PND = postnatal day.

F_1 Viability and Clinical Observations

Clinical observations were noted in individual pups in all groups, including the control groups, and were typically indicative of a pup not thriving (e.g., cold to the touch, no milk in the stomach) (Appendix E). There was no effect of 2H4MBP on pup survival (Table 13). The mean number of live pups per litter appeared to be reduced in the 0.05 ppm EE group on PND 4 relative to the control group. That reduction reflected three litters that did not survive to PND 4, resulting in a higher number of dead or missing (presumed dead) pups and a lower survival ratio for the PND 1–4 interval relative to the control group. On PND 28, there was a slight, but significant, decrease in mean litter size in the EE group relative to the control group.

Table 13. Summary of F_1 Litter Size and Pup Survival Following Perinatal Exposure to 2-Hydroxy-4-methoxybenzophenone

Postnatal Day	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[a]
No. of Live Pups (Litters)[b]					
0	273 (22)	263 (21)	282 (22)	234 (20)	221 (18)
Total Litter Size[c,d]					
0	12.8 ± 0.6 (22)	13.0 ± 0.6 (21)	13.3± 0.6 (22)	12.4 ± 0.4 (20)	13.2 ± 0.6 (18)
Live Litter Size[c,d]					
0	12.4 ± 0.6 (22)	12.5 ± 0.7 (21)	12.8 ± 0.5 (22)	11.7 ± 0.4 (20)	12.3 ± 0.6 (18)
1	12.3 ± 0.6 (22)	13.0 ± 0.5 (20)[e]	12.5 ± 0.5 (22)	11.7 ± 0.4 (20)	11.6 ± 0.8 (18)
4 (prestandardization)	12.2 ± 0.5 (22)	13.0 ± 0.5 (20)	12.5 ± 0.5 (22)	11.7 ± 0.4 (20)	11.4 ± 0.9 (16)
4 (poststandardization)	7.9 ± 0.1 (22)	7.9 ± 0.1 (20)	7.9 ± 0.1 (22)	8.0 ± 0.0 (20)	7.9 ± 0.1 (15)[f]
13	7.9 ± 0.1 (22)	7.9 ± 0.1 (20)	7.8 ± 0.1 (22)	7.9 ± 0.1 (20)	7.9 ± 0.1 (15)
21	7.9 ± 0.1 (22)	7.9 ± 0.1 (20)	7.7 ± 0.1 (22)	7.8 ± 0.1 (20)	7.9 ± 0.1 (15)
28	7.8 ± 0.1 (22)	7.9 ± 0.1 (20)	7.7 ± 0.1 (22)	7.8 ± 0.1 (20)	7.4 ± 0.2** (15)
No. of Dead Pups (Litters)[b]					
0	9 (4)	9 (7)	11 (9)	13 (7)	17 (5)
1–4	5 (4)	4 (4)	7 (4)	1 (1)	39 (5)
5–28	1 (1)	1 (1)	4 (3)	4 (4)	0 (0)
Dead per Litter[c,d]					
0	0.41 ± 0.28 (22)	0.43 ± 0.15 (21)	0.50 ± 0.14 (22)	0.65 ± 0.27 (20)	0.94 ± 0.47 (18)
1–4	0.23 ± 0.11 (22)	0.19 ± 0.09 (21)	0.32 ± 0.19 (22)	0.05 ± 0.05 (20)	2.17 ± 1.13 (18)
5–28	0.05 ± 0.05 (22)	0.05 ± 0.05 (20)	0.18 ± 0.11 (22)	0.20 ± 0.09 (20)	0.00 ± 0.00 (15)
Survival Ratio[c,d]					
0	0.97 ± 0.02 (22)	0.94 ± 0.03 (21)	0.96 ± 0.01 (22)	0.95 ± 0.02 (20)	0.93 ± 0.03 (18)
1–4	0.98 ± 0.01 (22)	0.94 ± 0.05 (21)	0.98 ± 0.01 (22)	1.00 ± 0.00 (20)	0.83 ± 0.09 (18)
5–28	0.99 ± 0.01 (22)	0.99 ± 0.01 (20)	0.98 ± 0.01 (22)	0.98 ± 0.01 (20)	1.00 ± 0.00 (15)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
**Statistically significant at $p \leq 0.01$.
EE = ethinyl estradiol.
[a]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[b]n = the number of pups examined (number of litters).
[c]Data are displayed as mean ± standard error of the litter means (n), where n = the number of litters. For F_1 pups, data are displayed as the mean of litter values ± standard error (n) of litter values (number of litters produced by F_0 dams).
[d]F_1 litter size and survival endpoints were analyzed using the Jonckheere (trend) and Shirley or Dunn tests (pairwise comparisons). All calculations were based on the last litter observation of the day.
[e]One whole litter loss occurred by postnatal day (PND) 1.
[f]Three whole litter losses occurred by PND 4 (one by PND 1).

F_1 Body Weights

Male Pups

An exposure concentration- and time-related reduction in male pup mean body weight per litter was observed during lactation in the 10,000 and 30,000 ppm 2H4MBP and the 0.05 ppm EE groups, relative to the control group (Table 14; Figure 12). From PND 1 through PND 28, mean body weight differences were significantly increased between the control group and the 30,000 ppm group and, to a lesser extent, the 10,000 ppm group. On PND 28, male pup mean body weights per litter were significantly decreased by 10%, 24%, and 11% compared to those of the control group in the 10,000 and 30,000 ppm 2H4MBP and 0.05 ppm EE groups, respectively.

Female Pups

An exposure concentration- and time-related reduction in female pup mean body weight per litter was observed during lactation in the groups exposed to 10,000 or 30,000 ppm 2H4MBP and 0.05 ppm EE, relative to the control group (Table 14; Figure 13). From PND 1 through PND 28, mean body weight differences became greater between the control group and the 30,000 ppm group and, to a lesser extent, the 10,000 ppm group. On PND 28, female pup mean body weights per litter were significantly decreased by 9%, 24%, and 7% compared to those of the control group in the 10,000 and 30,000 ppm 2H4MBP and 0.05 ppm EE groups, respectively.

Table 14. Summary of F_1 Male and Female Pup Mean Body Weights Following Perinatal Exposure to 2-Hydroxy-4-methoxybenzophenone[a,b]

Postnatal Day	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[c]
Male					
1	7.26 ± 0.10** 128 (22)[d]	7.17 ± 0.10 142 (20)	6.89 ± 0.11* 136 (21)	6.88 ± 0.10* 122 (20)	6.34 ± 0.19** 101 (18)
4[e]	10.76 ± 0.16** 126 (22)	10.49 ± 0.19 141 (20)	9.94 ± 0.17** 136 (21)	9.21 ± 0.23** 121 (20)	8.78 ± 0.35** 91 (17)
7	16.49 ± 0.31** 85 (22)	15.97 ± 0.35 80 (20)	15.22 ± 0.39* 82 (21)	14.19 ± 0.45** 83 (20)	14.10 ± 0.35** 57 (15)
13	31.37 ± 0.42** 85 (22)	30.79 ± 0.63 80 (20)	28.87 ± 0.51** 82 (21)	25.40 ± 0.74** 82 (20)	26.83 ± 0.58** 57 (15)
28	89.91 ± 1.08** 85 (22)	86.26 ± 1.53 80 (20)	81.11 ± 1.21** 82 (21)	67.93 ± 2.16** 80 (20)	80.46 ± 1.15** 57 (15)
Female					
1	6.88 ± 0.11* 143 (22)	6.87 ± 0.10 118 (20)	6.61 ± 0.11 140 (22)	6.63 ± 0.09 112 (20)	6.23 ± 0.12** 107 (17)
4[e]	10.13 ± 0.17** 142 (22)	9.92 ± 0.21 118 (20)	9.51 ± 0.18 139 (20)	8.93 ± 0.21** 112 (20)	8.39 ± 0.36** 102 (17)
7	15.51 ± 0.33** 88 (22)	14.86 ± 0.42 78 (20)	14.41 ± 0.39 90 (22)	13.61 ± 0.39** 76 (20)	13.56 ± 0.33** 61 (15)
13	29.80 ± 0.57** 88 (22)	29.31 ± 0.84 77 (20)	27.35 ± 0.62* 89 (22)	25.07 ± 0.64** 76 (20)	25.91 ± 0.46** 61 (15)
28	80.35 ± 1.19** 87 (22)	78.14 ± 1.62 77 (20)	73.04 ± 1.12** 88 (22)	60.70 ± 1.53** 76 (20)	74.64 ± 1.11** 54 (15)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
EE = ethinyl estradiol.
[a]Statistical analysis performed using mixed effects models with litter as a random effect for both trend and pairwise tests, and a Dunnett-Hsu adjustment for multiple pairwise comparisons. Pup weights were adjusted for covariate litter size: total live on postnatal day (PND) 1 for day 1 to day 4 and number of live pups poststandardization for later days.
[b]Data are displayed as mean ± standard error of the litter means. Body weight data are presented in grams.
[c]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[d]n = the number of pups examined (number of litters).
[e]PND 4 weights are prestandardization.

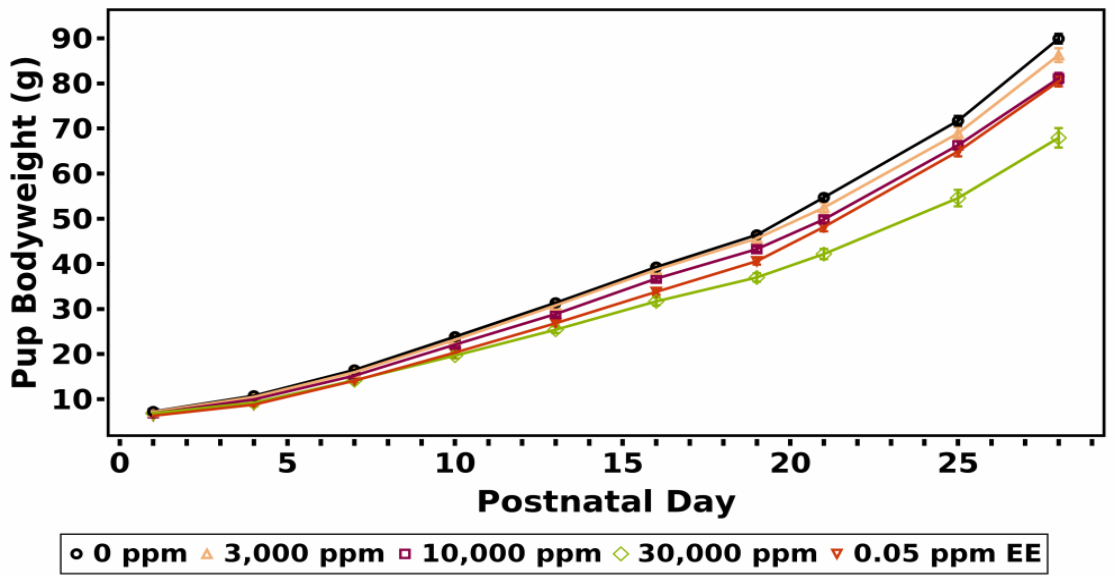


Figure 12. Lactation Growth Curves for F_1 Male Pups Following Perinatal Exposure to 2-Hydroxy-4-methoxybenzophenone

EE = ethinyl estradiol. Information for statistical significance in male pup weights is provided in Table 14.

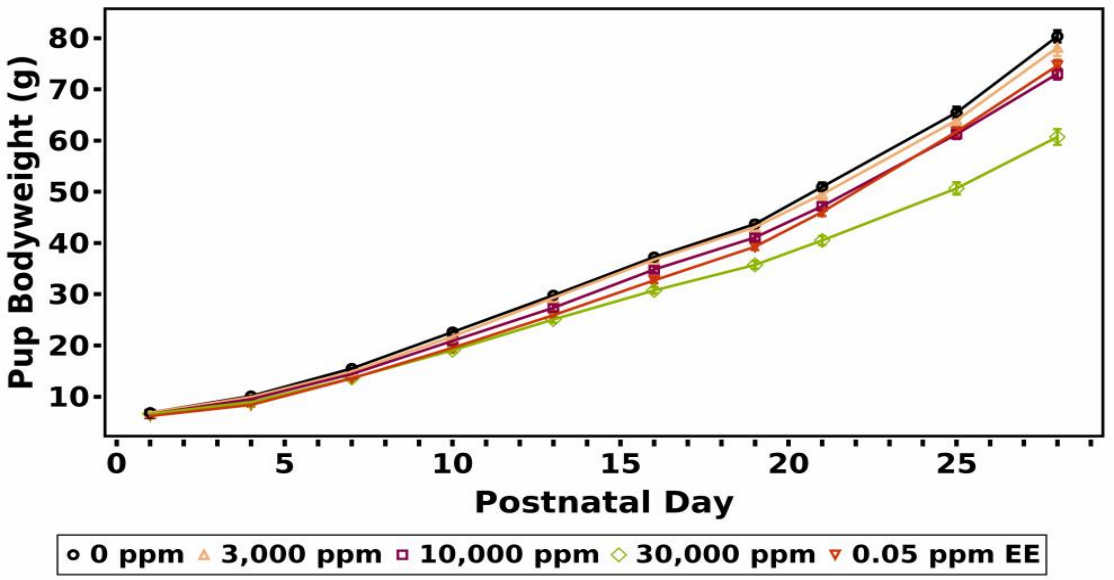


Figure 13. Lactation Growth Curves for F_1 Female Pups Following Perinatal Exposure to 2-Hydroxy-4-methoxybenzophenone

EE = ethinyl estradiol. Information for statistical significance in female pup weights is provided in Table 14.

F_0 Necropsy

F_0 dams were necropsied on PND 28 following pup weaning when the F_0 dams were 21 weeks of age. Gross findings of pale, discolored kidneys (unilateral/bilateral) were recorded for three females in the 30,000 ppm 2H4MBP group (Appendix E). Histopathological examination identified findings of renal tubule lumen dilatation, tubule epithelium regeneration, interstitial inflammation, papilla necrosis, nephropathy, and transitional epithelium hyperplasia. Similar findings were also observed in the F_1 and F_2 generations exposed to 2H4MBP (Appendix E).

F_1 Generation: Postweaning through Sexual Maturity

F_1 male and female rats were evaluated from postweaning through sexual maturity, as shown in Figure 14. Viability, clinical observations, mean body weights, feed consumption, and developmental endpoint results are presented below.

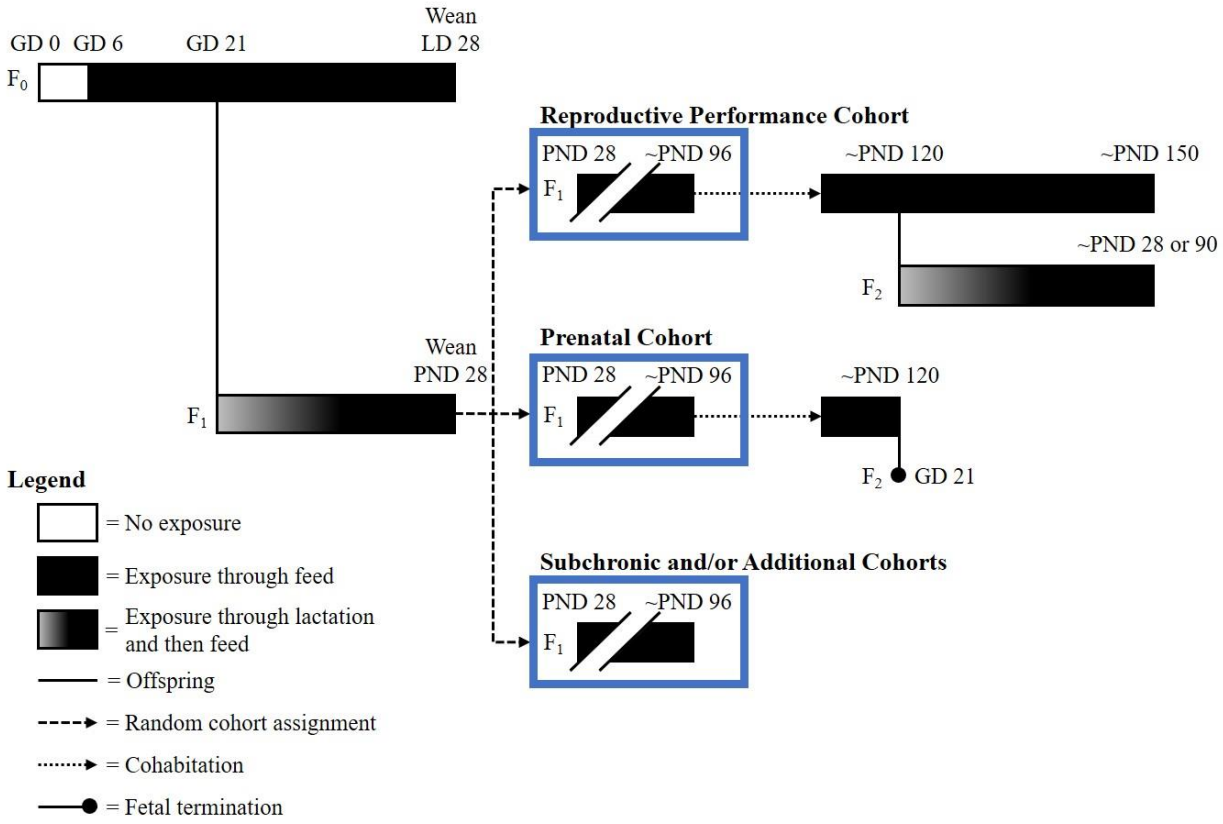


Figure 14. Design of the Modified One-Generation Study – F_1 Generation: Postweaning

GD = gestation day; LD = lactation day; PND = postnatal day.

F_1 Viability and Clinical Observations

Neither 2H4MBP nor EE exposure altered viability in the F_1 generation postweaning. Clinical observations were noted in all groups, including the control groups, on a sporadic basis (Appendix E). No clinical observations showed an increase in incidence or severity in association with exposure to 2H4MBP or EE.

F_1 Body Weights and Feed Consumption

Males (Postweaning)

Body weights between PND 28 and PND 91 were significantly decreased in males in the 10,000 and 30,000 ppm 2H4MBP and 0.05 ppm EE groups (Table 15; Figure 15). On PND 91, mean body weights of these groups were significantly decreased by 5%, 16%, and 18%, respectively, compared to those of the control group.

Overall, no adverse effects of 2H4MBP exposure on F_1 male feed consumption were found (Table 15). Sporadic small but significant decreases in absolute feed consumption (g/animal/day) were observed in the 30,000 ppm group between PND 28 and PND 84 (Appendix E) but did not affect overall feed consumption during the postweaning period. Relative feed consumption (g/kg/day) was significantly increased in the 10,000 and 30,000 ppm groups relative to the control group during the postweaning period, likely due to the lower body weights of the animals in these groups. A significant decrease in absolute feed consumption was observed in the 0.05 ppm EE group (14% below the control group) during the postweaning period, suggesting a continued effect of EE exposure on growth during the postweaning phase. 2H4MBP intake for F_1 males, based on feed consumption and dietary concentrations for PND 28 through PND 91, was approximately 267, 948, and 3,003 mg/kg/day at 3,000, 10,000, and 30,000 ppm 2H4MBP, respectively (Table 15). EE intake during the postweaning period was approximately 0.005 mg/kg/day.

Table 15. Summary of Postweaning Mean Body Weights, Body Weight Gains, and Feed and Test Article Consumption of All F_1 Male Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

Postnatal Day[a]	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[b]
Body Weight (g)[c,d]					
28	87.6 ± 1.1** 69 (22)	84.7 ± 1.5 65 (20)	79.5 ± 1.2** 67 (22)	65.7 ± 2.3** 65 (20)	78.2 ± 1.2** 45 (15)
91	393.0 ± 5.0** 64 (22)	387.6 ± 4.3 60 (20)	372.5 ± 5.2* 62 (21)	330.4 ± 6.8** 60 (20)	322.8 ± 4.5** 45 (15)
Body Weight Gain (g)[c,d]					
28–105	326.7 ± 4.5** 64 (22)	325.9 ± 3.9 60 (20)	319.2 ± 4.2 62 (21)	292.3 ± 5.2** 60 (20)	262.2 ± 4.3** 45 (15)
Postweaning Feed Consumption[e,f]					
28–91 (g/animal/day)	24.1 ± 0.4 (29)	23.9 ± 0.4 (28)	24.3 ± 0.3 (28)	23.0 ± 0.5 (26)	20.8 ± 0.3** (19)
28–91 (g/kg/day)	87.9 ± 1.5** (29)	89.0 ± 1.3 (28)	94.8 ± 1.0** (28)	100.1 ± 1.8** (26)	91.5 ± 1.1* (19)
Chemical Intake (mg/kg/day)[g,h]					
28–91	0.0 ± 0.0 (29)	267.1 ± 3.9 (28)	947.9 ± 10.4 (28)	3,002.5 ± 53.9 (26)	4.6 ± 0.1 (19)[i]

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
EE = ethinyl estradiol.
[a]Data are displayed as mean ± standard error (n).
[b]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[c]Statistical analysis performed using mixed effects models with litter as a random effect for both trend and pairwise tests, and a Dunnett-Hsu adjustment for multiple comparisons.
[d]n = the number of pups examined (number of litters).
[e]Statistical analysis performed using the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
[f]n = number of cages.
[g]Chemical intake calculated as: ([exposure concentration × feed consumption]/[average body weight of day range]).
[h]No statistical analysis performed on the chemical intake data.
[i]EE intake presented as µg/kg/day.

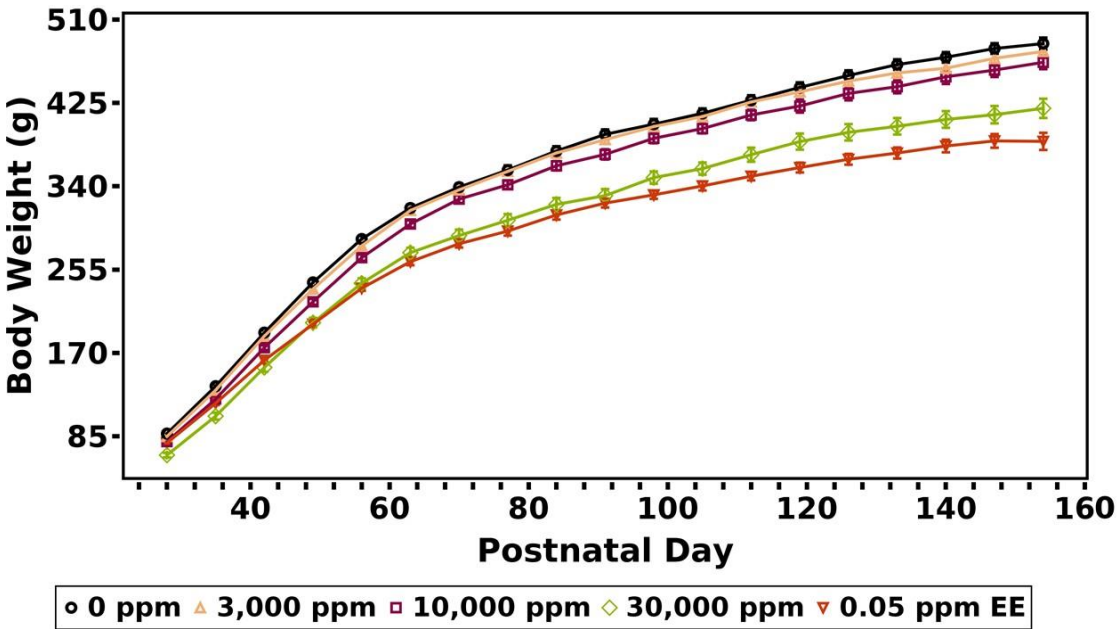


Figure 15. Postweaning Growth Curves for All F_1 Male Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

EE = ethinyl estradiol. Information for statistical significance in F_1 male rat weights is provided in Table 15.

Females (Postweaning)

PND 28 through PND 91 mean body weights were significantly decreased in females exposed to 30,000 ppm 2H4MBP or 0.05 ppm EE (Table 16; Figure 16). On PND 91, female mean body weights of the 30,000 ppm 2H4MBP and 0.05 ppm EE groups were significantly decreased by 14% and 17%, respectively, compared to those of the control group. The 10,000 ppm group displayed significantly decreased mean body weights (<10%) on PND 28 and PND 35 (Table 16; Appendix E), after which mean body weights were similar to those of the control group.

In general, 2H4MBP-exposed females displayed similar feed consumption values over the postweaning period (Table 16; Appendix E). There were small (approximately 15%), but significant, increases in absolute feed consumption (g/animal/day) recorded over two weekly intervals in the 30,000 ppm 2H4MBP group between PND 42 and PND 91. There was no overall reduction in absolute feed consumption during the postweaning period in the 30,000 ppm 2H4MBP group. Relative feed consumption (g/kg/day) was significantly increased in the 30,000 ppm group relative to the control group during the postweaning period, likely the result of lower body weights of the 2H4MBP-exposed animals. Absolute feed consumption by the EE group was similar to the control group; however, as these animals weighed less, their relative feed consumption was significantly increased compared to that of the control animals. 2H4MBP intake for F_1 females, based on feed consumption and dietary concentrations for PND 28 through PND 91, was approximately 287, 983, and 3,493 mg/kg/day at 3,000, 10,000, and 30,000 ppm 2H4MBP exposures, respectively. EE intake during the postweaning period was approximately 0.005 mg/kg/day.

Table 16. Summary of Postweaning Mean Body Weights, Body Weight Gains, and Feed and Test Article Consumption of All F_1 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

Postnatal Day[a]	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[b]
Body Weight (g)[c,d]					
28	78.0 ± 1.0** 78 (22)	75.6 ± 1.6 72 (20)	71.5 ± 1.3** 77 (22)	58.7 ± 1.6** 71 (20)	72.3 ± 1.1** 48 (15)
91	246.6 ± 3.5** 63 (22)	242.8 ± 3.2 60 (20)	236.9 ± 3.2 62 (22)	211.9 ± 2.7** 60 (20)	204.3 ± 3.0** 45 (15)
Body Weight Gain (g)[c,d]					
28–91	168.5 ± 3.0** 63 (22)	167.1 ± 2.5 60 (20)	165.4 ± 2.9 62 (22)	152.7 ± 2.7** 60 (20)	131.8 ± 3.1** 45 (15)
Postweaning Feed Consumption[e,f]					
28–91 (g/animal/day)	17.4 ± 0.3 (27)	17.2 ± 0.3 (27)	17.2 ± 0.3 (26)	18.3 ± 0.3 (27)	16.7 ± 0.5 (19)
28–91 (g/kg/day)	95.5 ± 1.5** (27)	95.5 ± 1.7 (27)	98.3 ± 1.5 (26)	116.4 ± 2.2** (27)	108.2 ± 4.1** (19)
Chemical Intake (mg/kg/day)[g,h]					
28–91	0.0 ± 0.0 (27)	286.5 ± 5.0 (27)	983.0 ± 15.3 (26)	3,493.2 ± 65.5 (27)	5.4 ± 0.2 (19)[i]

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
**Statistically significant at $p \leq 0.01$.
EE = ethinyl estradiol.
[a]Data are displayed as mean ± standard error (n).
[b]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[c]Statistical analysis performed using mixed effects models with litter as a random effect for both trend and pairwise tests, and a Dunnett-Hsu adjustment for multiple comparisons.
[d]n = the number of pups examined (number of litters).
[e]Statistical analysis performed using the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
[f]n = number of cages.
[g]Chemical intake calculated as: ([exposure concentration × feed consumption]/[average body weight of day range]).
[h]No statistical analysis performed on the chemical intake data.
[i]EE intake presented as µg/kg/day.

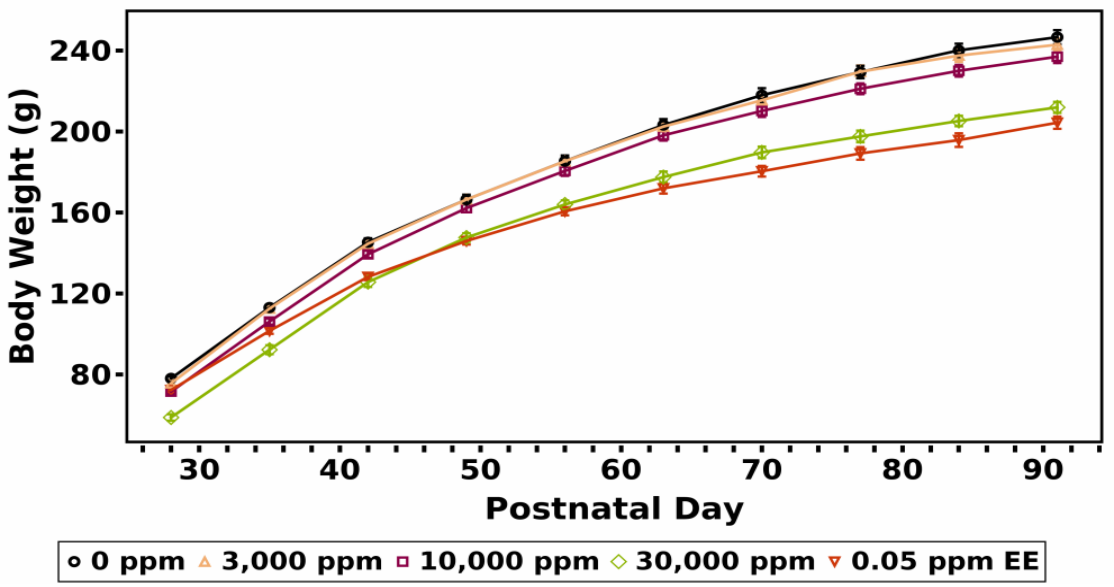


Figure 16. Postweaning Growth Curves for All F_1 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

EE = ethinyl estradiol. Information for statistical significance in F_1 female rat weights is provided in Table 16.

Developmental Endpoints

Anogenital Distance

F_1 and F_2 male and female offspring exposed to 2H4MBP or EE in feed did not display any alterations in mean PND 1 body-weight-adjusted AGD (Appendix E).

Areolae/Nipple Retention

F_1 and F_2 male offspring exposed to 2H4MBP or EE in feed did not display any signs of areolae/nipple retention (Appendix E).

Testicular Descent

F_1 males in the 30,000 ppm 2H4MBP group displayed a significant 1-day acceleration in the mean day of testicular descent (18.0 ± 0.2) compared to the control group (19.1 ± 0.2) (Appendix E). There was no difference in the mean day of testicular descent in the F_2 generation (Appendix E). The cumulative litter responses for the 30,000 ppm 2H4MBP group (F_1 generation) showed an earlier age at acquisition, whereas the F_2 generation did not display this response. The mean day of achieving testicular descent in control Sprague Dawley (Hsd:Sprague Dawley® SD®) rats in two other MOG studies conducted in the testing laboratory was PND 18.2 ± 0.2 and PND 18.0 ± 0.2. For NTP Reproductive Assessment by Continuous Breeding (RACB) studies, the mean day of testicular descent ranged from PND 15.3 ± 0.2 to PND 17.4 ± 0.5 over four studies.[94; 95]

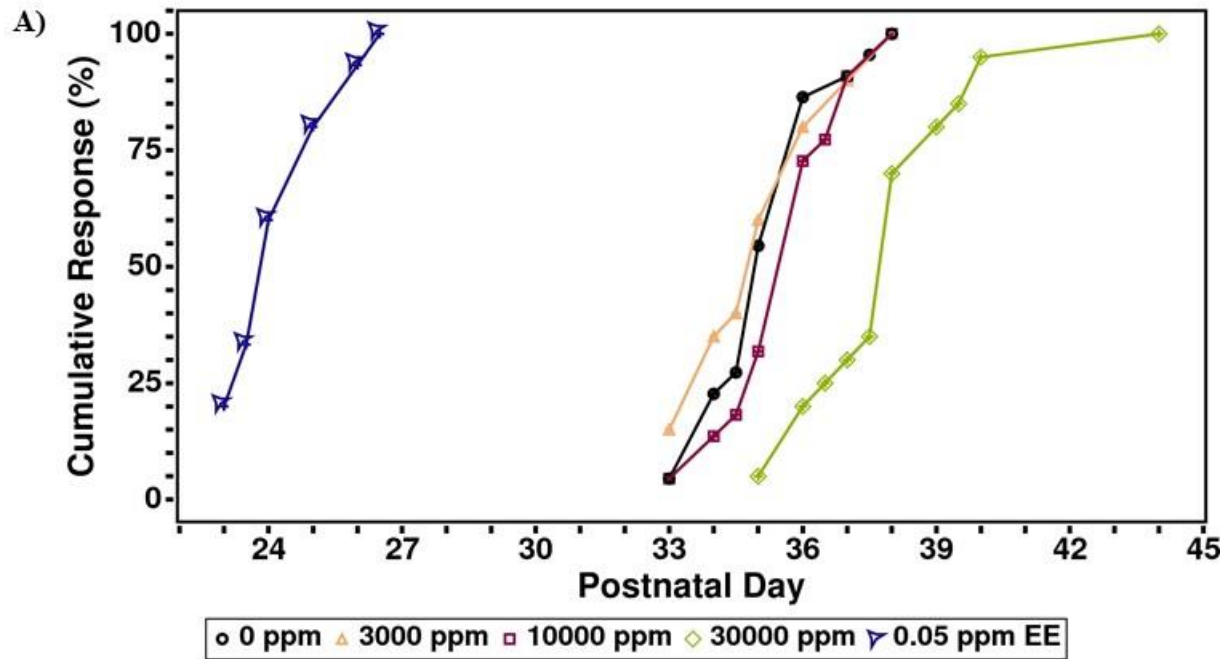
Vaginal Opening

Females exposed to 30,000 ppm 2H4MBP exhibited a significant delay in litter mean day of VO, relative to the control group (Table 17); however, when adjusted for body weight at weaning, this delay was somewhat mitigated, with the 30,000 ppm group displaying a 1-day delay. Figure 17 shows litter and adjusted litter cumulative response (%), or cumulative probability of attainment, plotted against PND for each exposure group. Exposure increases were associated with higher cumulative probabilities of attainment delays, particularly for the 30,000 ppm group, as seen in the exposure-related rightward shift of curves toward higher attainment days (Figure 17). These shifts were less pronounced after adjustment for body weight at weaning (Figure 17). The delay was associated with lower body weight, and these females also exhibited significantly decreased mean body weights during lactation and postweaning (Table 16; Figure 16). As expected, litter mean day of VO in the EE group was greatly accelerated (by approximately 11 days) compared to the control group (Table 17; Figure 17).

Table 17. Summary of Vaginal Opening of F_1 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

Parameter[a]	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[b]
No. Examined[c]	63 (22)	60 (20)	62 (22)	60 (20)	55 (15)
No. Not Attaining[d]	0	0	0	0	0
Day of VO					
Litter mean[e,f]	35.3 ± 0.2**	35.4 ± 0.4	35.9 ± 0.3	38.1 ± 0.4**	24.3 ± 0.3**
Adjusted litter mean[e,f,g]	35.9 ± 0.2*	35.8 ± 0.3	35.9 ± 0.3	37.0 ± 0.3	24.3 ± 0.3**
Mean Body Weight at Acquisition (g)[h]	115.7 ± 1.9**	114.3 ± 1.6	111.5 ± 1.6	109.0 ± 1.9*	59.0 ± 1.5**
Mean Body Weight at Weaning (g)[h]	80.6 ± 1.1**	78.1 ± 1.8	73.6 ± 1.3**	60.7 ± 1.6**	74.5 ± 1.2**

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
EE = ethinyl estradiol; VO = vaginal opening.
[a]Data are displayed as mean ± standard error unless otherwise noted; values are based on litter means, not individual pup values.
[b]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[c]No. Examined = the number of pups examined (number of litters).
[d]No. Not Attaining = number of pups that survived to the end of the observation period without attaining VO.
[e]Summary statistics and mixed model results are presented for animals that attained during the observation period.
[f]Statistical analysis performed using mixed effects models with litter as a random effect for both trend and pairwise tests, and a Dunnett-Hsu adjustment for multiple pairwise comparisons.
[g]Adjusted based on body weight at weaning.
[h]Analysis of body weight at acquisition and body weight at weaning for both linear trend and pairwise comparisons performed using mixed effects models with litter as a random effect and a Dunnett-Hsu adjustment for multiple pairwise comparisons.



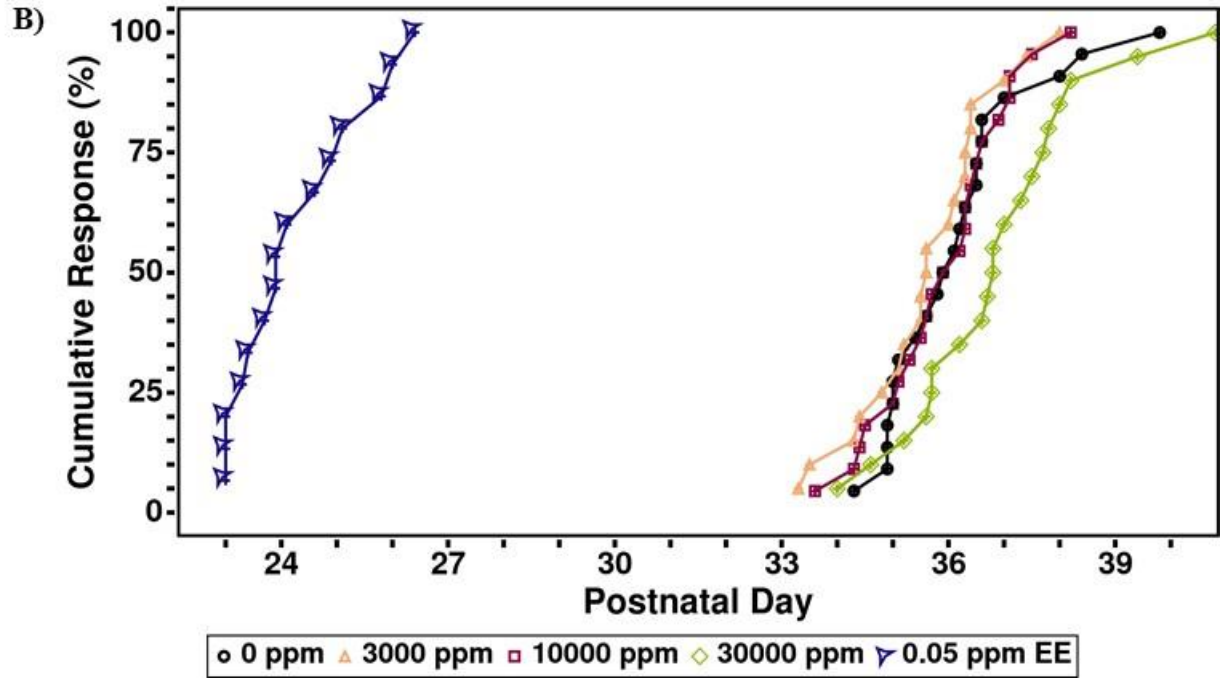


Figure 17. Time to Vaginal Opening of F_1 Female Offspring Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

EE = ethinyl estradiol. (A) Litter response and (B) litter response adjusted for body weight at weaning.

Balanopreputial Separation

Male rats in the 10,000 and 30,000 ppm 2H4MBP and 0.05 ppm EE groups displayed a significant delay in the mean day of attaining BPS (Table 18). Figure 18 shows litter and adjusted litter cumulative response (%), or cumulative probability of attainment, plotted against PND for each exposure group. An exposure-dependent rightward shift is seen for the 10,000 and 30,000 ppm 2H4MBP and 0.05 ppm EE groups, indicating higher cumulative probabilities of attainment at later PNDs (Figure 18). When litter mean day of attainment was adjusted for body weight on day of weaning, these delays were no longer significantly different from control males (Table 18; Figure 18). The observed delay in BPS in 2H4MBP- or EE-exposed animals is likely the consequence of growth retardation as evidenced by lower mean body weights and body weight gains (Table 15; Figure 15). Three males in the 30,000 ppm 2H4MBP group had not achieved BPS as of PND 59, when checks for this marker stopped. These males were from the same litter (dam 202). Two were assigned to the reproductive performance cohort (animals 1901 and 1907) and the other (animal 1903) was assigned to the prenatal cohort. None of them demonstrated evidence of mating or resultant evidence of pregnancy. At scheduled necropsy, two of the males had achieved BPS and the other (animal 1903) had a hypospadia.

Table 18. Summary of Balanopreputial Separation of F_1 Male Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

Parameter[a]	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[b]
No. Examined[c]	64 (22)	59 (20)	62 (21)	60 (20)	45 (15)
No. Not Attaining[d]	0 (0)	0 (0)	0 (0)	3 (1)	0 (0)
Day of BPS					
Litter mean[e,f]	43.7 ± 0.3**	44.0 ± 0.4	44.9 ± 0.3*	47.1 ± 0.4**	45.8 ± 0.3**
Adjusted litter mean[e,f,g]	44.7 ± 0.3	44.7 ± 0.3	44.8 ± 0.3	45.4 ± 0.3	44.8 ± 0.3
Proportional hazards model, p value[h]	0.112	0.956	0.956	0.852	0.138
Mean Body Weight at Acquisition (g)[i]	204.4 ± 2.9**	203.3 ± 2.9	196.4 ± 2.2	192.1 ± 2.8**	184.7 ± 2.2**
Mean Body Weight at Weaning (g)[i]	90.1 ± 1.1**	87.4 ± 1.6	81.4 ± 1.2**	68.6 ± 1.9**	80.3 ± 1.2**

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
EE = ethinyl estradiol; BPS = balanopreputial separation.
[a]Data are displayed as mean ± standard error unless otherwise noted; values are based on litter means, not individual pup values.
[b]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[c]No. Examined = number of pups examined (number of litters).
[d] No. Not Attaining = number of pups (number of litters) that survived to the end of the observation period without attaining BPS.
[e]Summary statistics and mixed model results are presented for animals that attained during the observation period.
[f]Statistical analysis performed using mixed effects models with litter as a random effect for both trend and pairwise tests, and a Dunnett-Hsu adjustment for multiple pairwise comparisons.
[g]Adjusted based on body weight at weaning.
[h]Statistical analysis performed using the proportional hazards model with exposure concentration and weaning weight as covariates, a random effect for litter for both trend and pairwise tests, and a Hommel adjustment for multiple comparisons. Time-to-event data for animals that did not achieve the event are included and treated as providing information up to the last day examined, with time counted as "greater than last day checked."
[i]Analysis of body weight at acquisition and body weight at weaning for both linear trend and pairwise comparisons performed using mixed effects models with litter as a random effect and a Dunnett-Hsu adjustment for multiple pairwise comparisons.

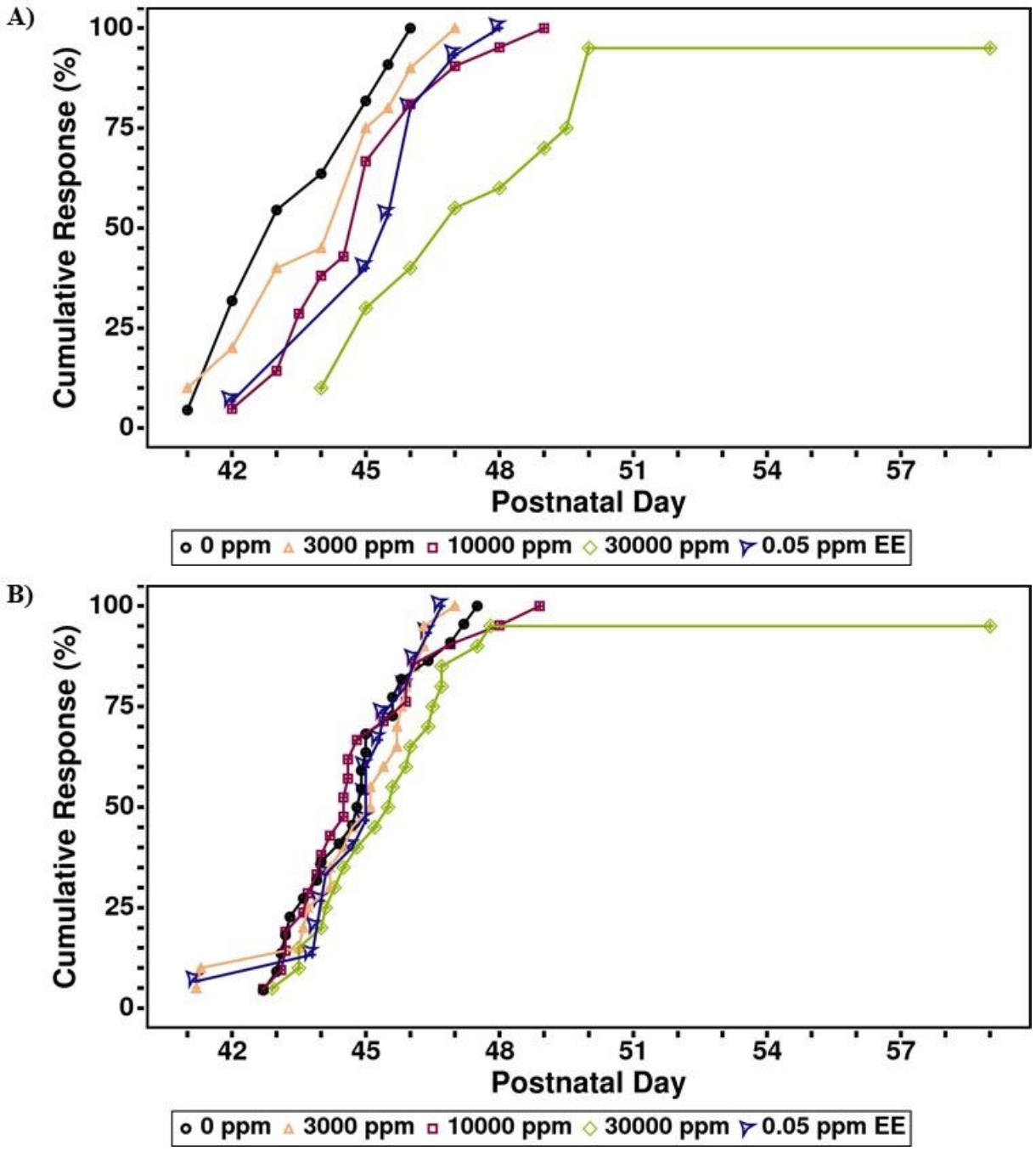


Figure 18. Time to Balanopreputial Separation of F_1 Male Offspring Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

EE = ethinyl estradiol. (A) Litter response and (B) litter response adjusted for body weight at weaning.

F_1 Cohort Data

Prenatal and Reproductive Performance Cohorts: Mating and Fertility

F_1 male and female rats from the prenatal and reproductive performance cohorts were mated and evaluated for reproductive endpoints, as shown in Figure 19. Viability, clinical observations, vaginal cytology, fertility, andrology, mean body weights, and feed consumption results are presented below.

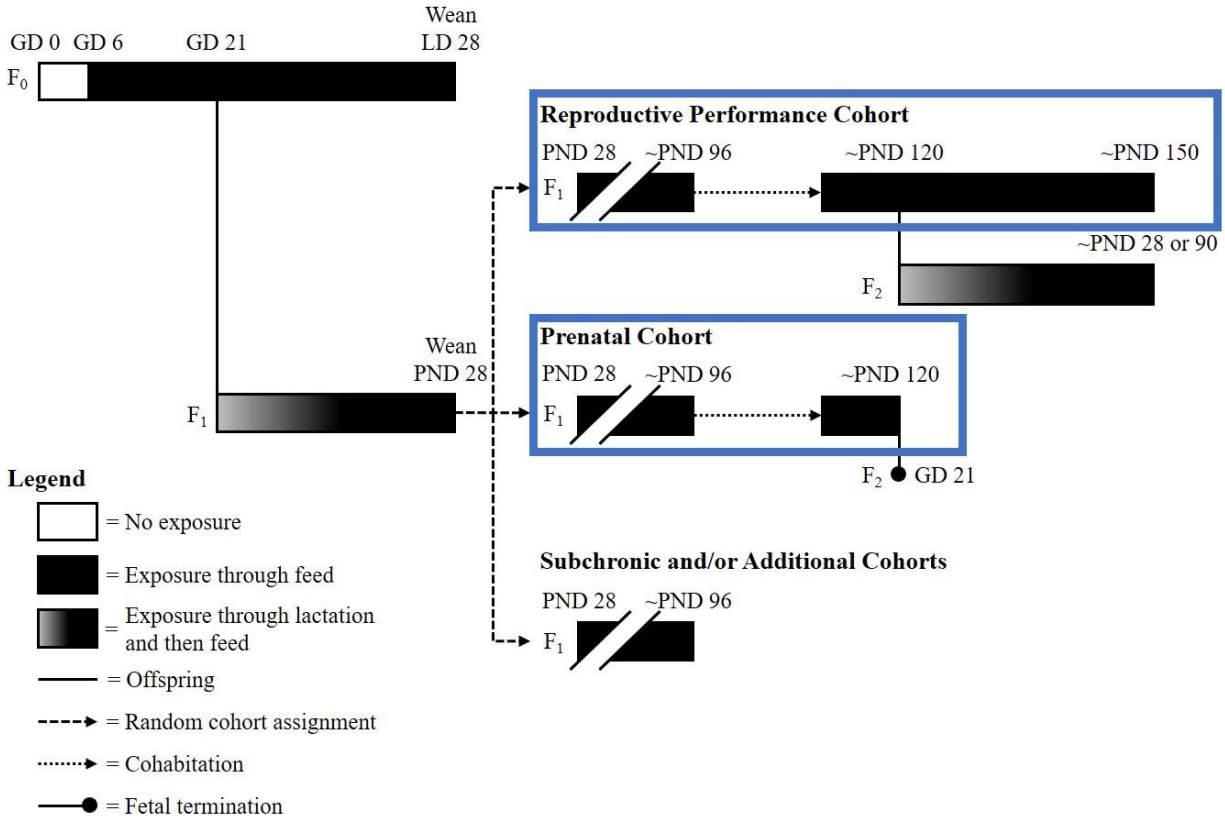


Figure 19. Design of the Modified One-Generation Study – Prenatal and Reproductive Performance Cohorts

GD = gestation day; LD = lactation day; PND = postnatal day.

Viability and Clinical Observations

There were no exposure-related deaths or clinical observations in F_1 male and female rats following exposure to 2H4MBP or EE in feed (Appendix E).

Selection and Mating

A male and a female, or two males and two females (1:1), from each litter were allocated to the prenatal and reproductive performance cohorts, respectively, avoiding sibling mating (Figure 19). Vaginal lavage samples were collected for approximately 2 weeks prior to cohabitation and continued until evidence of mating or until the cohabitation period was completed. Estrous cyclicity data are presented in Appendix E.

Vaginal Cytology

Rats in the 10,000 and 30,000 ppm 2H4MBP groups of both cohorts displayed a higher probability of extended estrus (Appendix E) and spent approximately 5% more time in estrus than did the control group. Analysis of estrous cyclicity using the continuous-time Markov model resulted in an increase in the stage length of estrus in the 10,000 and 3,000 ppm groups (approximately 5 hours), but only attained significance relative to the control group in the 10,000 ppm group. A significant decrease in the length of proestrus (approximately 2 hours) was observed in the 10,000 ppm group. These minimal estimated changes in stage length likely represent normal biological variability and are not considered biologically adverse. There were no EE exposure-related changes in estrous stage lengths.

Fertility

The precoital interval and number of females that mated (i.e., those that were sperm-positive, littered, or had implantation sites) were similar among the control, 2H4MBP, and EE groups in both cohorts, indicating that neither 2H4MBP nor EE exposure negatively affected mating behavior (Table 19). The number of pregnant females was also similar among the groups, indicating that F_1 male and female fertility were not affected by 2H4MBP or EE exposure at the concentrations examined. Respective responses observed were consistent between the cohorts.

F_1 Reproductive Performance Cohort Andrology

There were no 2H4MBP- or EE-related effects on motile sperm, progressively motile sperm, or testis spermatid head concentration (Appendix E). Males in the 30,000 ppm 2H4MBP group displayed lower cauda epididymal sperm counts (approximately 14%) and epididymis weight (approximately 6%) relative to control animals. Testis weight was lower in the 10,000 and 30,000 ppm 2H4MBP and 0.05 ppm EE groups (approximately 6%, 6%, and 9%, respectively), relative to control animals. These findings were not associated with histopathological changes (Appendix E) or significant changes in reproductive performance (Appendix E).

Gestation Body Weights

As previously mentioned, F_1 female rats exposed to 10,000 or 30,000 ppm 2H4MBP or 0.05 ppm EE displayed significantly decreased preweaning and postweaning mean body weights compared to the control group. Consequently, F_1 female mean body weights of the 30,000 ppm 2H4MBP and 0.05 ppm EE groups in both the prenatal and reproductive performance cohorts at the time of cohabitation were lower relative to control females. Gestation body weight curves of the exposed groups in both cohorts generally paralleled the control group (Figure 20, Figure 21). Dams in both cohorts exposed to 10,000 or 30,000 ppm 2H4MBP or 0.05 ppm EE, however, displayed significantly decreased GD 0–21 mean body weight gains (approximately 13%–14%, 25%–28%, and 22%–24%, respectively) relative to the respective control group (Table 20). This difference in mean body weight gain during pregnancy might be the result of a slight reduction in litter size of one to two fewer fetuses/pups observed in these groups (Appendix E). Respective responses observed were consistent between the two cohorts.

Gestation Feed Consumption

2H4MBP groups displayed similar absolute feed consumption (g/animal/day) during gestation as the respective control group. Relative feed consumption (g/kg/day) during gestation in the 3,000 and 10,000 ppm 2H4MBP groups was similar to the respective control group (Table 21; Appendix E). Pregnant females in the 30,000 ppm group of the prenatal cohort displayed a

significant increase in relative feed consumption between GD 0 and GD 21 (approximately 21%), but this is likely the result of the substantially lower body weights of this group. In the EE group of the reproductive performance cohort, absolute feed consumption between GD 0 and GD 21 was significantly decreased by approximately 19%, and relative feed consumption was similar to that of the control group. The opposite was true for the EE group in the prenatal cohort, in which relative feed consumption was significantly increased by approximately 25% relative to the control group. 2H4MBP intake of both cohorts during gestation, based on feed consumption and dietary concentrations, was approximately 240, 825, and 2,760 mg/kg/day at exposure concentrations of 3,000, 10,000, and 30,000 ppm 2H4MBP, respectively. EE intake was approximately 0.004 mg/kg/day. The respective dose consumed was similar between the two cohorts.

Table 19. Summary of Mating and Fertility Performance of F_1 Male and Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

Parameter	0 ppm		3,000 ppm		10,000 ppm		30,000 ppm		EE 0.05 ppm[a]	
	RPC	PC	RPC	PC	RPC	PC	RPC	PC	RPC	PC
No. Mating Pairs	41	22	40	20	40	22	40	20	30	15
No. Mated	40	19	37	19	35	21	35	19	29	15
No. Females Pregnant	35	18	37	18	33	20	33	19	28	15
Percent of Mated Females/Paired[b]	97.6	86.4	92.5	95.0	87.5	95.5	87.5	95.0	96.7	100.0
Precoital Interval[c,d]	4.7 ± 0.6 (22)	4.3 ± 0.7 (19)	4.8 ± 0.5 (20)	5.3 ± 1.0 (18)	5.1 ± 0.7 (19)	4.1 ± 0.8 (19)	4.2 ± 0.8 (20)	3.9 ± 0.6 (18)	4.0 ± 0.6 (15)	3.4 ± 0.5 (15)

EE = ethinyl estradiol; RPC = reproductive performance cohort; PC = prenatal cohort.
[a]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[b]Statistical analysis of the RPC performed using the Rao-Scott Cochran-Armitage test for both trend and pairwise comparisons to adjust for litter effects. Statistical analysis of the PC performed by the Cochran-Armitage (trend) and Fisher's exact (pairwise) tests.
[c]Statistical analysis of the RPC performed using a bootstrapped Jonckheere test for trend, and a Datta-Satten modified Wilcoxon test with Hommel adjustment for pairwise comparisons. Statistical analysis for the PC cohort performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
[d]Precoital interval in days is calculated for sperm-positive females; data are displayed as mean ± standard error (n).

Table 20. Summary of Gestation Mean Body Weight Gains for F_1 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed[a,b]

GD Interval	0 ppm		3,000 ppm		10,000 ppm		30,000 ppm		EE 0.05 ppm[c]	
	RPC	PC	RPC	PC	RPC	PC	RPC	PC	RPC	PC
0	252.3 ± 5.3** (33)	256.4 ± 4.2** (18)	255.0 ± 3.4 (36)	248.3 ± 6.1 (17)	248.2 ± 3.8 (32)	238.1 ± 3.7** (18)	219.9 ± 3.5** (31)	220.5 ± 3.4** (18)	209.3 ± 4.3** (28)	207.2 ± 2.7** (15)
6–21	141.6 ± 3.7** (33)	138.9 ± 4.2** (18)	136.2 ± 3.3 (36)	136.4 ± 3.0 (16)	123.3 ± 3.7** (32)	117.9 ± 6.3* (18)	101.1 ± 4.8** (31)	103.6 ± 7.4** (18)	112.9 ± 3.3** (27)	108.4 ± 4.4** (15)
0–21	173.0 ± 4.3** (33)	168.2 ± 4.5** (18)	166.8 ± 4.1 (36)	165.5 ± 3.9 (16)	149.8 ± 3.6** (32)	145.0 ± 6.6** (18)	124.6 ± 5.9** (31)	126.3 ± 8.2** (18)	134.1 ± 3.4** (27)	128.0 ± 5.3** (15)
0–3	17.6 ± 0.9** (33)	16.7 ± 1.2 (18)	16.9 ± 0.7 (36)	15.7 ± 1.5 (17)	15.6 ± 0.8 (32)	16.3 ± 1.4 (18)	13.0 ± 1.4** (31)	14.7 ± 1.1 (18)	11.7 ± 0.9** (28)	11.4 ± 1.3** (15)
3–6	13.8 ± 0.8** (33)	12.7 ± 0.9** (18)	13.7 ± 0.7 (36)	12.9 ± 0.8 (17)	10.9 ± 0.7** (32)	10.8 ± 0.6 (18)	10.4 ± 0.7** (31)	8.0 ± 1.2** (18)	9.5 ± 0.4 ** (28)	8.3 ± 0.5** (15)
6–9	13.0 ± 0.6** (33)	13.2 ± 0.9** (18)	11.9 ± 0.7 (36)	11.3 ± 1.1 (17)	11.7 ± 0.7 (32)	10.1 ± 0.7* (18)	9.8 ± 0.7** (31)	10.1 ± 0.8* (18)	9.6 ± 0.4 ** (28)	8.6 ± 0.6** (15)
9–12	14.2 ± 0.7** (33)	13.9 ± 0.8** (18)	12.9 ± 0.6 (36)	15.2 ± 1.0 (17)	10.9 ± 0.5** (32)	12.2 ± 0.9 (18)	8.4 ± 1.1** (31)	10.6 ± 1.1* (18)	10.7 ± 0.8** (28)	12.5 ± 0.7 (15)
12–15	20.4 ± 0.8** (33)	21.6 ± 0.9** (18)	21.1 ± 0.7 (36)	23.5 ± 1.8 (17)	18.5 ± 1.0 (32)	18.0 ± 1.1 (18)	17.1 ± 0.7* (31)	18.0 ± 1.5 (18)	15.6 ± 0.7** (28)	16.1 ± 0.9** (15)
15–18	46.3 ± 1.2** (33)	47.6 ± 2.4** (18)	44.3 ± 1.4 (36)	43.1 ± 1.5 (17)	40.3 ± 1.7* (32)	36.4 ± 3.0** (18)	29.9 ± 2.7** (31)	31.8 ± 2.6** (18)	37.5 ± 1.0** (28)	35.2 ± 2.0** (15)
18–21	47.8 ± 2.0** (33)	42.6 ± 2.3** (18)	45.9 ± 1.6 (20)	45.7 ± 2.1 (16)	41.9 ± 1.7* (32)	41.2 ± 2.3 (18)	36.0 ± 1.9** (31)	33.1 ± 3.3* (18)	39.1 ± 2.2** (27)	36.0 ± 1.6* (15)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
GD = gestation day; EE = ethinyl estradiol; RPC = reproductive performance cohort; PC = prenatal cohort.
[a]Data are displayed as mean ± standard error (n), where n = number of litters. Body weight data are reported in grams.
[b]Statistical analysis for the RPC performed using mixed effects models with litter as a random effect for both trend and pairwise tests, and a Dunnett-Hsu adjustment for multiple pairwise comparisons. Statistical analysis for the PC performed using the Jonckheere (trend) and Williams or Dunnett (pairwise) tests.
[c]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.

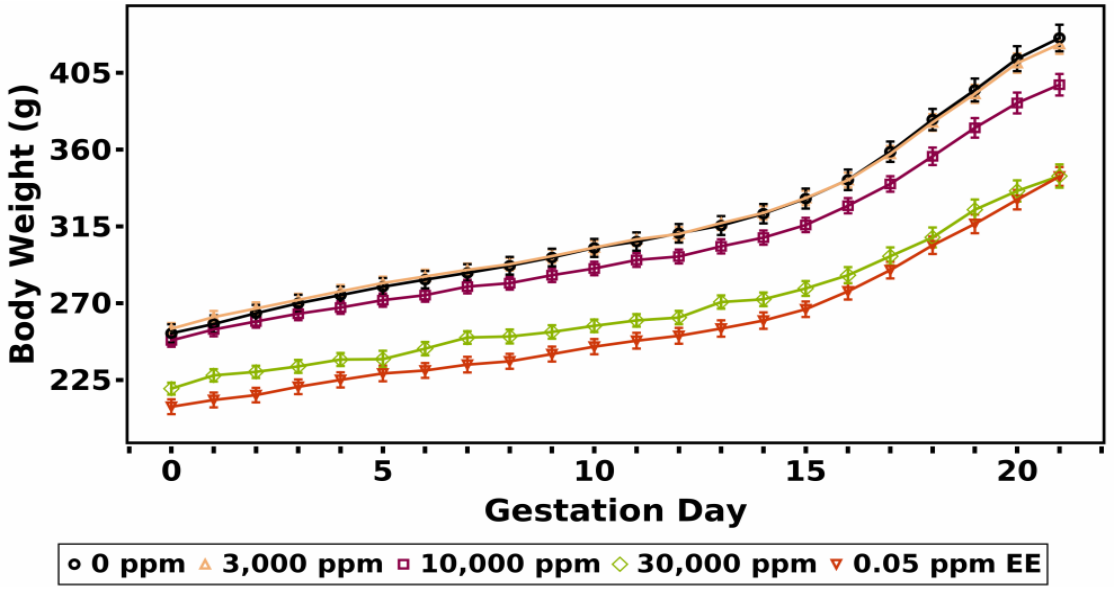


Figure 20. Gestation Growth Curves for F_1 Female Rats in the Reproductive Performance Cohort Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

EE = ethinyl estradiol. Information for statistical significance in F_1 female rat weights is provided in Table 20.

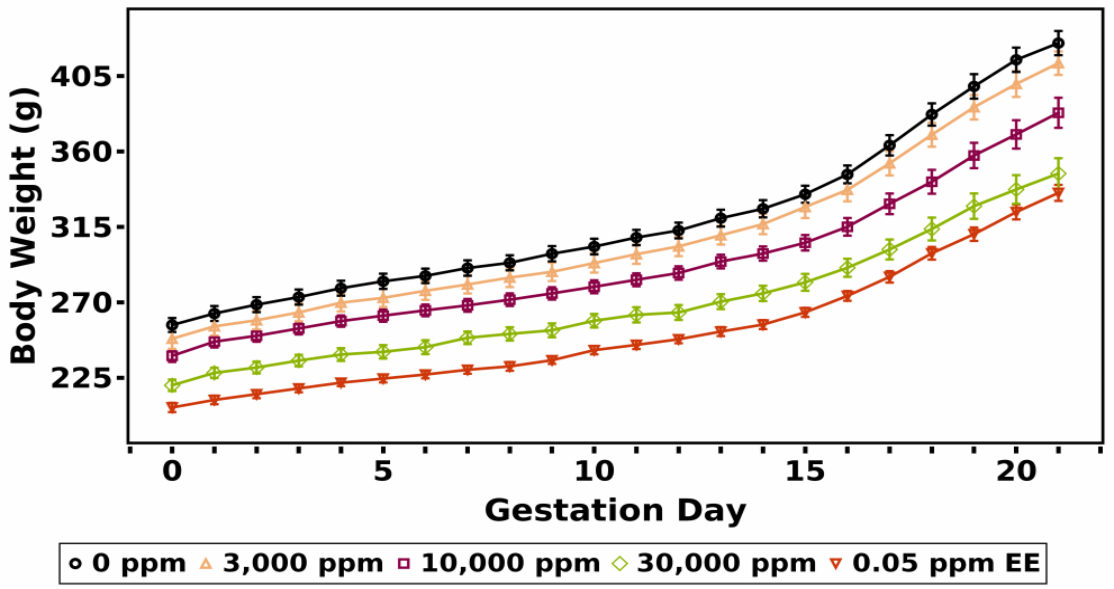


Figure 21. Gestation Growth Curves for F_1 Female Rats in the Prenatal Cohort Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

EE = ethinyl estradiol. Information for statistical significance in F_1 female rat weights is provided in Table 20.

Table 21. Summary of Gestation Feed and Test Article Consumption for F_1 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed[a]

GD Interval	0 ppm		3,000 ppm		10,000 ppm		30,000 ppm		EE 0.05 ppm[b]	
	RPC	PC	RPC	PC	RPC	PC	RPC	PC	RPC	PC
Feed Consumption (g/animal/day)[c]										
0–21	27.8 ± 0.8 (22)	23.5 ± 0.4 (17)	26.6 ± 0.7 (20)	22.7 ± 0.6 (13)	26.1 ± 0.8 (19)	23.2 ± 0.7 (14)	25.4 ± 0.6 (19)	24.1 ± 0.9 (14)	22.5 ± 0.9** (15)	23.1 ± 1.4 (14)
0–3	26.4 ± 1.7 (22)	19.7 ± 0.7** (18)	25.8 ± 1.3 (20)	20.5 ± 0.9 (17)	24.9 ± 1.3 (19)	19.0 ± 0.6 (13)	26.6 ± 1.1 (18)	26.3 ± 2.2** (16)	21.7 ± 1.8* (15)	24.3 ± 2.9 (13)
3–6	25.1 ± 1.4** (22)	21.2 ± 0.4* (18)	22.7 ± 0.6 (20)	20.4 ± 0.5 (17)	21.2 ± 0.5 (19)	21.6 ± 1.5 (18)	19.3 ± 0.5** (18)	19.3 ± 1.2 (15)	17.3 ± 0.7** (15)	16.2 ± 0.5** (14)
6–9	30.7 ± 1.3 (22)	23.8 ± 0.9 (18)	29.2 ± 1.6 (20)	21.5 ± 0.8 (15)	27.5 ± 1.5 (18)	23.0 ± 1.4 (16)	31.4 ± 2.0 (17)	29.1 ± 1.6 (17)	25.8 ± 2.1* (15)	26.0 ± 3.2 (14)
9–12	22.4 ± 0.4** (22)	21.6 ± 0.4* (18)	22.6 ± 0.5 (20)	21.2 ± 0.6 (17)	20.7 ± 0.5 (19)	21.1 ± 1.1 (18)	19.3 ± 0.6** (19)	19.6 ± 0.9 (17)	17.6 ± 0.4** (15)	17.4 ± 0.3** (15)
12–15	31.5 ± 1.0 (22)	25.2 ± 0.9** (17)	30.2 ± 1.4 (19)	23.8 ± 0.6 (15)	31.9 ± 1.9 (18)	29.9 ± 2.6 (17)	35.8 ± 1.8 (18)	34.3 ± 2.2** (15)	27.0 ± 2.2* (15)	28.5 ± 2.6 (15)
15–18	26.8 ± 0.5** (22)	26.6 ± 0.5** (18)	25.5 ± 0.4 (20)	25.1 ± 0.5 (17)	25.0 ± 0.4** (18)	24.1 ± 1.1** (17)	21.4 ± 0.8** (20)	23.3 ± 1.3** (15)	21.9 ± 0.5** (15)	28.4 ± 2.9* (15)
18–21	31.5 ± 1.0 (22)	26.6 ± 1.4 (18)	30.5 ± 1.3 (20)	27.2 ± 1.2 (16)	31.4 ± 1.4 (19)	30.9 ± 2.3 (17)	30.3 ± 1.5 (20)	23.6 ± 1.3 (15)	26.2 ± 1.3** (15)	24.2 ± 1.4 (15)
Feed Consumption (g/kg/day)[c]										
0–21	88.5 ± 2.8 (22)	73.7 ± 1.3** (17)	84.3 ± 2.1 (20)	74.7 ± 1.7 (13)	86.0 ± 2.3 (19)	79.2 ± 2.5 (14)	94.8 ± 2.6 (19)	89.5 ± 3.6** (14)	88.7 ± 4.6 (15)	91.9 ± 6.1** (14)
0–3	101.0 ± 6.7 (22)	74.0 ± 2.0** (18)	98.2 ± 4.9 (20)	80.3 ± 3.4 (17)	96.9 ± 4.5 (19)	77.1 ± 2.5 (13)	116.9 ± 5.3* (18)	115.6 ± 9.9** (16)	102.2 ± 9.9 (15)	115.0 ± 14.7** (13)
3–6	91.4 ± 5.3 (22)	75.8 ± 1.5 (18)	81.2 ± 1.7 (20)	75.4 ± 1.6 (17)	78.7 ± 2.0 (19)	83.7 ± 6.4 (18)	81.2 ± 2.0 (18)	80.6 ± 4.2 (15)	76.9 ± 3.7* (15)	72.7 ± 1.9 (14)
6–9	107.4 ± 5.3 (22)	81.5 ± 3.0** (18)	101.3 ± 5.7 (20)	76.8 ± 2.8 (15)	97.3 ± 4.5 (18)	84.7 ± 4.5 (16)	127.0 ± 9.0 (17)	116.8 ± 7.0** (17)	112.2 ± 11.6 (15)	111.7 ± 13.5 (14)
9–12	73.7 ± 1.2 (22)	70.9 ± 1.4 (18)	74.2 ± 1.3 (20)	71.4 ± 1.3 (17)	70.9 ± 1.7 (19)	74.9 ± 3.9 (18)	74.2 ± 1.8 (19)	75.0 ± 3.4 (17)	71.7 ± 1.0 (15)	71.8 ± 1.1 (15)
12–15	100.2 ± 5.2** (22)	78.3 ± 3.1** (17)	95.2 ± 4.7 (19)	76.8 ± 2.5 (15)	104.4 ± 5.8 (18)	100.2 ± 8.6* (17)	133.8 ± 7.8** (18)	126.9 ± 9.7** (15)	106.4 ± 11.0 (15)	111.8 ± 10.5** (15)

GD Interval	0 ppm		3,000 ppm		10,000 ppm		30,000 ppm		EE 0.05 ppm[b]	
	RPC	PC	RPC	PC	RPC	PC	RPC	PC	RPC	PC
15–18	76.0 ± 1.1 (22)	74.6 ± 1.1 (18)	72.7 ± 0.8 (20)	72.9 ± 1.9 (17)	74.8 ± 1.4 (18)	73.9 ± 2.8 (17)	72.9 ± 2.1 (20)	78.4 ± 4.6 (15)	77.2 ± 1.4 (15)	102.1 ± 11.1** (15)
18–21	78.9 ± 2.8* (22)	65.7 ± 3.2 (18)	76.8 ± 3.6 (20)	69.6 ± 2.5 (16)	83.3 ± 3.9 (19)	86.9 ± 8.3 (17)	93.8 ± 5.8* (20)	72.9 ± 5.2 (15)	82.0 ± 4.8 (15)	76.4 ± 4.4 (15)
Chemical Intake (mg/kg/day)[d,e]										
0–21	0.0 ± 0.0 (22)	0.0 ± 0.0 (17)	252.8 ± 6.3 (20)	224.2 ± 5.0 (13)	859.7 ± 23.2 (19)	791.8 ± 25.2 (14)	2,844.2 ± 79.2 (19)	2,684.4 ± 107.5 (14)	4.4 ± 0.2[e] (15)	4.6 ± 0.3[f] (14)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
GD = gestation day; EE = ethinyl estradiol; RPC = reproductive performance cohort; PC = prenatal cohort.
[a]Data are displayed as mean ± standard error (n), where n = number of litters. Consumption is not reported for the nonpregnant animals during gestation and lactation.
[b]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[c]Statistical analysis of the RPC cohort performed using a bootstrapped Jonckheere test for trend, and a Datta-Satten modified Wilcoxon test with Hommel adjustment for pairwise comparisons. Statistical analysis of the PC performed using the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
[d]Chemical intake calculated as: ([exposure concentration × feed consumption]/[average body weight of day range]).
[e]No statistical analysis was performed on the chemical intake data.
[f]EE intake presented as µg/kg/day.

Prenatal Cohort Findings

F_1 rats and F_2 fetuses from the prenatal cohort were evaluated for maternal reproductive performance and fetal findings, respectively, as shown in Figure 22.

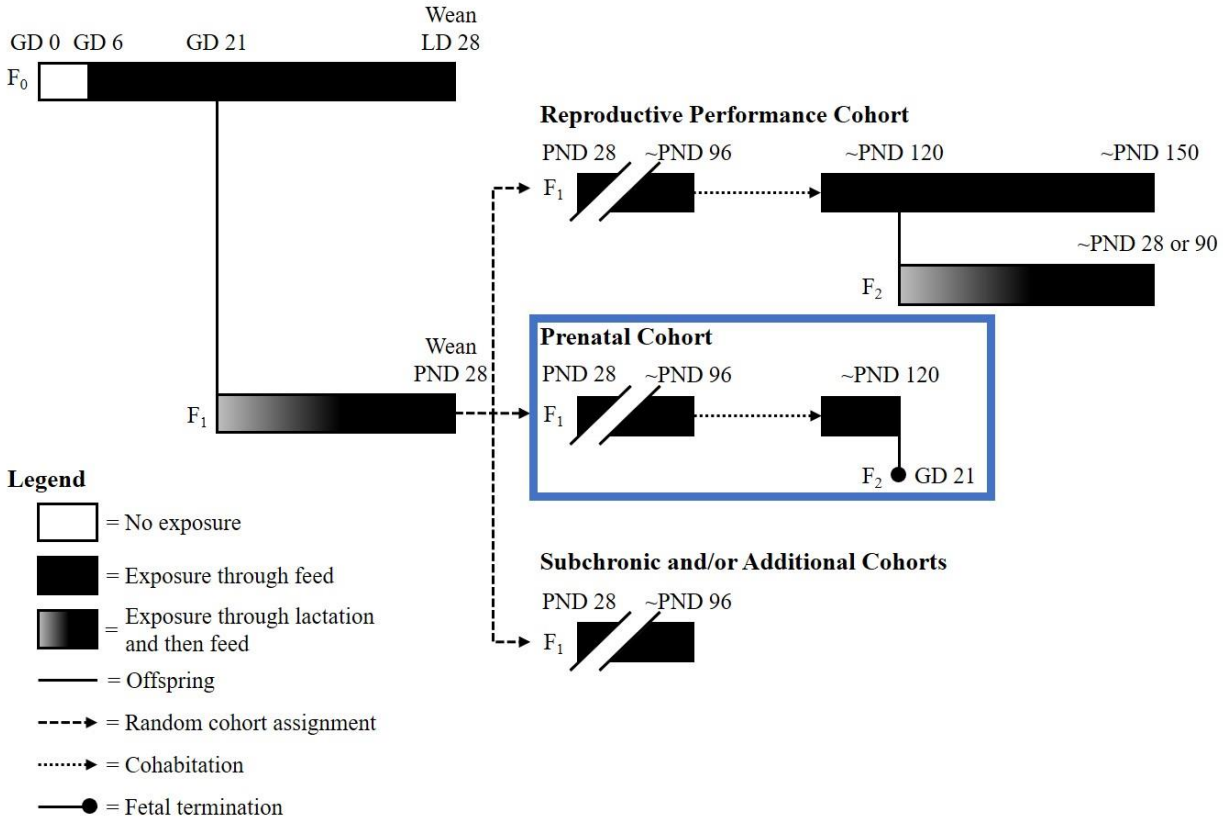


Figure 22. Design of the Modified One-Generation Study – Prenatal Cohort

GD = gestation day; LD = lactation day; PND = postnatal day.

Maternal Reproductive Performance and Uterine Data

In the prenatal cohort, females were between 109 and 132 days of age at the time of laparotomy. Pregnant females exposed to 10,000 or 30,000 ppm 2H4MBP displayed lower gravid uterine weights (15% and 17%, respectively), fewer implants, and fewer live fetuses (approximately 2 fewer/litter) than control animals; significant decreases were observed for gravid uterine weight and number of implantations at 30,000 ppm (Table 22). In the 30,000 ppm 2H4MBP group, these findings correlated with significant decreases in the mean number of corpora lutea (approximately 4 fewer/litter) relative to the control group and are consistent with the reduction in live litter size on PND 0 relative to control animals observed in the reproductive performance cohort (Appendix E). Females in the 0.05 ppm EE group exhibited significantly decreased gravid uterine weight (20% lower than the control group), mean number of corpora lutea, implantations, and live fetuses (Table 22). Dams exposed to 2H4MBP or EE did not display any adverse changes in postimplantation loss, mean live fetal weights, or fetal sex ratio.

Table 22. Summary of Uterine Content Data for F_1 Female Rats in the Prenatal Cohort Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[a]
Pregnancy Summary[b]					
Paired Females	22	20	22	20	15
Mated Females	19	19	21	19	15
Pregnant Females[c]	18	18	20	19	15
Pregnant Females Examined on GD 21	18	16	18	18	15
Preimplantation Loss[d,e]					
Mean No. of Corpora Lutea/Female	18.56 ± 0.77** (18)	17.56 ± 0.77 (18)	17.40 ± 0.89 (20)[f]	14.89 ± 0.87** (19)	13.53 ± 0.47** (15)
Implantations/Female	15.61 ± 0.65** (18)	14.94 ± 0.67 (16)	13.28 ± 1.17 (18)	12.94 ± 0.88* (18)	12.13 ± 0.79** (15)
Preimplantation Loss (%)	14.51 ± 3.73 (18)	14.58 ± 3.38 (16)	24.91 ± 5.80 (18)	15.89 ± 3.49 (18)	11.47 ± 4.89 (15)
Intrauterine Deaths[e]					
Postimplantation Loss (%)[d,g]	5.33 ± 2.38 (18)	1.85 ± 0.84 (16)	7.86 ± 3.16 (18)	8.45 ± 5.46 (18)	4.19 ± 1.26 (15)
Total Resorptions per Litter[d]	0.67 ± 0.26 (18)	0.31 ± 0.15 (16)	0.61 ± 0.16 (18)	0.44 ± 0.12 (18)	0.53 ± 0.17 (15)
Early Resorptions per Litter[d]	0.50 ± 0.25 (18)	0.31 ± 0.15 (16)	0.61 ± 0.16 (18)	0.39 ± 0.12 (18)	0.47 ± 0.13 (15)
Late Resorptions per Litter[d]	0.17 ± 0.09 (18)	0.00 ± 0.00 (16)	0.00 ± 0.00 (18)	0.06 ± 0.06 (18)	0.07 ± 0.07 (15)
Dead Fetuses per Litter[d]	0.00 ± 0.00 (18)	0.00 ± 0.00 (16)	0.00 ± 0.00 (18)	0.00 ± 0.00 (18)	0.00 ± 0.00 (15)
No. of Early Resorptions	9	5	11	7	7
No. of Late Resorptions	3	0	0	1	1
No. of Whole Litter Resorptions[b]	0	0	0	1	0
No. of Dead Fetuses	0	0	0	0	0
Live Fetuses[e]					
No. of Live Fetuses[g]	269 (18)	234 (16)	228 (18)	225 (17)	174 (15)
Live Fetuses per Litter[d]	14.94 ± 0.82 (18)	14.63 ± 0.59 (16)	12.67 ± 1.17 (18)	13.24 ± 0.57 (17)	11.60 ± 0.76** (15)
Live Male Fetuses per Litter[d]	7.83 ± 0.58 (18)	7.38 ± 0.47 (16)	6.72 ± 0.74 (18)	6.76 ± 0.42 (17)	6.07 ± 0.56* (15)
Live Female Fetuses per Litter[d]	7.11 ± 0.76 (18)	7.25 ± 0.49 (16)	6.29 ± 0.60 (17)	6.41 ± 0.54 (17)	5.53 ± 0.54 (15)
Live Male Fetuses per Litter (%)[d]	53.19 ± 3.69 (18)	50.37 ± 2.60 (16)	55.21 ± 3.94 (18)	52.09 ± 3.17 (17)	51.48 ± 3.59 (15)
Fetal Weight (g)[d,h]					
Fetal Weight per Litter	5.06 ± 0.06 (18)	5.15 ± 0.09 (16)	5.08 ± 0.10 (18)	5.01 ± 0.10 (17)	5.23 ± 0.08 (15)

	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[a]
Male Fetal Weight per Litter	5.15 ± 0.07 (18)	5.34 ± 0.08 (16)	5.17 ± 0.09 (18)	5.13 ± 0.10 (17)	5.37 ± 0.09* (15)
Female Fetal Weight per Litter	4.95 ± 0.06 (18)	4.96 ± 0.09 (16)	4.98 ± 0.11 (17)	4.89 ± 0.09 (17)	5.10 ± 0.08 (15)
Gravid Uterine Weight (g)[d,h]					
Gravid Uterine Weight	107.08 ± 5.01** (18)	107.03 ± 3.26 (16)	90.65 ± 7.42 (18)	88.78 ± 6.11* (18)	85.58 ± 4.96** (15)
Terminal Body Weight	423.9 ± 7.3** (18)	412.6 ± 7.1 (16)	381.6 ± 9.0** (18)	345.5 ± 9.2** (18)	335.0 ± 4.8** (15)
Adjusted Body Weight[i]	316.82 ± 5.34** (18)	305.56 ± 5.91 (16)	290.98 ± 3.02** (18)	256.75 ± 4.58** (18)	249.45 ± 3.35** (15)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
EE = ethinyl estradiol; GD = gestation day.
[a]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[b]Statistical analysis performed by the Cochran-Armitage (trend) and Fisher's exact (pairwise) tests.
[c]Includes animals that had any evidence of pregnancy but were removed from the study before GD 21.
[d]Data are reported per litter as mean ± standard error (n) and do not include nonmated, nonpregnant, or unexamined animals or those that did not survive to the end of the study.
[e]Statistical analysis performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
[f]Includes two dams with total litter loss.
[g]n = the number of pups examined (number of litters).
[h]Statistical analysis performed by the Jonckheere (trend) and Williams or Dunnett (pairwise) tests.
[i]Body weight adjusted for gravid uterus weight.

Fetal Findings

Placental Morphology

There was no effect of 2H4MBP or EE exposure on the incidence of placental abnormalities (Appendix E). Fused placentae between two adjacent fetuses were noted for a single litter in the control group and the 10,000 ppm 2H4MBP group. Fused placentae were observed in two litters in the 30,000 ppm 2H4MBP group; one litter had a fusion between two adjacent fetuses, and the other litter had multiple fused placentae. The significant increase in incidence in placental abnormalities in the 30,000 ppm group was not considered 2H4MBP-related as most of the fusions were limited to a single litter and fused placentae have been observed in control litters of different stocks of Sprague Dawley rats.

External

There was no effect of 2H4MBP or EE at the exposures tested on the incidence of fetal external abnormalities (Appendix E), which were limited to a single fetus in the 30,000 ppm group that displayed anal atresia, clubbed hind limbs, tail agenesis, and a hematoma on the torso. This fetus also had multiple visceral and skeletal abnormalities.

Visceral

Male and female fetuses (combined) exposed to 30,000 ppm 2H4MBP displayed a higher incidence of enlarged liver, a malformation (Table 23), which had not been observed in NTP historical controls.

The 30,000 ppm 2H4MBP group displayed a higher incidence of unilateral or bilateral (combined) hydronephrosis, a malformation, relative to the control group (Table 23). This higher incidence was observed in 2.22% of the fetuses (29.41% of the litters), whereas it was observed in 1.12% and 1.15% of the fetuses (16.67% and 13.33% of the litters) from the control group and EE group, respectively. The NTP historical control range for unilateral or bilateral hydronephrosis is 0.00% to 0.81% for fetuses; (0.00% to 16.67% for litters). The incidence of bilateral distended ureter, a variation, was higher in all 2H4MBP-exposed groups as well as the EE group, relative to the control group. When unilateral and bilateral distended ureters were combined, the fetal incidence was 10.68%, 12.72%, and 8.44% (62.50%, 50.00%, and 35.29% of the litters) in the 3,000, 10,000, and 30,000 ppm groups versus 4.83% and 12.64% (44.44% and 46.67% of the litters) in the control and EE groups, respectively. Historical control incidence for distended ureter in fetuses is 10.90% (4.83% to 15.36%) and for litters is 56.70% (43.75% to 68.18%). Hydroureter of the left kidney was observed in one fetus in the control group and in two fetuses in the 3,000 ppm group, but given the low incidence, these were not considered related to 2H4MBP exposure (Appendix E). The NTP historical control range for hydroureter is up to 2.83% and 21.05% for fetuses and litters, respectively. Hydronephrosis and other abnormalities associated with the kidney and ureter (e.g., dilated renal pelvis, distended ureter, hydroureter) are common findings in this strain of rat; therefore, these collective findings may or may not be related to the 2H4MBP-associated microscopic findings observed in the kidney of adult F_1 males and females exposed to 30,000 ppm 2H4MBP (Appendix E).

Other malformations observed in 2H4MBP-exposed fetuses include ventricular septal defects in two fetuses in the 10,000 ppm group and in one fetus in the 30,000 ppm group (Table 23). This finding was not considered related to 2H4MBP due to the low incidence and lack of a clear exposure concentration-response and because it had been observed in a control fetus in a previous study (1/1,385). A single fetus (dam 1950, fetus 01) in the 30,000 ppm 2H4MBP group displayed adrenal gland agenesis, malpositioned kidneys, distended stomach, and agenesis of the gonads (Appendix E). This fetus also had external and skeletal malformations. None of the visceral findings associated with this fetus was considered 2H4MBP-related due to their singular occurrence. One fetus in the 10,000 ppm group displayed small, round kidneys, which were not considered 2H4MBP-related due to the singular occurrence.

There were no additional effects of EE exposure on the incidence of fetal visceral variations.

Table 23. Summary of Select Visceral Findings in Fetuses Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[a]
No. Litters Examined	18	16	18	17	15
No. Fetuses Examined	269	234	228	225	174
Fetal Findings[b,c]					
Enlarged liver – [M][d]					
Fetuses	0 (0.0)	1 (0.43)	2 (0.88)	7 (3.11)	0 (0.0)
Litters	0 (0.00)	1 (6.25)	1 (5.56)	2 (11.76)	0 (0.00)
Hydronephrosis – [M][e]					
Fetuses	3 (1.12)	1 (0.43)	0 (0.0)	5 (2.22)	2 (1.15)
Litters	3 (16.67)	1 (6.25)	0 (0.00)	5(29.41)	2 (13.33)
Distended ureter, bilateral – [V][f]					
Fetuses	4 (1.49)	11 (4.7)	15 (6.58)[#]	10 (4.44)	12 (6.9)[#]
Litters	3 (16.67)	6 (37.50)	8 (44.44)	5 (29.41)	7 (46.67)
Distended ureter – [V][g]					
Fetuses	13 (4.83)	25 (10.68)	29 (12.72)	19 (8.44)	22 (12.64)
Litters	8 (44.44)	10 (62.50)	9 (50.00)	6 (35.29)	7 (46.67)
Ventricular septum, septum defect – [M][h]					
Fetuses	0 (0.0)	0 (0.0)	2 (0.88)	1 (0.44)	0 (0.0)
Litters	0 (0.00)	0 (0.00)	2 (11.11)	1 (5.88)	0 (0.00)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
[#]Statistically significant at $p \leq 0.05$ (litter-based analysis).
EE = ethinyl estradiol; [M] = malformation; [V] = variation.
[a]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[b]Upper row denotes number of affected fetuses (%) and lower row the number of affected litters (%).
[c]Statistical analysis for fetal data including litter effects performed using a Rao-Scott modification to the Cochran-Armitage test where the litter was the random effect for both trend and pairwise analyses.
[d]Historical control incidence: fetuses – 0/1,385; litters – 0/97.
[e]Historical control incidence: fetuses – 4/1,385 (0.29%), range 0.00% to 0.81%; litters – 4/97 (4.12%), range 0.00% to 16.67%.
[f]Historical control incidence: fetuses – 60/1,385 (4.33%), range 1.28% to 7.85%; litters – 28/97 (28.87%), range 12.50% to 43.18%.
[g]Historical control incidence: fetuses – 151/1,385 (10.90%), range 4.83% to 15.36%; litters – 55/97 (56.70%), range 43.75% to 68.18%.
[h]Historical control incidence: fetuses – 1/1,385 (0.07%), range 0.00% to 0.17%; litters – 1/97 (1.03%), range 0.00% to 2.27%.

Head

There was no effect of 2H4MBP or EE exposure on the incidence of fetal head abnormalities at the exposures tested. Fetal head abnormalities were limited to a single fetus in the 3,000 ppm group that displayed anophthalmia of the right eye (Appendix E).

Skeletal

There was no effect of 2H4MBP or EE exposure on the incidence of fetal skeletal abnormalities at the exposures tested (Appendix E). Skeletal malformations in exposed groups were limited to fused sternebrae, multiple rib abnormalities, and vertebral abnormalities in a single fetus in the 30,000 ppm 2H4MBP group. Full lumbar 1 ribs were observed in several fetuses in the 3,000 and 10,000 ppm 2H4MBP groups. Given the low incidence and exposure response, these findings were not considered 2H4MBP-related.

Skeletal variations observed in 2H4MBP- and/or EE-exposed groups included incomplete ossification of the parietal skull, sternebrae extra ossification sites, misaligned sternebrae, incomplete sternebrae ossification (II, III, IV, V, VI), rudimentary rib (lumbar 1), thoracic centrum bipartite ossification, and thoracic centrum dumbbell ossification. With the exception of the lumbar 1 rudimentary rib variation, the incidences of the variations were limited to <3 fetuses per group. The incidences of the skeletal variations were not considered related to the test article because there was no exposure-related trend and/or the incidences were similar to the concurrent control group (Appendix E).

Reproductive Performance Cohort Findings

F_1 and F_2 rats from the reproductive performance cohort were evaluated for maternal reproductive performance and offspring effects, respectively, as shown in Figure 23. Littering, mean body weights, and feed consumption results from the F_1 rats as well as viability, clinical observations, mean body weights, and gross pathology results from the F_2 rats are presented below.

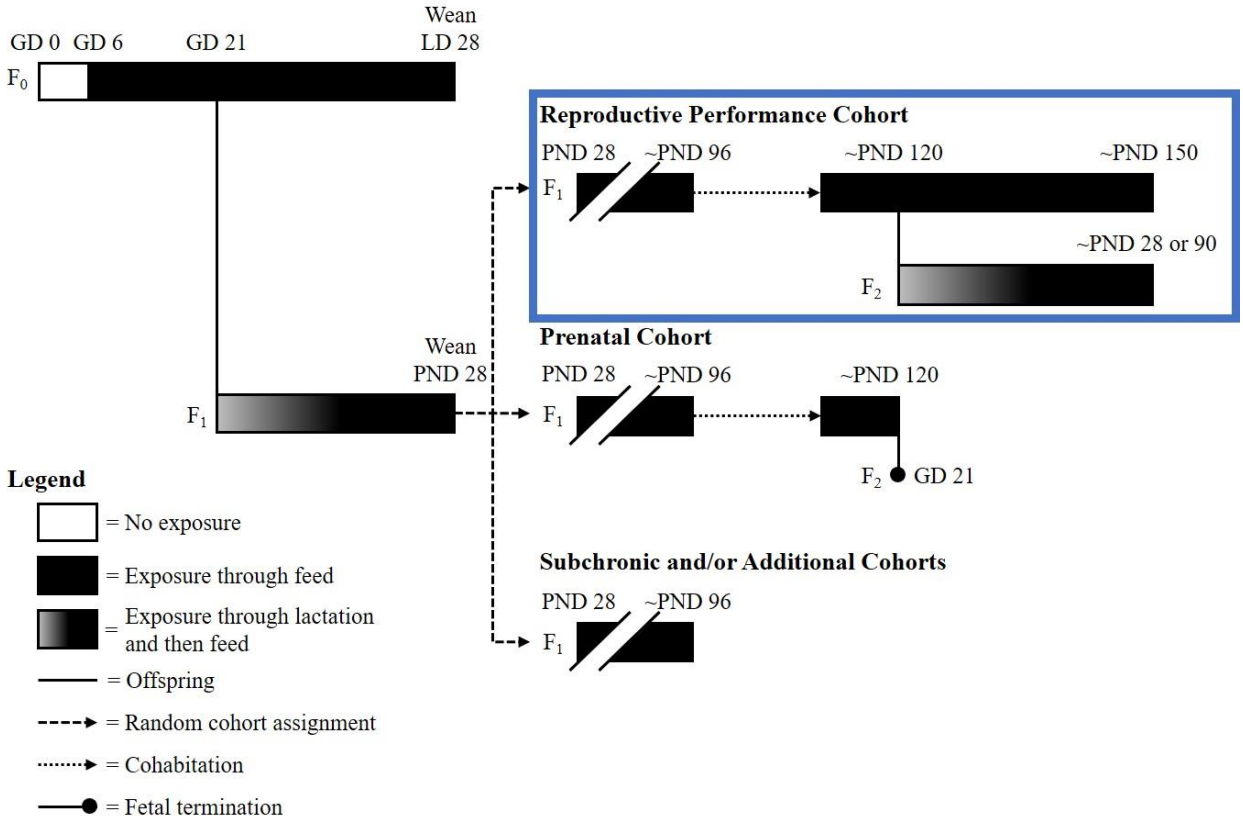


Figure 23. Design of the Modified One-Generation Study – Reproductive Performance Cohort

GD = gestation day; LD = lactation day; PND = postnatal day.

Reproductive Performance and Littering

Reproductive performance and littering parameters for the reproductive performance cohort are presented in Table 24. Gestation length was similar among the 2H4MBP-exposed groups and the control group. The EE group displayed a significant decrease (approximately 0.4 days) in gestation length compared to the control group.

Table 24. Summary of Reproductive Parameters of F_1 Female Rats in the Reproductive Performance Cohort Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[a]
No. Females Paired	41	40	40	40	30
No. Females Mated	40	37	35	35	29
No. Females Littering	35	37	33	32	28
Percent of Mated Females/Paired[b,c]	97.6	92.5	87.5	87.5	96.7
Percent of Littered Females/Paired[b,c]	85.4	92.5	82.5	80.0	93.3
Percent of Littered Females/Mated[b,c]	87.5	100.0	94.3	91.4	96.6
Precoital Interval (days)[d,e,f]	4.7 ± 0.6 (22)	4.8 ± 0.5 (20)	5.1 ± 0.7 (19)	4.2 ± 0.8 (20)	4.0 ± 0.6 (15)
Gestation Length (days)[d,e,g]	22.4 ± 0.1 (22)	22.5 ± 0.1 (20)	22.6 ± 0.1 (19)	22.2 ± 0.1 (20)	22.0 ± 0.1** (15)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
**Statistically significant at $p \leq 0.01$.
EE = ethinyl estradiol.
[a]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[b]Statistical analysis performed using the Rao-Scott Cochran-Armitage test for both trend and pairwise comparisons to adjust for litter effects (unless otherwise noted).
[c]Animals removed from the study between mating and littering were excluded from calculations of percent littered females.
[d]Statistical analysis performed using a bootstrapped Jonckheere test for trend, and a Datta-Satten modified Wilcoxon test with Hommel adjustment for pairwise comparisons.
[e]Data are displayed as mean ± standard error (n).
[f]Precoital interval calculated for sperm-positive females.
[g]Gestation length calculated for sperm-positive females that delivered a litter.

Lactation Body Weights and Feed Consumption

Consistent with their premating and gestation weights, F_1 female mean body weights during lactation were significantly decreased in the 10,000 and 30,000 ppm 2H4MBP and 0.05 ppm EE groups relative to the control group (Table 25; Figure 24). On LDs 1 and 28, female mean body weights of the 10,000 and 30,000 ppm 2H4MBP and 0.05 ppm EE groups were significantly decreased by 5%–7%, 18%–20%, and 19%–21%, respectively, compared to the control group. Body weight gain between LD 1 and LD 28 in the 10,000 and 30,000 ppm 2H4MBP and 0.05 ppm EE groups was higher relative to the control group. In general, feed consumption values during lactation in the groups exposed to 2H4MBP or EE were similar to the control group (Table 25). 2H4MBP intake during lactation in the 3,000, 10,000, and 30,000 ppm 2H4MBP groups, based on feed consumption and dietary concentrations for the LD 1–13 interval, was approximately 426, 1,621, and 5,944 mg/kg/day, respectively (Table 25). EE intake during the postweaning period was approximately 0.009 mg/kg/day.

Table 25. Summary of Mean Body Weights, Body Weight Gains, and Feed and Test Article Consumption for F_1 Female Rats in the Reproductive Performance Cohort Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed during Lactation

Lactation Day[a]	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[b]
Body Weight (g)[c]					
1	309.2 ± 6.0** (22)	309.4 ± 4.1 (20)	288.3 ± 4.7* (20)	248.1 ± 5.7** (20)	243.6 ± 4.9** (15)
13	333.9 ± 5.4** (22)	333.4 ± 4.2 (20)	310.8 ± 4.2** (20)	263.4 ± 5.1** (20)	272.0 ± 4.5** (15)
28	317.8 ± 5.1** (22)	316.4 ± 4.0 (20)	300.9 ± 3.9* (20)	260.9 ± 4.0** (20)	255.9 ± 4.7** (15)
Body Weight Gain (g)[c]					
1–28	8.6 ± 2.9 (22)	7.0 ± 2.7 (20)	12.6 ± 3.2 (20)	12.8 ± 4.0 (20)	12.3 ± 2.5 (15)
Feed Consumption[d]					
1–13 (g/animal/day)	44.8 ± 1.1* (21)	45.9 ± 1.3 (20)	48.6 ± 1.7 (20)	50.4 ± 2.1 (20)	45.6 ± 1.6 (15)
1–13 (g/kg/day)	139.1 ± 3.5** (21)	142.1 ± 4.5 (20)	162.1 ± 6.0** (20)	198.1 ± 9.0** (20)	177.1 ± 8.4** (15)
Chemical Intake (mg/kg/day)[e,f]					
1–13	0.0 ± 0.0 (21)	426.2 ± 13.5 (20)	1,620.8 ± 60.0 (20)	5,944.0 ± 268.8 (20)	8.9 ± 0.4 (15)[g]

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
EE = ethinyl estradiol.
[a]Data are displayed as mean ± standard error (n), where n = number of litters.
[b]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[c]Statistical analysis performed using mixed effects models with litter as a random effect for both trend and pairwise tests, and a Dunnett-Hsu adjustment for multiple comparisons.
[d]Statistical analysis performed using a bootstrapped Jonckheere test for trend, and a Datta-Satten modified Wilcoxon test with Hommel adjustment for pairwise comparisons.
[e]Chemical intake calculated as: ([exposure concentration × feed consumption]/[average body weight of day range]).
[f]No statistical analysis performed on the chemical intake data.
[g]EE consumption presented as µg/kg/day.

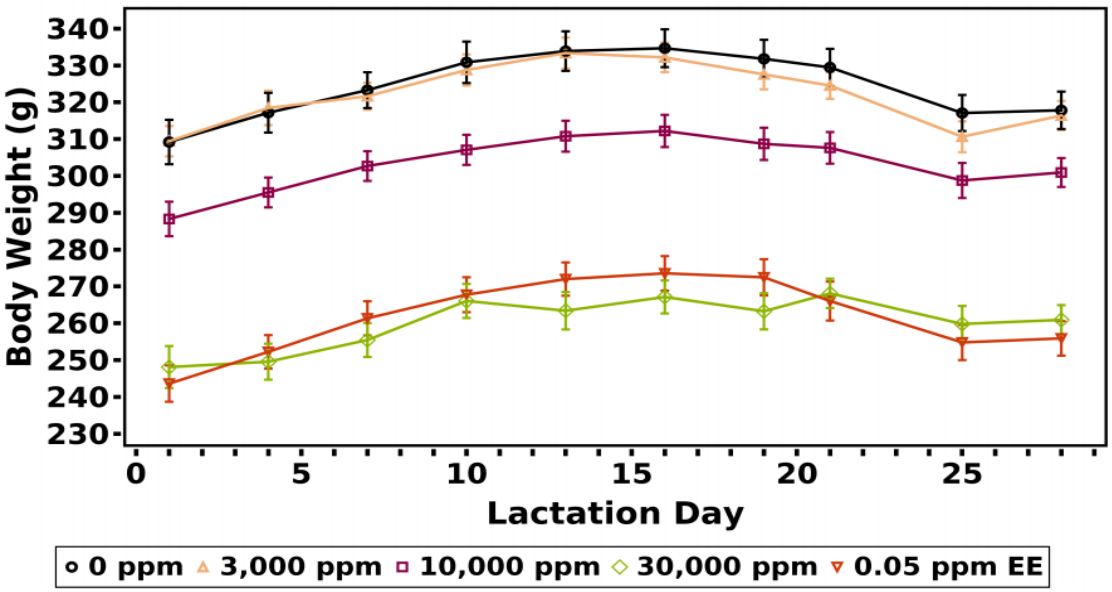


Figure 24. Lactation Growth Curves for F_1 Female Rats in the Reproductive Performance Cohort Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

EE = ethinyl estradiol. Information for statistical significance in F_1 female rat weights is provided in Table 25.

F_2 Viability and Clinical Observations

Clinical observations noted in individual pups in all groups, including the control group, were typically indicative of an individual pup not thriving (e.g., cold to the touch, no milk in the stomach). Exposure-related reductions in mean total and live litter size were observed in the 2H4MBP- and EE-exposed groups. Dams in the 10,000 and 30,000 ppm 2H4MBP groups had lower total and live litter size than the control group on PND 0 (by ~1 pup/litter). Total and live litter size in the EE-exposed group were significantly decreased (by ~2 pups/litter) on PND 0 than in the control group (Table 26). Although the reductions in mean live litter size in the 2H4MBP-exposed groups did not achieve statistical significance compared to the control group after PND 0, the findings were consistent with the reductions in the mean number of live fetuses/pregnant females that were observed in the prenatal cohort (Table 22).

Table 26. Summary of F_2 Litter Size and Pup Survival Following Perinatal Exposure to 2-Hydroxy-4-methoxybenzophenone

Postnatal Day	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[a]
No. of Live Pups (Litters)[b]					
0	477 (35)	462 (37)	404 (33)	386 (32)	314 (28)
Total Litter Size[c,d]					
0	14.6 ± 0.5* (22)	13.8 ± 0.5 (20)	13.7 ± 0.7 (20)	12.9 ± 0.5* (20)	11.8 ± 0.4** (15)
Live Litter Size[c,d]					
0	13.6 ± 0.5* (22)	12.9 ± 0.6 (20)	12.4 ± 0.9 (20)	12.0 ± 0.4* (20)	11.3 ± 0.5** (15)
1	13.4 ± 0.5* (22)	12.7 ± 0.6 (20)	12.2 ± 0.9 (20)	12.0 ± 0.4 (20)	10.9 ± 0.5** (15)
4 (prestandardization)	13.1 ± 0.4* (22)	12.6 ± 0.6 (20)	11.9 ± 0.8 (20)	11.5 ± 0.4 (20)	10.8 ± 0.5** (15)
4 (poststandardization)	7.8 ± 0.2 (22)	7.6 ± 0.2 (20)	7.6 ± 0.3 (20)	7.9 ± 0.1 (20)	7.6 ± 0.2 (15)
7	6.8 ± 0.4 (21)	6.9 ± 0.3 (20)	6.8 ± 0.3 (20)	6.8 ± 0.4 (20)	7.3 ± 0.3 (15)
13	5.7 ± 0.4 (20)	6.1 ± 0.3 (19)	5.8 ± 0.3 (20)	6.2 ± 0.4 (18)	6.8 ± 0.3* (15)
21	5.7 ± 0.4 (20)	5.9 ± 0.3 (19)	5.7 ± 0.3 (20)	6.0 ± 0.4 (18)	6.8 ± 0.3* (15)
28	5.7 ± 0.4 (20)	5.9 ± 0.3 (19)	5.7 ± 0.3 (20)	5.9 ± 0.3 (18)	6.7 ± 0.3* (15)
No. of Dead Pups (Litters)[c,d]					
0	34 (18)	41 (13)	42 (18)	29 (17)	16 (12)
1–4	27 (13)	13 (9)	17 (9)	13 (11)	14 (8)
5–28	83 (26)	69 (25)	58 (23)	79 (24)	35 (13)
Dead per Litter[c,d]					
0	0.95 ± 0.27 (22)	1.03 ± 0.49 (20)	1.67 ± 0.70 (20)	0.94 ± 0.25 (20)	0.47 ± 0.15 (15)
1–4	0.84 ± 0.32 (22)	0.35 ± 0.12 (20)	0.45 ± 0.13 (20)	0.48 ± 0.16 (20)	0.53 ± 0.19 (15)
5–28	2.73 ± 0.45 (22)	1.93 ± 0.40 (20)	1.84 ± 0.29 (20)	2.60 ± 0.51 (20)	1.18 ± 0.28** (15)
Survival Ratio[c,d]					
0	0.94 ± 0.02 (22)	0.94 ± 0.03 (20)	0.86 ± 0.05 (20)	0.93 ± 0.02 (20)	0.95 ± 0.02 (15)
1–4	0.92 ± 0.03 (22)	0.98 ± 0.01 (20)	0.97 ± 0.01 (20)	0.96 ± 0.01 (20)	0.95 ± 0.02 (15)
5–28	0.68 ± 0.06 (22)	0.75 ± 0.05 (20)	0.77 ± 0.04 (20)	0.67 ± 0.06 (20)	0.85 ± 0.04** (15)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
EE = ethinyl estradiol.
[a]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[b]n = the number of pups examined (number of F_1 litters).
[c]Data are displayed as the mean of litter values ± standard error of litter values (n = number of litters produced by F_0 dams); n is dependent on the number of litters produced by the F_0 generation in which up to two nonindependent F_1 offspring/sex/litter were selected to produce F_2 pups through nonsibling mating.
[d]Statistical analysis performed using a bootstrapped Jonckheere test for trend, and a Datta-Satten modified Wilcoxon test with Hommel adjustment for pairwise comparisons.

F_2 Body Weights

Male Pups

Male pups exposed to 30,000 ppm 2H4MBP displayed lower mean body weights (litter means) over time compared to the control group (Table 27; Figure 25; Appendix E). On PND 21, male pup mean body weights per litter of the 30,000 ppm group were lower by approximately 8% and by PND 28 they were significantly decreased 15% relative to the control group. A significant decrease in pup mean body weight was first observed in male offspring on PND 25. These effects are consistent with what was observed in the F_1 generation, but the magnitude of change with exposure concentration is not as severe. EE exposure had no adverse effect on male pup mean body weights.

Female Pups

Female pups exposed to 30,000 ppm 2H4MBP also displayed lower mean body weights (litter means) relative to the control group (Table 27; Figure 26; Appendix E). On PND 21 and PND 28, female pup mean body weights per litter of the 30,000 ppm group were significantly decreased by approximately 12% and 21% relative to the control group, respectively. A significant decrease in pup mean body weight was first observed in female offspring on PND 16. These effects are consistent with what was observed in the F_1 generation, but the magnitude of reduction with exposure concentration is not as severe. There was no adverse effect of EE exposure on female pup mean body weights.

Table 27. Summary of F_2 Male and Female Pup Mean Body Weights and Body Weight Gains Following Perinatal Exposure to 2-Hydroxy-4-methoxybenzophenone[a,b]

Postnatal Day	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.05 ppm[c]
Male					
Body Weight					
1	6.95 ± 0.12 213 (33)[d]	7.17 ± 0.14 226 (36)	7.06 ± 0.14 192 (32)	6.75 ± 0.09 185 (32)	6.53 ± 0.10** 144 (28)
4	9.18 ± 0.24 205 (33)	9.72 ± 0.30 223 (36)	9.72 ± 0.25 187 (32)	8.87 ± 0.16 184 (32)	9.22 ± 0.18 141 (28)
21	42.56 ± 1.28** 91 (30)	47.72 ± 1.32** 110 (34)	45.95 ± 1.10 101 (32)	39.18 ± 1.33 88 (30)	46.30 ± 0.95* 88 (27)
28	72.28 ± 1.90** 91 (30)	80.42 ± 2.01** 110 (34)	75.41 ± 1.76 101 (32)	61.82 ± 2.46** 88 (30)	76.78 ± 1.19 87 (27)
Body Weight Gain					
4–28	62.96 ± 1.77** 91 (30)	70.20 ± 1.79* 110 (34)	65.52 ± 1.56 101 (32)	52.43 ± 2.38** 88 (30)	66.92 ± 1.09 87 (27)
Female					
Body Weight					
1	6.67 ± 0.13** 255 (35)	6.90 ± 0.12 230 (35)	6.52 ± 0.13 207 (32)	6.37 ± 0.09 199 (32)	6.22 ± 0.10** 160 (27)
4	8.72 ± 0.24* 245 (34)	9.15 ± 0.28 226 (35)	8.62 ± 0.24 200 (32)	8.29 ± 0.18 190 (32)	8.36 ± 0.20 159 (27)
21	42.55 ± 1.23** 94 (30)	43.14 ± 1.31 95 (32)	42.09 ± 1.13 85 (32)	37.64 ± 1.27** 87 (28)	44.12 ± 0.82 91 (26)
28	69.12 ± 1.70** 94 (30)	70.49 ± 1.96 95 (32)	66.19 ± 1.70 85 (32)	54.49 ± 2.09** 86 (28)	71.12 ± 1.03 91 (26)
Body Weight Gain					
4–28	60.08 ± 1.50** 94 (30)	61.12 ± 1.81 95 (32)	57.15 ± 1.57 85 (32)	45.62 ± 1.93** 86 (28)	61.75 ± 0.97 91 (26)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
EE = ethinyl estradiol.
[a]Data are displayed as mean ± standard error of the litter means. Body weight data are presented in grams.
[b]Statistical analysis performed using mixed effects models with litter as a random effect for both trend and pairwise tests, and a Dunnett-Hsu adjustment for multiple pairwise comparisons. Pup weights were adjusted for covariate litter size: total live on postnatal day 1 for day 1 to day 4 and number of live pups poststandardization for later days.
[c]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[d]n = number of pups examined (number of F_1 litters).

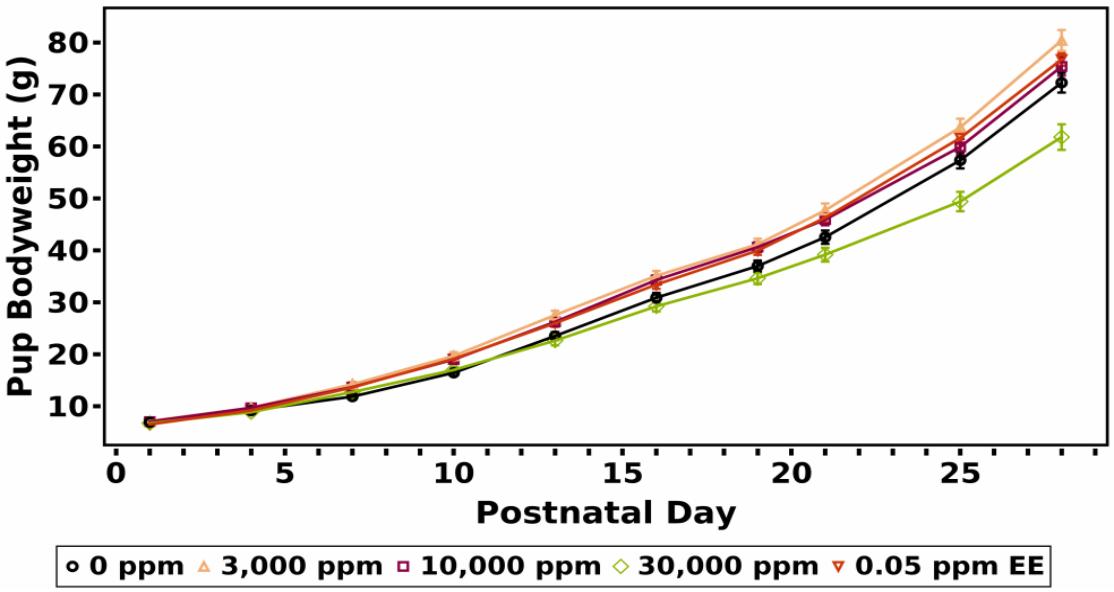


Figure 25. Lactation Growth Curves for F_2 Male Pups Following Perinatal Exposure to 2-Hydroxy-4-methoxybenzophenone

EE = ethinyl estradiol. Information for statistical significance in F_2 male rat weights is provided in Table 27.

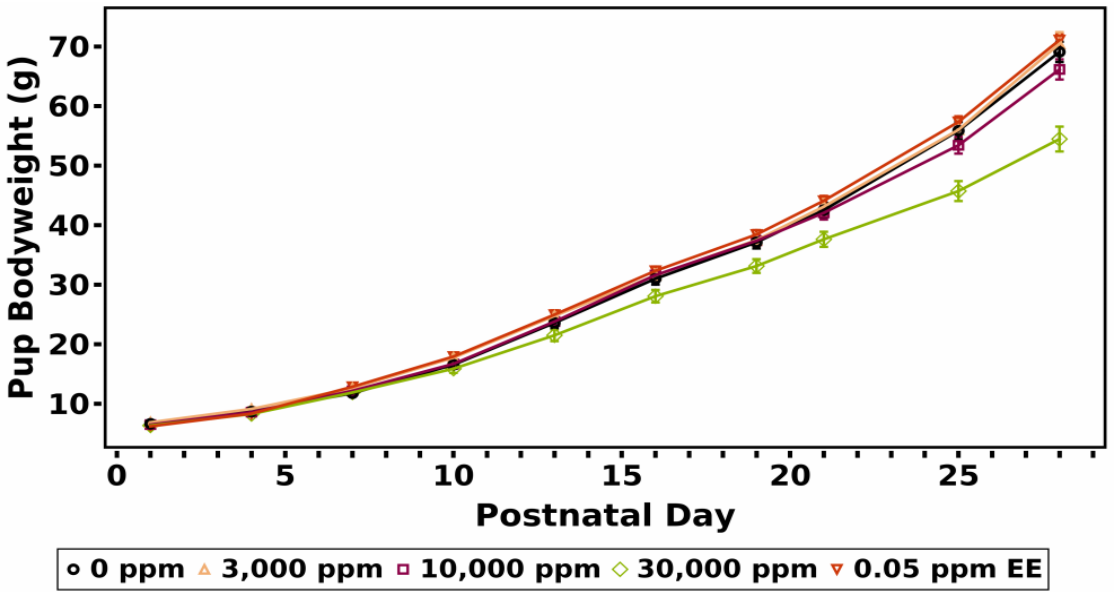


Figure 26. Lactation Growth Curves for F_2 Female Pups Following Perinatal Exposure to 2-Hydroxy-4-methoxybenzophenone

EE = ethinyl estradiol. Information for statistical significance in F_2 female rat weights is provided in Table 27.

Prenatal and Reproductive Performance Cohorts: Necropsies

F_1 Male Necropsies

F_1 males in the reproductive performance cohort were euthanized following the mating period at 153–155 days of age. The F_1 males in the prenatal cohort were euthanized following completion of pairing at 111–113 days of age.

Male rats exposed to 30,000 ppm 2H4MBP displayed a higher incidence of discolored (pale or dark) or enlarged kidneys and discolored (brown) urinary bladders (Table 28). Necropsy mean body weights of rats exposed to 30,000 ppm 2H4MBP or 0.05 ppm EE in both cohorts were significantly decreased by 14% and 15%−20%, respectively, compared to control animals (Table 29). Rats in both cohorts from all 2H4MBP-exposed groups displayed higher left and right absolute and relative kidney weights (Table 29). Absolute kidney weights were 5%−12%, 10%−14%, and 13%−22% higher and relative weights were 7%−10%, 15%−16%, and 30%−42% higher than those of control animals in the 3,000, 10,000, and 30,000 ppm groups, respectively. Gross findings in the kidney and bladder correlated with histopathological changes consistent with a retrograde nephropathy. One male rat in the 30,000 ppm 2H4MBP group in the reproductive performance cohort exhibited a diaphragmatic hernia. These hernias were also observed in F_1 females and in the F_2 generation. One male in the 30,000 ppm 2H4MBP group displayed hypospadias and another displayed bilateral smaller testes (Appendix E).

Male rats in all 2H4MBP-exposed groups in both cohorts displayed higher absolute and relative liver weights compared to the control animals (Table 29). Absolute liver weights of males exposed to 3,000 ppm 2H4MBP in the reproductive performance and prenatal cohorts were higher by 6% and 11%, respectively, relative to control animals. Absolute liver weights of males in both cohorts exposed to 10,000 and 30,000 ppm were significantly increased 14%−20% relative to control animals. Relative liver weights of the 3,000, 10,000, and 30,000 ppm 2H4MBP groups in both cohorts were significantly increased approximately 7%−9%, 20%−23%, and 32%−34%, respectively, relative to the control group. The reproductive performance and prenatal cohorts displayed generally similar responses.

Rats in both cohorts exposed to 30,000 ppm 2H4MBP displayed slightly lower right and left absolute testis weights (approximately 4%−6%) (Table 29). Rats exposed to 30,000 ppm in the reproductive performance cohort exhibited a slight but significant decreased (5%−6%) right and left absolute epididymis weights. Absolute ventral prostate gland weights of the 30,000 ppm 2H4MBP groups were significantly decreased 19% and 10% relative to control animals in the reproductive performance and prenatal cohorts, respectively. This difference in cohort response might be due to duration of exposure being longer in the reproductive performance cohort. No 2H4MBP-related histopathological effects in the testis or epididymis were found. No exposure-related changes in sperm motility, sperm concentration, or testicular sperm head concentration were found (Appendix E). Rats in the 30,000 ppm 2H4MBP group in both cohorts displayed significantly decreased absolute levator ani/bulbocavernosus (LABC) muscle weights (10%−12%); however, when adjusted for body weight, this difference was negligible (Table 29). No gross pathological findings in the males exposed to 0.05 ppm EE were considered to be related to exposure. In general, male rats exposed to EE displayed lower absolute weights of the testes, epididymides, prostate gland, kidney, liver, seminal vesicles with coagulating glands, and

LABC. These observations are likely the result of animals weighing 15%−20% less than control animals.

Table 28. Summary of Gross Necropsy Findings in Adult F_1 Male Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

	0 ppm		3,000 ppm		10,000 ppm		30,000 ppm		EE 0.05 ppm	
	RPC	**PC**	**RPC**	**PC**	**RPC**	**PC**	**RPC**	**PC**	**RPC**	**PC**
No. of Animals Examined (No. of Litters)	41 (22)	23 (22)	40 (20)	20 (20)	40 (21)	22 (21)	40 (20)	20 (20)	30 (15)	15 (15)
Kidney[a]										
Dilation										
Unilateral	1 (1)	0	0	0	0	2 (2)	1 (1)	0	0	0
Enlarged										
Unilateral	0	0	0	0	0	0	0	1 (1)	0	0
Bilateral	0	0	0	0	1 (1)	0	1 (1)	5 (5)	0	0
Discolored, dark										
Unilateral	0	0	0	0	0	0	4 (4)	0	0	0
Bilateral	0	0	0	0	0	0	15 (12)	4 (4)	0	0
Unilateral or bilateral	0	0	0	0	0	0	19 (14)	4 (4)	0	0
Discolored, pale										
Unilateral	0	0	0	0	0	0	4 (4)	4 (4)	0	0
Bilateral	0	0	0	0	0	0	1 (1)	0	0	0
Unilateral or bilateral	0	0	0	0	0	0	5 (5)	4 (4)	0	0
Discolored, mottled										
Unilateral	0	0	0	0	0	0	0	0	0	0
Bilateral	0	0	0	0	0	0	0	1 (1)	0	0
Unilateral or bilateral	0	0	0	0	0	0	0	1 (1)	0	0
Urinary Bladder[a]										
Discoloration, brown	0	0	0	0	0	0	16 (14)	9 (9)	0	0

EE = ethinyl estradiol; RPC = reproductive performance cohort; PC = prenatal cohort.
[a]Incidence presented as number of animals with lesion (number of litters). No statistical analysis was performed.

Table 29. Summary of Organ Weights of Adult F_1 Male Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed[a,b]

	0 ppm		3,000 ppm		10,000 ppm		30,000 ppm		EE 0.05 ppm[c]	
	RPC	**PC**	**RPC**	**PC**	**RPC**	**PC**	**RPC**	**PC**	**RPC**	**PC**
No. of Litters Examined	22	23	20	20	21	22	20	20	15	15
Necropsy Body Wt. (g)	485.5 ± 5.0**	422.3 ± 6.6**	478.5 ± 4.6	430.9 ± 7.1	468.3 ± 5.2	414.2 ± 5.0	419.6 ± 6.9**	365.1 ± 5.4**	389.5 ± 6.2**	353.8 ± 5.8**
Liver										
Absolute (g)	18.25 ± 0.30**	18.80 ± 0.50**	19.29 ± 0.32	20.81 ± 0.42**	21.19 ± 0.35**	22.58 ± 0.49**	21.09 ± 0.34**	21.41 ± 0.32**	15.41 ± 0.29**	15.70 ± 0.46**
Relative (mg/g)[d]	37.55 ± 0.35**	44.43 ± 0.67**	40.27 ± 0.44**	48.36 ± 0.76**	45.24 ± 0.52**	54.49 ± 0.91**	50.37 ± 0.52**	58.72 ± 0.69**	39.56 ± 0.47**	44.34 ± 0.91
R. Kidney										
Absolute (g)	1.65 ± 0.02**	1.57 ± 0.04**	1.74 ± 0.03	1.71 ± 0.02**	1.84 ± 0.03**	1.76 ± 0.04**	2.02 ± 0.04**	1.77 ± 0.05**	1.41 ± 0.02**	1.35 ± 0.02**
Relative (mg/g)	3.41 ± 0.04**	3.71 ± 0.07**	3.64 ± 0.04*	3.99 ± 0.06*	3.92 ± 0.05**	4.25 ± 0.07**	4.84 ± 0.10**	4.85 ± 0.13**	3.64 ± 0.04**	3.82 ± 0.07
L. Kidney										
Absolute (g)	1.65 ± 0.02**	1.53 ± 0.04**	1.74 ± 0.03	1.72 ± 0.03**	1.84 ± 0.04**	1.74 ± 0.04**	2.01 ± 0.04**	1.73 ± 0.04**	1.42 ± 0.02**	1.34 ± 0.02**
Relative (mg/g)	3.39 ± 0.04**	3.63 ± 0.06**	3.64 ± 0.04	4.01 ± 0.11**	3.93 ± 0.06**	4.19 ± 0.07**	4.82 ± 0.11**	4.73 ± 0.07**	3.65 ± 0.06**	3.79 ± 0.07
R. Testis										
Absolute (g)	2.10 ± 0.02**	1.95 ± 0.04**	2.08 ± 0.02	2.03 ± 0.03	1.98 ± 0.03*	1.91 ± 0.03	1.98 ± 0.04*	1.87 ± 0.03	1.92 ± 0.03**	1.87 ± 0.04
L. Testis										
Absolute (g)	2.10 ± 0.02**	1.97 ± 0.03**	2.07 ± 0.02	2.03 ± 0.03	1.98 ± 0.03**	1.91 ± 0.04	1.98 ± 0.03**	1.86 ± 0.03*	1.92 ± 0.02**	1.87 ± 0.03*
R. Epididymis										
Absolute (g)	0.69 ± 0.01**	0.65 ± 0.01	0.69 ± 0.01	0.66 ± 0.01	0.66 ± 0.01	0.62 ± 0.01	0.66 ± 0.01*	0.63 ± 0.01	0.64 ± 0.01**	0.61 ± 0.01
L. Epididymis										
Absolute (g)	0.70 ± 0.01**	0.65 ± 0.01	0.68 ± 0.01	0.67 ± 0.01	0.67 ± 0.01	0.63 ± 0.01	0.65 ± 0.01**	0.62 ± 0.01	0.64 ± 0.01**	0.61 ± 0.02*
Seminal Vesicles with Coagulating Gland[e]										
Absolute (g)	1.51 ± 0.04	1.49 ± 0.05	1.50 ± 0.04	1.53 ± 0.06	1.45 ± 0.04	1.44 ± 0.04	1.42 ± 0.03	1.44 ± 0.05	1.33 ± 0.05*	1.34 ± 0.05
Dorso-lateral Prostate										
Absolute (g)	0.45 ± 0.01**	0.49 ± 0.02	0.47 ± 0.02	0.47 ± 0.03	0.45 ± 0.02	0.50 ± 0.02	0.40 ± 0.02	0.43 ± 0.02	0.41 ± 0.01	0.40 ± 0.02**

	0 ppm		3,000 ppm		10,000 ppm		30,000 ppm		EE 0.05 ppm[c]	
	RPC	**PC**	**RPC**	**PC**	**RPC**	**PC**	**RPC**	**PC**	**RPC**	**PC**
Ventral Prostate										
Absolute (g)	0.74 ± 0.02**	0.57 ± 0.03	0.74 ± 0.02	0.54 ± 0.03	0.66 ± 0.02*	0.54 ± 0.02	0.60 ± 0.02**	0.52 ± 0.02	0.67 ± 0.03	0.52 ± 0.02
Levator Ani/bulbocavernosus Muscle Complex										
Absolute (g)	1.24 ± 0.02**	1.25 ± 0.03**	1.21 ± 0.02	1.24 ± 0.03	1.18 ± 0.02	1.12 ± 0.03*	1.09 ± 0.03**	1.13 ± 0.03**	1.08 ± 0.02**	1.14 ± 0.03*
Relative (mg/g)	2.56 ± 0.04	2.96 ± 0.06	2.54 ± 0.03	2.88 ± 0.09	2.53 ± 0.03	2.77 ± 0.06	2.61 ± 0.05	3.09 ± 0.08	2.78 ± 0.05**	3.23 ± 0.07**

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
EE = ethinyl estradiol; RPC = reproductive performance cohort; PC = prenatal cohort.
[a]Data are displayed as mean ± standard error of the litter means.
[b]Statistical analysis for the RPC performed using the Jonckheere (trend) and Williams or Dunnett (pairwise) tests. Statistical analysis for the PC performed using mixed models with a random effect for litter and a Dunnett-Hsu adjustment for multiple pairwise comparisons.
[c]The EE group was not included in any trend analysis, it was included in the pairwise analysis to the vehicle control group.
[d]Relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body weight.
[e]For the PC, n = 22, 19, 20, and 16 litters for the 0, 3,000, 10,000, and 30,000 ppm groups, respectively. For the RPC, n = 19 litters for the 3,000 ppm group.

F_1 Female Necropsies

F_1 females (and F_2 offspring) in the reproductive performance cohort were euthanized and necropsied on PND 28, when the F_1 females were between 127 and 168 days of age. F_1 females in the prenatal cohort were between 109 and 132 days of age at the time of necropsy and the collection of organ weight data.

There were no gross observations in the prenatal cohort attributed to 2H4MBP exposure. Females in the reproductive performance cohort exposed to 30,000 ppm 2H4MBP displayed a higher individual and litter incidence of abnormal kidney findings (dilation, discoloration) (Table 30). These findings were also observed at a low incidence in the 3,000 ppm group and are consistent with what was observed in the F_1 males. This difference in response between the two cohorts might have been the result of duration of exposure and is consistent with what was observed in the F_1 males.

The reproductive performance and prenatal cohorts exposed to 10,000 or 30,000 ppm 2H4MBP displayed terminal/adjusted body weights that were significantly decreased 5%−8% and 18%−19%, respectively, compared to the control females (Table 31). Females in all 2H4MBP-exposed groups from both cohorts displayed significantly increased relative liver weights (10%–14%, 17%–32%, and 28%–53% in the 3,000, 10,000, and 30,000 ppm groups, respectively) compared to the control females (Table 31). Rats in the reproductive performance cohort exposed to 3,000, 10,000, and 30,000 ppm 2H4MBP displayed higher (approximately 5%−7%, 11%, and 24%−30%, respectively) relative right and left kidney weights compared to the control group. Absolute kidney weights were significantly decreased (12%−14%) compared to the control group in females in the reproductive performance cohort exposed to 0.05 ppm EE. Relative liver weights were significantly increased in the 0.05 ppm EE groups in both cohorts compared to the control groups, likely because necropsy body weights were lower than those of the control group.

Females exposed to 10,000 or 30,000 ppm 2H4MBP in both cohorts displayed lower absolute right and left ovarian weights (Table 31). Females in the reproductive performance cohort exposed to 30,000 ppm 2H4MBP displayed significantly decreased absolute adrenal gland weight compared to the control group. Both cohorts of the EE groups had lower absolute ovarian and adrenal cortical weights.

Table 30. Summary of Gross Necropsy Findings in Adult F_1 Female Rats in the Reproductive Performance Cohort Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.5 ppm
No. of Animals Examined (No. of Litters)	41 (22)	40 (20)	40 (21)	40 (20)	30 (15)
Kidney[a]					
Dilation					
Unilateral	0	1 (1)	0	2 (2)	0
Enlarged					
Unilateral	0	0	0	1 (1)	0
Discolored, dark					
Unilateral	0	0	0	1 (1)	0
Bilateral	0	0	0	1 (1)	0
Unilateral or bilateral	0	0	0	2 (2)	0
Discolored, pale					
Unilateral	0	0	0	4 (3)	0
Bilateral	0	0	0	3 (3)	0
Unilateral or bilateral	0	0	0	7 (6)	0
Discolored, mottled					
Unilateral	0	0	0	0	0
Bilateral	0	2 (2)	0	0	0
Unilateral or bilateral	0	2 (2)	0	0	0

[a]Incidence presented as number of animals with lesion (number of litters). No statistical analysis was performed.

Table 31. Summary of Organ Weights of Adult F_1 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed[a,b]

	0 ppm		3,000 ppm		10,000 ppm		30,000 ppm		EE 0.05 ppm[c]	
	RPC	PC	RPC	PC	RPC	PC	RPC	PC	RPC	PC
No. of Litters Examined	22	19	20	17	20	19	20	18	15	15
Necropsy Body Wt. (g)[d]	316.7 ± 4.6**	315.5 ± 5.2**	315.1 ± 3.4	304.5 ± 5.7	302.1 ± 3.1*	291.6 ± 2.9**	262.1 ± 3.6**	256.8 ± 4.6**	255.7 ± 3.8**	249.5 ± 3.4**
Liver										
Absolute (g)	14.02 ± 0.41**	15.18 ± 0.46	15.82 ± 0.36**	16.15 ± 0.44	17.53 ± 0.37**	16.38 ± 0.23	17.60 ± 0.54**	15.80 ± 0.44	13.93 ± 0.32	12.83 ± 0.30**
Relative (mg/g)[e]	44.12 ± 1.08**	48.07 ± 1.08**	50.19 ± 1.02**	53.02 ± 1.01**	58.14 ± 1.25**	56.17 ± 0.58**	67.40 ± 2.11**	61.42 ± 0.99**	54.57 ± 1.16**	51.43 ± 0.96*
R. Kidney										
Absolute (g)	1.14 ± 0.02	–	1.19 ± 0.02	–	1.21 ± 0.01	–	1.22 ± 0.04	–	0.98 ± 0.02**	–
Relative (mg/g)	3.61 ± 0.05**	–	3.78 ± 0.05	–	4.01 ± 0.04*	–	4.70 ± 0.19**	–	3.85 ± 0.06**	–
L. Kidney										
Absolute (g)	1.12 ± 0.02	–	1.19 ± 0.01*	–	1.18 ± 0.01*	–	1.14 ± 0.02	–	0.98 ± 0.02**	–
Relative (mg/g)	3.53 ± 0.05**	–	3.79 ± 0.04**	–	3.92 ± 0.04**	–	4.37 ± 0.07**	–	3.86 ± 0.07**	–
Adrenal Glands										
Absolute (g)	0.071 ± 0.001**	0.073 ± 0.002	0.067 ± 0.001	0.066 ± 0.002	0.068 ± 0.001	0.066 ± 0.003	0.060 ± 0.002**	0.070 ± 0.003	0.059 ± 0.001**	0.056 ± 0.002**
Relative (mg/g)	0.23 ± 0.01	0.23 ± 0.01**	0.21 ± 0.00	0.22 ± 0.001	0.22 ± 0.001	0.23 ± 0.01	0.23 ± 0.01	0.27 ± 0.01**	0.23 ± 0.01	0.22 ± 0.01
R. Ovary										
Absolute (g)	0.075 ± 0.003**	0.106 ± 0.005**	0.068 ± 0.002	0.092 ± 0.005*	0.066 ± 0.003	0.093 ± 0.005*	0.058 ± 0.003**	0.084 ± 0.003**	0.055 ± 0.003**	0.075 ± 0.004**
Relative (mg/g)	0.24 ± 0.01	0.33 ± 0.01	0.22 ± 0.01	0.30 ± 0.02	0.22 ± 0.01	0.32 ± 0.02	0.22 ± 0.01	0.32 ± 0.01	0.21 ± 0.01	0.30 ± 0.02
L. Ovary[f]										
Absolute (g)	0.071 ± 0.002**	0.096 ± 0.006*	0.071 ± 0.002	0.101 ± 0.003	0.068 ± 0.003	0.085 ± 0.005	0.059 ± 0.003**	0.085 ± 0.005	0.063 ± 0.004	0.069 ± 0.005**
Relative (mg/g)	0.23 ± 0.01	0.31 ± 0.02	0.22 ± 0.01	0.33 ± 0.01	0.23 ± 0.01	0.29 ± 0.02	0.23 ± 0.01	0.33 ± 0.02	0.25 ± 0.02	0.27 ± 0.02

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
EE = ethinyl estradiol; RPC = reproductive performance cohort; PC = prenatal cohort.
[a]Data displayed as mean ± standard error of the litter means.
[b]Statistical analysis for the RPC performed using the Jonckheere (trend) and Williams or Dunnett (pairwise) tests. Statistical analysis for the PC performed using mixed models with a random effect for litter and a Dunnett-Hsu adjustment for multiple pairwise comparisons.
[c]The EE group was not included in any trend analysis; it was included in the pairwise analysis to the vehicle control group.
[d]The terminal body weight for the prenatal females is the final body weight minus the gravid uterine weight.
[e]Relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body weight.
[f]n = 19 for the 10,000 ppm group in the RPC. The decrease is due to one female's value being excluded because it was an outlier.

F_2 Necropsy

Pups were euthanized on PND 28; gross pathology findings are reported in Appendix E. One male each in the 3,000 and 30,000 ppm 2H4MBP groups exhibited bilateral undescended testes. Three males each in the 3,000 and 10,000 ppm 2H4MBP groups exhibited unilateral undescended testes. Several females in the 30,000 ppm 2H4MBP group displayed dilated, discolored, or enlarged kidneys consistent with what was observed in adults. Diaphragmatic hernias were observed in three males in the 30,000 ppm 2H4MBP group and in one male in the EE group (Appendix E). Diaphragmatic hernias were also observed in 2H4MBP- or EE-exposed F_1 rats in the reproductive performance cohort (Appendix E). The collective EE group had two males with diaphragmatic hernias. No hernias were observed in control animals or in the F_0 females (Appendix E). These hernias consist of a small protrusion of the liver through the diaphragm and are sometimes recorded grossly as diaphragmatic hernias and sometimes as hepatodiaphragmatic hernias.

Pathology

This section describes the statistically significant or biologically noteworthy changes in the incidences of nonneoplastic lesions. Summaries of the incidences of nonneoplastic lesions mentioned in this section are presented as supplemental data in Appendix E.

Kidney: The kidney was the primary target of 2H4MBP exposure (Table 32; Appendix E). In the F_1 reproductive performance cohort, the incidences of renal tubule epithelial regeneration were significantly increased in the 30,000 ppm males and females relative to their respective control groups; a higher incidence of this lesion was also noted in the 10,000 ppm females. When compared to control animals, both male and female rats exposed to 30,000 ppm had significantly increased incidences of interstitial chronic active inflammation, renal tubule concretions, renal tubule dilation, urothelial hyperplasia, and urothelial ulcers. In the F_1 reproductive performance cohort, pelvic concretion and papillary necrosis was significantly increased compared to control animals in the 30,000 ppm males, and there was a positive trend for pelvic concretion and papillary necrosis in the females. F_1 females in the reproductive performance cohort also had significantly increased incidences of renal tubule epithelial degeneration (30,000 ppm), chronic progressive nephropathy (3,000 and 10,000 ppm), and mineralization (3,000 and 10,000 ppm) compared to control animals, and there was a positive trend for pelvic dilation. Renal lesions were also observed in the F_0 and other cohorts (see below).

Interstitial chronic active inflammation was characterized by a mixture of inflammatory cell types, including neutrophils, lymphocytes, and macrophages, with some fibrosis. This lesion was distinct from the interstitial infiltrates of mononuclear cells that accompanies chronic progressive nephropathy. When the renal papilla was necrotic, it was frequently no longer visible in the section of tissue, with just eosinophilic amorphous material present where the tip of the papilla should be. When the necrotic papilla was still present in the section, it was characterized by a pale, washed out, eosinophilic color and lack of cellular detail. Renal tubule dilation was the most frequently observed change in the kidneys of male and female rats and was frequently accompanied by intratubular accumulations of round or angular pale-brown to red-brown material, often with a laminated appearance. These renal tubular concretions were similar to the pelvic concretions. Other dilated renal tubules contained proteinaceous casts, characterized by homogenous, bright eosinophilic material, or cell debris. Renal tubule dilation was generally a

focal change, most often involving the poles of the kidney, which affected the entire length of the nephron. The epithelium lining the dilated tubules was flattened and frequently showed evidence of degeneration (females) or regeneration (males and females).

Renal tubule epithelial degeneration was characterized by the absence of epithelial cells or the presence of individual necrotic epithelial cells, whereas renal tubule epithelial regeneration was characterized by plump epithelial cells with basophilic cytoplasm that projected into the tubular lumen. Regeneration most likely occurred after degeneration, and the lack of observed degeneration in the males might imply a quicker onset or a more severe course of renal tubular epithelial degeneration in male rats relative to female rats. Urothelial hyperplasia consisted of an increased number of cell layers of the epithelium lining the renal pelvis and occurred as either a focal (males) or diffuse (males and females) change. The severity of the lesion was based on the thickness of the hyperplasia as well as on the amount of pelvis involved, with focal lesions being less severe than those involving the entire renal pelvis (diffuse). Urothelial hyperplasia was usually of minimal to mild severity, but in one female rat, moderate urothelial hyperplasia was accompanied by squamous metaplasia of the urothelium. Ulceration of the urothelium was characterized by a focal area devoid of epithelium. Roughly half of the animals with ulcers of the urothelium also had urothelial hyperplasia. One male rat had necrosis of the urothelium; focal necrosis typically develops into an ulcer as the necrotic epithelium is sloughed off. Pelvic dilation was characterized by an increased space between the renal papilla and the renal pelvis. In most cases, papillary necrosis was evidenced by the absence of the tip of the papilla and accumulations of pale, eosinophilic material where the tip of the papilla should be. Occasionally, the tip of the papilla was still in place but was pale and lacked nuclear detail. Most occurrences of chronic progressive nephropathy were of minimal or mild severity; minimal nephropathy consisted of basophilic tubules with a thickened basement membrane, whereas mild cases of nephropathy typically also had tubular proteinaceous casts and mixed mononuclear cell inflammation within the interstitium. Mineralization was characterized by small focal deposits of deeply basophilic granular material, typically along the corticomedullary junction; evidence of minimal secondary renal tubule necrosis was occasionally associated with mineral deposition but not recorded separately.

The various renal lesions associated with exposure to 30,000 ppm 2H4MBP were consistent with an obstructive nephropathy. Obstructive nephropathy occurs when something restricts the outflow of urine, such as crystals, with subsequent inflammation or a lower urinary tract blockage. Retrograde nephropathy, which is a form of obstructive nephropathy, is due to urine backflow into the kidney, causing tubule dilation that ascends from the papilla to the cortex.[96; 97]

F_0 females, F_1 males in the prenatal cohort, and F_2 males and females were also necropsied; however, only lesions that were grossly visible at the time of necropsy were examined histologically. Only one F_1 female from the prenatal cohort, a 3,000 ppm 2H4MBP group animal, was examined histologically, and there were no gross or histological lesions of the kidney. In the F_0 females, 0, 0, 1, and 7 animals from the 0, 3,000, 10,000, and 30,000 ppm 2H4MBP groups had gross lesions, and 0, 0, 0, and 3 had gross lesions of the kidneys, respectively. The three F_0 females in the 30,000 ppm group had pale kidneys observed at necropsy; this observation was associated histologically with various kidney lesions, including renal tubule dilation, renal tubule epithelial regeneration, interstitial chronic active inflammation, papillary necrosis, and urothelial hyperplasia. In F_1 males from the prenatal cohort, 2, 2, 2, and 15 animals from the 0, 3,000, 10,000, and 30,000 ppm 2H4MBP groups had gross lesions, of

which 0, 0, 2, and 13 animals had gross lesions of the kidneys, respectively. Gross lesions included enlarged and discolored kidneys in the 30,000 ppm group and dilated pelvis in the 10,000 ppm group. Histologically, the kidneys from the 30,000 ppm group had papillary necrosis and pelvic concretions, renal tubule dilation and concretions, renal tubule epithelial regeneration, and hyperplasia and ulceration of the urothelium; the kidneys from females in the 10,000 ppm group had pelvic dilation.

In the F_2 males, 2, 5, 6, and 6 animals from the respective 0, 3,000, 10,000, and 30,000 ppm 2H4MBP groups had gross lesions, of which 1, 0, 3, and 0 had gross lesions of the kidneys. Gross lesions included discoloration and pelvic dilation, which were seen histologically as congestion and pelvic dilation. In the F_2 females, 2, 1, 0, and 8 animals had gross lesions from the respective 0, 3,000, 10,000, and 30,000 ppm 2H4MBP groups, of which 1, 0, 0, and 7 had gross lesions of the kidneys. Histological findings associated with these gross findings included renal tubule and pelvic dilation (Appendix E).

Table 32. Incidences of Nonneoplastic Lesions of the Kidney in Adult F_1 Male and Female Rats in the Reproductive Performance Cohort Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed[a]

	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.5 ppm
Male[b]	41 (22)	40 (20)	40 (21)	40 (20)	0 (15)
Renal tubule, epithelium, regeneration[c]	0**	0	0	33 (17)** [1.2][d]	–[e]
Interstitium, inflammation, chronic active	0**	0	0	22 (14)** [1.7]	–
Renal tubule, concretion	0**	0	0	35 (19)** [1.4]	–
Pelvis, concretion	0**	0	0	17 (13)** [1.5]	–
Renal tubule, dilation	0**	0	0	37 (20)** [1.5]	–
Urothelium, hyperplasia, total	0**	1 (1) [1.0]	0	18 (15)** [1.3]	–
Urothelium, ulcer	0**	0	0	12 (9)** [1.0]	–
Papilla, necrosis	0**	0	0	10 (10)** [1.3]	–
Female	35 (22)	37 (20)	33 (20)	32 (20)	0 (15)
Renal tubule, epithelium, regeneration	0**	0	3 (3) [1.0]	13 (12)** [1.5]	–
Interstitium, inflammation, chronic active	0**	0	0	8 (8)* [1.4]	–
Renal tubule, concretion	0**	0	0	13 (12)** [1.4]	–
Pelvis, concretion	0**	0	0	9 (5) [1.0]	–
Renal tubule, dilation	0**	0	0	28 (19)** [1.4]	–
Urothelium, hyperplasia, diffuse	0**	0	0	15 (12)** [1.3]	–
Urothelium, ulcer	0**	0	0	6 (6)* [1.0]	–
Papilla, necrosis	0*	0	0	4 (3) [1.0]	–
Renal tubule, epithelium, degeneration	0**	0	0	21 (14)** [1.1]	–
Pelvis, dilation, total	0*	1 (1) [3.0]	0	5 (5) [2.0]	–
Chronic progressive nephropathy	18 (14) [1.1]	35 (19)** [1.1]	29 (19)** [1.0]	22 (17) [1.0]	–
Mineralization	9 (8) [1.0]	28 (17)** [1.0]	24 (18)** [1.0]	10 (8) [1.2]	–

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
EE = ethinyl estradiol.
[a]Statistical analysis performed using the Cochran-Armitage test with a Poly-3 adjustment for age and a Rao-Scott modification for the random effect due to litter.
[b]Number of animals (number of litters) with tissue examined microscopically.
[c]Number of animals (number of litters) with lesion.
[d]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.
[e]Nonneoplastic lesions were not evaluated in the EE group.

Urinary Bladder: In F_1 males from the reproductive performance cohort exposed to 30,000 ppm 2H4MBP, there was an increase, although not significant, in the incidences of urinary bladder concretions (Appendix E). Most of these animals had gross observations of brown discoloration in the urinary bladder.

Liver: Hepatodiaphragmatic hernias (HDN) occurred at a low incidence in the 10,000 and 30,000 ppm males and females and in the 3,000 ppm females in the F_1 reproductive performance cohort (0, 0, 1, 1 for the 0, 3,000, 10,000, and 30,000 ppm males, respectively; 0, 2, 1, 4 for the 0, 3,000, 10,000, and 30,000 ppm females, respectively). Although none of the incidences was statistically different from control animals, no occurrences of HDN were observed in either the male or female control groups (Table 33). All but two of the HDNs (one in the 10,000 ppm males and one in the 30,000 ppm females) correlated with gross observations of diaphragmatic hernias at necropsy. HDNs were rounded protrusions of the liver that were histologically similar to normal liver.

Table 33. Incidences of Diaphramatic Hernias and Hepatodiaphragmatic Hernias in Adult F_1 Male and Female Rats in the Reproductive Performance Cohort and F_2 Male Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed

	0 ppm	3,000 ppm	10,000 ppm	30,000 ppm	EE 0.5 ppm
F_1 Male					
Diaphragm, hernia[a]	0 41 [22][c]	0 40 [20]	0 40 [21]	1 (1)[b] 40 [20]	1 (1) 30 [15]
Hepatodiaphragmatic hernia[d]	0 41 [22][e]	0 40 [20]	1 (1)[b] 40 [21]	1 (1) 40 [20]	1 (1) 2 [15]
F_1 Female					
Diaphragm, hernia	0 41 [22]	2 (2) 40 [20]	1 (1) 40 [21]	3 (3) 40 [20]	0 30 [15]
Hepatodiaphragmatic hernia	0 35 [22]	2 (2) 2 [20]	1 (1) 1 [20]	4 (3) 32 [20]	0 0 [15]
F_2 Male					
Diaphragm, hernia	0 91 [30]	0 110 [34]	0 101 [32]	3 (3) 88 [30]	1 (1) 87 [27]

EE = ethinyl estradiol.
[a]No statistical analysis was performed.
[b]Number of animals with lesion (number of litters).
[c]Number of animals examined for gross lesions [number of litters].
[d]Statistical analysis performed using the Cochran-Armitage test with a Poly-3 adjustment for age and a Rao-Scott modification for the random effect due to litter.
[e]Number of animals with tissue examined microscopically [number of litters].

Preputial Gland: There was a significant increase in the incidence of preputial gland, duct ectasia in F_1 males in the reproductive performance cohort exposed to 30,000 ppm 2H4MBP (Appendix E). This lesion consists of a dilation of the ducts of the preputial gland and is a common background change seen in rats, especially as they age. In its most severe form, ectatic ducts become cystic or even rupture, inciting a marked inflammatory reaction. The average severities of these lesions were between minimal and mild in the control group and exposed groups. The biological importance of this lesion is unknown.

Discussion

The objective of the present study was to characterize the potential for 2-hydroxy-4-methoxybenzophenone (2H4MBP), a common component of sunscreen and personal care products, to adversely affect any phase of rat development, maturation, and ability to reproduce. Mechanistic screening studies have shown that 2H4MBP and its metabolites are capable of activating the estrogen receptor and antagonizing the androgen receptor to varying degrees.[98; 99] In this study, Sprague Dawley (Hsd:Sprague Dawley® SD®) rats were exposed to 2H4MBP in 5K96 feed, using the National Toxicology Program (NTP) modified one-generation (MOG) study design. As disposition is similar following oral and dermal exposure, 2H4MBP exposure via the diet was selected for this study, rather than topical application, to sustain internal exposure; if applied topically, internal dose would be influenced by intra- and interanimal grooming behavior. To minimize the potential endocrine activity of phytoestrogens that are often present in rodent diets, 5K96 feed was used because it provides a diet low in phytoestrogens. This report complements ICH[c] S5r2 guideline studies (fertility and early embryonic development, embryo-fetal development, and pre- and postnatal developmental studies in rats) on 2H4MBP[53] conducted by the U.S. Food and Drug Administration's National Center for Toxicological Research (NCTR), an interagency NTP partner, and allows for the comparison of study designs and outcomes.

Exposure concentration selection was informed by a dose range-finding study that demonstrated that 25,000 ppm was well tolerated in pregnant rats and did not affect parturition, litter size, or pup viability. In that study, pup body weights of the 25,000 ppm group were significantly decreased compared to the control group, suggesting potential growth retardation; this response was severe at the 50,000 ppm exposure concentration and viable litter size was also affected. Therefore, 30,000 ppm was selected as the highest exposure concentration for the MOG study. The exposure concentrations of 3,000 and 10,000 ppm were selected to aid in identifying potential exposure concentration-response relationships. This spacing would ideally avoid significant overlap of the respective mg 2H4MBP/kg body weight (mg/kg) exposure concentrations, recognizing that the amount of feed consumed is dependent on pregnancy state (e.g., prior to mating versus lactation), sex, and age. Because 2H4MBP has been reported to induce estrogen-like activity, a low exposure concentration (0.05 ppm) of ethinyl estradiol (EE), a synthetic form of estrogen, was included as a positive control group. NTP studies have shown that comparing plasma concentrations of 2H4MBP in rats following feed exposure of 3,000–30,000 ppm to plasma concentrations in humans following repeated dermal application of 20 g/m^2 revealed rat-to-human dose multiples of 0.1 to 4.[19] Collectively, these data demonstrate similar external (5- to 57-fold) and internal (0.1- to 4-fold) exposure of 2H4MBP in rats and humans.

Exposure of F_0 females to 2H4MBP or EE via the diet began on gestation day (GD) 6 (implantation). F_1 offspring were exposed to 2H4MBP or EE at the same exposure concentration as their respective dams. Upon weaning, F_1 offspring in each group were randomly assigned to one of three cohorts: (1) reproductive performance cohort (2/sex/litter), (2) prenatal cohort

[c]ICH = International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use.

(1/sex/litter), and (3) biological sampling cohort (1/sex/litter). Upon sexual maturity, nonsibling F_1 rats allocated to the prenatal and reproductive performance cohorts were paired for mating to evaluate reproductive performance and F_2 prenatal and postnatal development. The likelihood of identifying potential 2H4MBP-induced adverse effects (similarity and magnitude thereof) at any phase of growth or development was increased by examining related endpoints in multiple pups within a litter during both preweaning and postweaning periods.

The concentrations of free (unconjugated compounds) and total (free and all conjugated forms) 2H4MBP, 2,4-dihydroxybenzophenone (DHB), 2,3,4-trihydroxybenzophenone (THB), and 2,5-dihydroxy-4-methoxybenzophenone (D2H4MBP) were quantified in plasma from the biological sampling cohort at postnatal days (PNDs) 28 and 56.[64] Free plasma 2H4MBP and DHB concentrations were similar to each other and increased with increasing exposure concentration, with no age or sex differences except in the 10,000 ppm group, as concentrations of both analytes were significantly increased in PND 56 animals relative to PND 28 animals. Free D2H4MBP and THB were not detected in these animals. The concentrations of total 2H4MBP and DHB were higher (approximately 100- to 300-fold) than the free 2H4MBP and DHB concentrations, demonstrating extensive conjugation of 2H4MBP and its metabolites. The rank order of the total concentrations was 2H4MBP ≈ DHB > D2H4MBP >> THB. Free and total analyte plasma concentrations were not sex-dependent in either PND 28 or PND 56 pup plasma.

In the current MOG study, 2H4MBP exposure was associated with lower F_1 and F_2 mean body weights (8%–24%). Lower preweaning F_1 pup mean body weights have also been observed in CD-1 mice exposed to 2H4MBP.[41] The lower F_1 body weights observed postweaning to sexual maturity were not associated with lower feed consumption. Pregnant F_0 females and females in both F_1 cohorts exposed to 2H4MBP also did not display decreases in gestational or lactational feed consumption. Collectively, this suggests that 2H4MBP could have altered utilization of the consumed diet (and, thus, affected growth) and could have reduced or delayed preweaning growth. The observed lower mean body weights of the 2H4MBP groups, in the absence of effects on feed consumption, is consistent with findings reported in Fischer 344 (F344)/N rats administered 25,000 ppm 2H4MBP.[40]

2H4MBP did not result in any significant effects on mating, pregnancy, or littering indices, nor did it result in adverse histopathological findings in the testis or changes in sperm parameters at concentrations up to 30,000 ppm. These observations contrast with those reported in the NTP Reproductive Assessment by Continuous Breeding study[41] in CD-1 mice, in which 2H4MBP was associated with smaller litter sizes and decreases in pup viability. In a previous study, 50,000 ppm was associated with lower sperm density in both F344/N rats and mice. No effects on sperm parameters were apparent at the next lower exposure concentration (12,500 ppm) in rats, however, percent of sperm cell abnormalities were significantly increased in mice at this exposure concentration.[40] These findings were collectively attributed to stress-induced toxicity, potentially by affecting metabolism or digestive processes, as evidenced by lower mean body weights. Chronic stress is known to affect rat spermatogenesis.[100; 101] The absence of similarly robust effects on sperm parameters and reproductive performance in the current study might reflect strain and stock differences. Alternatively, it is possible that if higher 2H4MBP exposure concentrations could have been used in this study, a similar magnitude of response to that observed in the CD-1 mouse and F344/N rat in previous studies may have been observed, either as a stress-related response or as a more direct effect from 2H4MBP exposure.

Examining data across cohorts in the 30,000 ppm group, mean numbers of corpora lutea and F_2 implants on GD 21 were significantly decreased (3.7 and 2.7, respectively) relative to the control group, and the mean number of live fetuses was also lower (1.7). Mean live F_2 litter size on PND 0 was significantly decreased (1.5 pups) in the reproductive performance cohort, and F_1 live litter size on PND 0 was also slightly lower (<1 pup). These observations suggest that 2H4MBP exposure might have affected litter size, although the magnitude of this effect was small. The slightly smaller litter size might have been due to a direct effect (the decrease of the number of ova ovulated, as evidenced by the lower number of corpora lutea enumerated in the prenatal cohort) or an indirect effect of a stress-induced response (reflected in the lower mean body weights). 2H4MBP, administered at 50,000 ppm in the diet from GD 6 through PND 23, has been shown to delay follicular development, but this was not observed at 25,000 ppm.[18] 2H4MBP has also been shown to affect early follicular assembly in rat ovary cultures.[102] Thus, the observed decrease in corpora lutea is consistent with alterations in follicular development.[18] No subsequent 2H4MBP-related effects on live litter size were observed. Collectively, given the minimal apparent response that may or may not be a direct effect of 2H4MBP, this was considered equivocal evidence of an adverse effect on reproductive performance.

EE exposure did not affect F_1 live litter size on PND 0; however, mean live F_2 litter size on PND 0 in the reproductive performance cohort and the mean number of live F_2 fetuses per litter on GD 21 in the prenatal cohort were both significantly decreased (approximately 2–3 pups per litter) relative to the control group. Fewer corpora lutea and total F_2 implants were observed in the EE prenatal cohort. Rat follicular development has been shown to be affected by EE (200 µg/kg) when exposed on PND 0 and examined on PND 21.[103] F_2 live litter size on PND 0 through PND 4 in the EE F_2 reproductive performance cohort was significantly decreased relative to the control group (approximately 2 pups per litter) in part because 3 of the 18 EE litters had 0 pups. After litter standardization on PND 4, survival in the EE group appeared higher than in the control group, but this was likely the result of several control litters that exhibited excessive pup loss. In a previously conducted multigenerational study, EE exposure at 0.05 ppm was not reported to significantly decrease (or increase) the number of live pups born.[93] Upon inspection of the NCTR study data, however, there is an apparent minimal nonsignificant decrease in mean live born (approximately 1 pup per litter) that is consistent with what was observed in the EE group in the current study.[93] A similar decrease in number of implants was observed in the NCTR Segment 1 study.[104]

Progressively lower relative preweaning F_1 body weights were observed in males and females exposed to 30,000 ppm 2H4MBP. On PND 4, both males and females displayed significantly decreased mean body weights of approximately 12%–14%, relative to the control group, and by PND 28, body weights of both males and females were significantly decreased by approximately 24%. In contrast, F_2 males and females did not exceed a 10% lower relative body weight until PND 25 and PND 28, respectively. The reason for this difference in F_1 versus F_2 generational response is unclear, but it could be related to increased 2H4MBP metabolism in the F_1 dams resulting from sustained 2H4MBP exposure. The no-observed-effect level (NOEL) for 2H4MBP-related effects on body weight is 3,000 ppm based on lower body weights in both sexes in both generations. The considerable effects on body weights associated with exposure to 2H4MBP were considered some evidence of developmental toxicity.

2H4MBP did not accelerate vaginal opening (VO), as would be expected if it displayed estrogenic activity, consistent with the expected robust acceleration of VO that was observed

with EE. The day of VO attainment was delayed in the 30,000 ppm group, and body weights on day of acquisition were similar to those of the control group. When weaning weight was used as a covariate, addressing growth retardation, the apparent delay was mitigated. A similar VO delay, concomitant with lower mean body weight, has been reported for corticosterone administered in drinking water.[105] Intrauterine growth retardation—after ligation of the uterine artery on GD 17 and resulting in 16% lower body weight on PND 2 and lower postnatal body weights relative to the control group—has been shown to delay VO.[106] Postnatal dietary restriction also has been shown to delay VO, with similar body weights relative to the control group at time of VO.[107] The lower PND 4 pup and postnatal mean body weights and the delay in VO observed in the current study are consistent with these findings.

2H4MBP exposure did not significantly alter any apical androgen-sensitive endpoints, demonstrating that it does not appear to affect androgen-mediated lengthening of anogenital distance or advancement of balanopreputial separation (BPS). 2H4MBP did not affect areola/nipple retention at the tested concentrations, indicating an absence of androgen-receptor antagonism. BPS was delayed in the 10,000 and 30,000 ppm 2H4MBP groups, as well as in the 0.05 ppm EE positive control group. Similar to VO, body weights on day of acquisition were comparable to those of the control group, and, when adjusted for weaning weight, there were also no differences relative to the control group. Intrauterine growth retardation and postnatal feed restriction, resulting in lower postnatal body weights, have been shown to delay BPS.[106] It is plausible that, like VO, the similar weights on day of attainment observed in the current study suggest a weight or body mass requirement for the attainment of BPS.

Diaphragmatic hernias were observed at a low incidence in 2H4MBP-exposed animals in both the F_1 and F_2 generations but were not observed in any control animals. They were also not observed in control animals in two other MOGs (EHMC and BPAF).[108; 109] This finding was also observed in the male F_1 and F_2 EE groups. Diaphragmatic hernias have been shown to be induced by 2,4-dichlorophenyl-p-nitrophenyl ether, which displays some similarity to 2H4MBP.[110; 111] The presence of gross diaphragmatic hernias correlated with histologic hepatodiaphragmatic hernias in all but two animals. Although these incidences occurred only in exposed groups, there was no exposure response and no pairwise significance, and they have been observed in control groups in other developmental and reproductive toxicity studies. Therefore, it is unclear whether the occurrence of diaphragmatic and hepatodiaphragmatic hernias were related to 2H4MBP exposure.

No malformations observed at adult necropsy were consistent with perturbation of normal androgen-receptor-mediated development (grossly normal prostate, seminal vesicles, and epididymis). There was, however, a single incidence of hypospadias, a lesion commonly seen when androgen action is attenuated.[112; 113] Given the singular incidence and the absence of corresponding changes in androgen-dependent processes, the hypospadias was likely not related to 2H4MBP exposure. In F_1 adult males in the reproductive performance cohort, the weights of androgen-dependent reproductive tissues (testes, epididymides, ventral prostate gland) and levator ani/bulbocavernosus muscle complex were all slightly lower in the 30,000 ppm group compared to the control group. All of those organ weight changes occurred concurrently with lower body weights, however, and are likely secondary to the apparent growth retardation. Moreover, there were no apparent 2H4MBP-related histopathological findings in the reproductive tissues, nor was the ability of males to successfully mate and induce pregnancy adversely affected in either the prenatal or reproductive performance cohorts. Sperm and

spermatid counts, which are androgen-sensitive endpoints, were also not affected. In totality, unlike what has been reported in cell models, 2H4MBP exposure had no apparent effect on androgen-receptor-dependent processes, nor did it affect mating or pregnancy indices.

2H4MBP exposure was associated with greater kidney weights and histologic lesions consistent with obstructive nephropathy, including renal tubule epithelial regeneration, renal tubule degeneration (females only), interstitial chronic active inflammation, renal tubule and pelvic concretions, renal tubule dilation, papillary necrosis, urothelial hyperplasia, and urothelial ulcers. In addition, increased chronic progressive nephropathy, pelvic dilation, and renal mineralization were present in females. These findings are consistent with renal effects previously reported following subchronic exposure[40] and those observed with chronic exposure.[34] F_1 males and females exposed to 2H4MBP also displayed greater liver weights. This finding is consistent with the fetal malformation finding of enlarged liver. The absolute weights of the adrenal glands were significantly decreased in the female 30,000 ppm reproductive performance cohort. Chronic stress would be expected to increase corticosterone levels and result in lower adrenal gland weights due to negative feedback; however, sustained elevated adrenocorticotropic hormone (or equivalent) would be expected to increase both adrenal gland weight and the levels of corticosterone.[114] The NOEL for adult general toxicity necropsy findings is 3,000 ppm based on histopathological findings in the urinary system consistent with chronic obstructive nephropathy.

There was no effect of 2H4MBP exposure on the incidence of fetal skeletal abnormalities. Fetal findings were limited to an increase in the incidences of hydronephrosis of the kidney and enlarged liver in the 30,000 ppm group. A relatively high background incidence was found in this strain of rat for hydronephrosis (fetus incidence and range: 4/1,385 and 0.00%–0.81%), along with dilated renal pelvis (fetus incidence and range: 6/1,385 and 0.00%–1.06%), distended ureter (fetus incidence and range: 151/1,385 and 4.83%–15.36%), and hydroureter (fetus incidence and range: 11/1,385 and 0.17%–2.83%). Moreover, the background incidence of some findings (e.g., dilated renal pelvis and/or ureter) could be greater in fetuses than in pups, suggesting that these changes might be transient.[18; 115; 116] At necropsy of the F_2 offspring on PND 28, dilation of the renal pelvis was observed grossly in six rats in the 30,000 ppm group and in one F_2 rat in the control group. No incidences of hydronephrosis were observed in F_2 pups at necropsy; nevertheless, the observed fetal findings are consistent with the finding that the kidney and liver are target tissues for 2H4MBP-mediated toxicity.

In the current study, 2H4MBP exposure was associated with minimal apparent responses on litter size (fetal or PND 0) and fewer corpora lutea. A similar decrease in the numbers of corpora lutea and implants has also been observed at 30,000 ppm in the NCTR fertility and early embryonic development study, in which female dosing started two weeks prior to cohabitation through GD 6. No apparent responses were observed in the NCTR embryo-fetal toxicity study in which dosing is for a shorter duration (GD 6–15).[93] If 2H4MBP-related, this difference in response may be the result of the longer duration of exposure. The observed EE exposure-related decreases on PND 0 live F_2 litter size in the reproductive performance cohort, and GD 0 in the prenatal cohort (as well as total number of implants) is consistent with what has been observed in the 0.05 ppm EE group in the NCTR fertility and early embryonic development study.[93] These similarities demonstrate the consistency of responses observed with conducting a single study versus conducting three independent studies that would necessitate the use of more animals.

Conclusions

Under the conditions of this modified one-generation (MOG) study, there was *equivocal evidence of reproductive toxicity* of 2-hydroxy-4-methoxybenzophenone (2H4MBP) in Hsd:Sprague Dawley® SD® rats based on a decrease in F_2 litter size in both the prenatal and reproductive performance cohorts.

Under the conditions of this MOG study, there was *some evidence of developmental toxicity* of 2H4MBP in Hsd:Sprague Dawley® SD® rats based on the observed postnatal growth retardation. The relationship of the increased occurrence of diaphragmatic and hepatodiaphragmatic hernias in F_1 adults and F_2 pups to 2H4MBP exposure is unclear.

Exposure to 2H4MBP was not associated with signals consistent with alterations in estrogenic, androgenic, or antiandrogenic action. Exposure to 2H4MBP was associated with lower F_1 and F_2 mean body weights; this effect on body weight contributed to the apparent 2H4MBP-related decreases in male reproductive organ weights. Mating and littering were not significantly affected by 2H4MBP exposure. Exposure to 2H4MBP was associated with nonneoplastic kidney lesions in the F_0, F_1, and F_2 generations. Expected estrogenic responses were observed in the EE group.

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Appendix A. Chemical Characterization and Dose Formulation Studies

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A.1. Procurement and Characterization

A.1.1. 2-Hydroxy-4-methoxybenzophenone

2-Hydroxy-4-methoxybenzophenone (2H4MBP) was obtained from Ivy Fine Chemicals (Cherry Hill, NJ) in a single lot (20100801), which was used for the dose range-finding and modified one-generation (MOG) studies. Identity, purity, and stability analyses were conducted by the analytical chemistry and study laboratory at Battelle (Columbus, OH). Reports on analysis performed in support of the 2H4MBP studies are on file at the National Institute of Environmental Health Sciences.

Lot 20100801 of the chemical was a light-yellow powder. The lot identity was confirmed using infrared (IR) spectroscopy and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The IR spectrum (Figure A-1) was in good agreement with the anticipated structure and the reference spectrum (BP #824 from the Sadtler Basic Monomers and Polymers Library [Bio-Rad Laboratories, Hercules, CA]). Reference ^{1}H and ^{13}C NMR spectra for 2H4MBP were obtained from the National Institute of Advanced Industrial Science and Technology (NIAIST) (Tokyo, Japan) Spectral Database for Organic Compounds (SDBS No. 5800HSP-01-137 and 5800CDS-04-696, respectively). The Advanced Chemistry Development (ACD, Toronto, Canada) HNMR spectral prediction program (Version 12.01) was also used to predict these NMR spectra. Both the ^{1}H and ^{13}C NMR spectra obtained for lot 20100801 were consistent with these references. Additionally, a ^{1}H-^{1}H correlated spectroscopy (COSY) two-dimensional spectrum, Distortionless Enhancement by Polarization Transfer (DEPT) ^{13}C spectral series, and ^{1}H-^{13}C heteronuclear multiple quantum coherence (HMQC) two-dimensional spectrum collected for lot 20100801 were in good agreement with the anticipated spectra for 2H4MBP.

The purity of 2H4MBP lot 20080801 was determined using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, as well as gas chromatography (GC) with flame ionization detection (FID). The HPLC/UV analysis showed a single impurity with a peak area <0.1%, indicating an 2H4MBP purity of approximately 100.0%. The chromatogram obtained from GC/FID consisted of a single major peak consistent with a purity of 100.0%. Lot 20080801 was screened for common residual volatile solvents using GC with electron capture detection (ECD) and FID; no significant volatile impurities were found. Differential scanning calorimetry (DSC) was also used to determine the purity of the test article. Analysis using a PerkinElmer (Shelton, CT) diamond DSC yielded a purity of 99.9% with a melting point of approximately 62°C. In addition, Karl Fisher titration of 2H4MBP lot 20080801 was conducted to estimate moisture content, which was found to be insignificant (<0.5%) in an analysis conducted by Galbraith Laboratories, Inc. (Knoxville, TN). Thus, the overall purity of 2H4MBP lot 20100801 was determined to be >99.9%. Additional details on the chromatography systems used are provided in Table A-1.

Although the entirety of 2H4MBP came from lot 20100801, the chemical was received in eight drums (25 kg each) and not homogenized. Homogeneity analysis conducted on three samples taken during chemical handling using HPLC/UV found that the samples were statistically equivalent to the purity of the standard.

To ensure stability, the test chemical was stored in sealed amber glass bottles at room temperature (approximately 25°C). Periodic analysis of 2H4MBP lot 20100801 by the study

laboratory using HPLC/UV showed no degradation of the bulk 2H4MBP chemical prior to and during the animal studies relative to a frozen reference sample.

A.1.2. Ethinyl Estradiol

Ethinyl estradiol (EE) was obtained in a single lot (090M1241V) from Sigma-Aldrich (St. Louis, MO) via Government Scientific Source, Inc. (Reston, VA). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at Battelle (Columbus, OH).

EE lot 090M1241V was a white powder. The lot identity was confirmed using IR spectroscopy and ^{1}H and ^{13}C spectroscopy. The IR spectrum (Figure A-2) was consistent with the available reference spectrum in the Sadtler Steroids, Androgens, Progestins, and Estrogens Library (Bio-Rad Laboratories, Hercules, CA). Reference ^{1}H and ^{13}C NMR spectra for EE were obtained from the NIAIST (Tokyo, Japan) Spectral Database for Organic Compounds. The ACD (Toronto, Canada) spectral prediction program (Version 12.01) was also used to predict these NMR spectra. Both the ^{1}H and ^{13}C NMR spectra obtained for lot 09M1241V were consistent with these references. Additionally, a ^{1}H-^{1}H COSY two-dimensional spectrum, DEPT ^{13}C spectral series, and ^{1}H-^{13}C HMQC two-dimensional spectrum collected for lot 09M1241V were in good agreement with the anticipated spectra for EE. Elemental analysis indicated that the sample was approximately 80.4% carbon, 11.5% oxygen, 7.9% hydrogen, and >0.5% nitrogen, which is consistent with theoretical values.

Purity assessment by HPLC/UV showed one impurity with a relative area of 0.23% of the total peak area, indicating an EE purity of 99.8% for lot 090M1241V. Analysis for volatiles using headspace GC/FID found that the sample contained approximately 0.023% acetone; no other volatiles were detected. DSC yielded a purity of 99.7% and a melting point of 184°C. Karl Fischer analysis indicated that the water content of lot 090M1241V was approximately 0.4%. These data indicate that the EE purity of lot 090M1241V was ≥99.7%, consistent with the manufacturer-reported purity of 99%. Additional details on the systems used are provided in Table A-1.

HPLC/UV analysis was used to determine the partition coefficient (log P_{ow}) for EE lot 090M1241V, and the average determined log P_{ow} was 1.2, which is approximately one-third of the published log P_{ow} value for EE of 3.7. However, calculation of the log P_{ow} against additional comparison hormones produced a log P_{ow} of 3.8, consistent with the published value.

To ensure stability, the EE positive control was stored in sealed glass containers at room temperature (approximately 25°C). Prior to the study and at study termination, lot 090M1241V was analyzed using HPLC/UV to ensure chemical stability.

A.2. Preparation and Analysis of Dose Formulations

A.2.1. 2-Hydroxy-4-methoxybenzophenone

Dosed feed formulations were prepared monthly (dose range-finding study) or eight times (MOG study) (Table A-2) using irradiated low-phytoestrogen feed (5K96 Casein diet). Formulations were stored at approximately 5°C for up to 42 days in amber glass bottles. Prior to beginning the study, the homogeneity of 1,000–50,000 ppm 2H4MBP formulations in 5K96 feed was confirmed using HPLC/UV. The analytical chemistry laboratory at Battelle (Columbus, OH)

conducted the homogeneity evaluation and all additional dose formulation analysis throughout the study.

Stability analysis was conducted on the 1,000 ppm formulation using HPLC/UV. When sealed and stored in amber plastic bags, the 2H4MBP formulations stored for 42 days at room temperature (approximately 25°C), refrigerated (approximately 5°C), or frozen (−20°C) were within 10% of the day 0 values. There was a slight declining trend in concentration (0.1%–0.2% per day) at all temperatures. To simulate conditions in the animal room, the 1,000 ppm formulation was stored in open glass containers with and without rodent urine and feces for 7 days; no significant loss in 2H4MBP was found when analyzed with HPLC/UV relative to the day 0 values. The preadministration dose formulations were analyzed three times over the course of the study (Table A-3) using HPLC/UV. All preadministration samples were within 10% of the targeted dose; the largest variation was a 10,000 ppm formulation that was 5.3% above the targeted dose. For one set of dose formulations, postadministration samples were collected from the animal room approximately one month after preparation. These formulations were within 10% of the target dose.

A.2.2. Ethinyl Estradiol

Dosed feed formulations were prepared eight times (Table A-2) using 5K96 feed. Formulations were stored at −20°C for <57 days in sealed amber plastic bags. The homogeneity of 0.05 ppm EE formulations in 5K96 feed was confirmed before conducting the studies.

Stability analysis conducted on the 0.05 ppm formulation found that it was stable for 57 days when stored in sealed amber plastic bags at −20°C and usable for 57 days when store in sealed amber plastic bags at approximately 5°C and room temperature. An animal room simulation of the 0.05 ppm formulation in open glass containers without rodent urine and feces for 8 days showed formulations were within 10% of the day 0 value; however, when urine and feces were present, a slight decline in EE occurred.

The preadministration dosed feed formulations were analyzed three times over the course of the dose range-finding study (Table A-3) and four times over the course of the MOG study (Table A-4) using HPLC/UV. All preadministration samples were within 10% of the target concentration with the exception of two formulations, one of which was that were 11% below and the other 12% above. Postadministration samples were collected from the animal room at the end of the exposure period and sent to Battelle (Columbus, OH) for analysis. The concentration of the animal room sample was within 10% of the preadministration analyses and, therefore, demonstrated acceptable stability during its use in the study.

Table A-1. Chromatography Systems Used in the Modified One-Generation Study of 2-Hydroxy-4-methoxybenzophenone

Chromatography	Detection System	Column	Mobile Phase
System A			
HPLC	UV (289 nm)	Phenomenex, Synergi Fusion RP; 100 × 4.6 mm, 4 μm	40/60 acetonitrile:ASTM Type I water; flow rate 1.2 mL/min
System B			
HPLC	UV (289 nm)	Phenomenex, Synergi Fusion RP; 100 × 3 mm, 2.5 μm	40/60 acetonitrile:ASTM Type I water; flow rate 0.8 mL/min
System C			
HPLC	UV (289 nm)	Phenomenex, Synergi Fusion RP; 100 × 3 mm, 2.5 μm	40/60 acetonitrile:ASTM Type I water; flow rate 0.8 mL/min
System D			
GC	FID	Restek, Rtx-5; 30 m × 0.32 mm, 1.0 μm film thickness	Helium; flow rate of ~3 mL/min
System E			
GC	FID; ECD	Restek, Rtx-624; 30 m × 0.53 mm, 3 μm film thickness	Helium; flow rate of ~5 mL/min
System F			
HPLC	UV (280 nm)	Phenomenex, Luna; 250 mm × 4.6 mm, 5 μm film thickness	50/50 acetonitrile:ASTM Type 1 water; flow rate 1.0 μL/min
System G			
HPLC	UV (205 nm)	Thermo, BDS Hypersil; 100 mm × 4.6 mm, 3 μm film thickness	65/35 acetonitrile:ASTM Type 1 water; flow rate 1 mL/min

HPLC = high-performance liquid chromatography; UV = ultraviolet; ASTM = American Society for Testing and Materials; GC = gas chromatography; FID = flame ionization detection; ECD = electron capture detection.

Table A-2. Preparation and Storage of Dose Formulations in the Modified One-Generation Study of 2-Hydroxy-4-methoxybenzophenone

Preparation
Stock solutions of 2H4MBP or EE were prepared by weighing the appropriate amount of lot 20100801 (2H4MBP) or lot 090M1241V (EE) into volumetric flasks and bringing to volume with methanol. Flasks were sealed and mixed well to ensure the test articles thoroughly dissolved. Irradiated 5K96 feed was weighed into amber glass bottles to which stock solution and methanol were added to create the proper 2H4MBP or EE concentration. Bottles were sealed and rotated end-over-end for 30 minutes to ensure homogeneity. Over the course of the study, eight dose formulations were prepared.
Chemical Lot Number
20100801 (2H4MBP) 090M1241V (EE)
Maximum Storage Time
42 days (2H4MBP) 57 days (EE)

Preparation
Storage Conditions
Stored in sealed amber glass bottles at approximately 5°C (2H4MBP) Stored in sealed amber plastic bags at −20°C (EE)
Study Laboratory
Battelle (Columbus, OH)

2H4MBP = 2-hydroxy-4-methoxybenzophenone; EE = ethinyl estradiol.

Table A-3. Results of Analyses of Dose Formulations Administered to Rats in the Dose Range-finding Study of 2-Hydroxy-4-methoxybenzophenone

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)[a]	Difference from Target (%)
2H4MBP				
June 30, 2011	July 8–9, 2011	3,000	3,010	0.0
		10,000	10,100	1.0
		25,000	25,100	0.0
		50,000	51,500	3.0
July 21, 2011	July 27–28, 2011	3,000	3,030	1.0
		10,000	10,100	1.0
		25,000	25,400	2.0
		50,000	50,400	1.0
August 29, 2011	September 1–2, 2011	3,000	2,980	−0.7
		10,000	9,980	−0.2
		25,000	25,600	2.0
		50,000	50,200	0.0
Animal Room Samples				
June 30, 2011	August 16–17, 2011	3,000	2,830	−5.8
		10,000	9,840	−1.6
		25,000	26,000	4.0
		50,000	49,100	−1.7
July 21, 2011	September 7–8, 2011	3,000	2,870	−4.3
		10,000	9,760	−2.4
		25,000	28,800	15.2
		50,000	51,600	3.3

2H4MBP = 2-hydroxy-4-methoxybenzophenone.
[a]Average of triplicate analysis.

Table A-4. Results of Analyses of Dose Formulations Administered to Rats in the Modified One-Generation Study of 2-Hydroxy-4-methoxybenzophenone

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)[a]	Difference from Target (%)
2H4MBP				
February 6, 2012	February 8–9, 2012	3,000	2,960	−1.3
		10,000	10,000	0.0
		30,000	30,100	0.3
April 16, 2012	April 20–21, 2012[b]	3,000	3,075	2.5
		10,000	10,225	2.3
		30,000	30,300	1.0
July 2, 2012	July 10–11, 2012[b]	3,000	3,020	0.7
		10,000	10,185	1.9
		30,000	31,583	5.3
Animal Room Samples				
February 6, 2012	March 22–23, 2012	3,000	2,990	−0.3
		10,000	9,600	−4.0
		30,000	31,300	4.3
EE				
February 3, 2012	February 10–19, 2012	0.05	0.0503	0.6
April 13, 2012	April 20–21, 2012	0.05	0.0488	−2.4
		0.05[c]	0.0449	−11.0
April 30, 2012	May 11–12, 2012	0.05[c]	0.0563	12.6
June 28, 2012	July 11–12, 2012	0.05	0.0448	−10.4
		0.05	0.0524	4.8

2H4MBP = 2-hydroxy-4-methoxybenzophenone; EE = ethinyl estradiol.
[a]Average of triplicate analysis.
[b]Average of two samples with triplicate analysis per sample.
[c]Not used due to an unacceptable concentration.

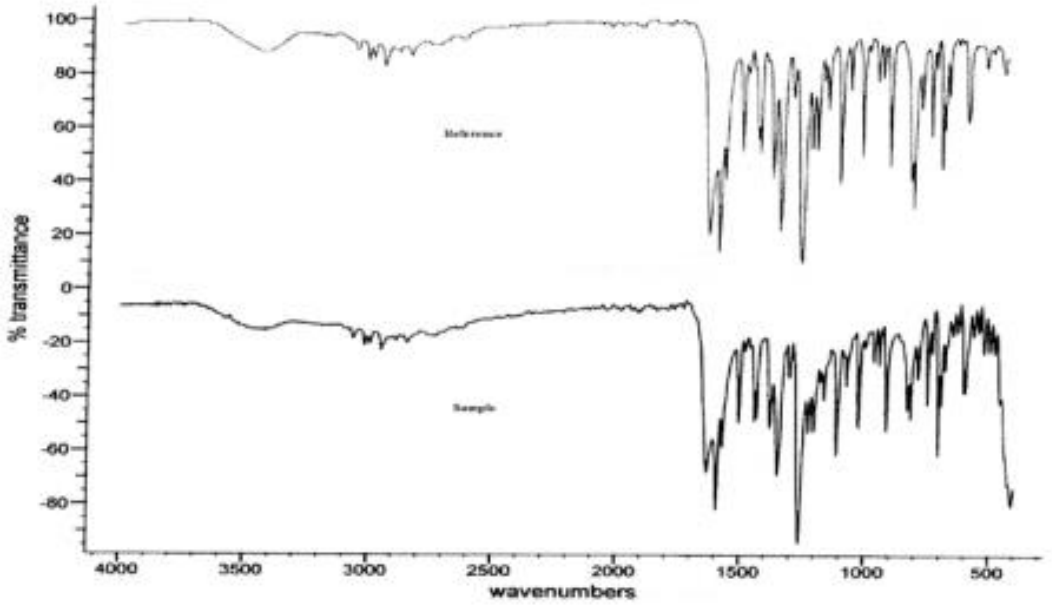

Figure A-1. Reference (Top) and Sample (Bottom) Infrared Absorption Spectra for 2-Hydroxy-4-methoxybenzophenone

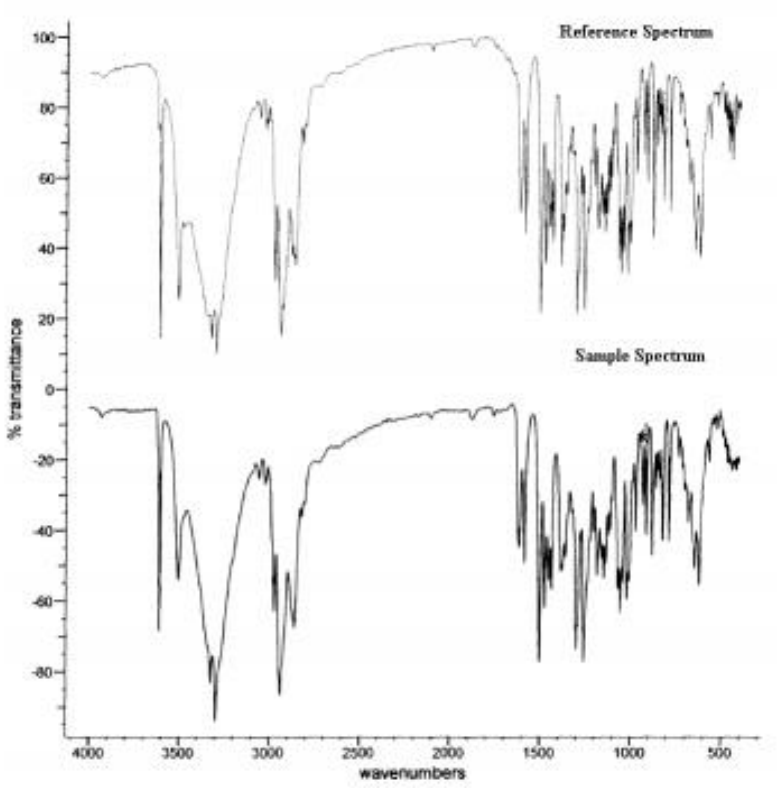

Figure A-2. Reference (Top) and Sample (Bottom) Infrared Absorption Spectra for Ethinyl Estradiol

Appendix B. Ingredients, Nutrient Composition, and Contaminant Levels in 5K96 Rat Ration

Tables

Table B-1. Nutrient Composition of 5K96 Rat Ration B-2
Table B-2. Contaminant Levels in 5K96 Rat Ration B-2

Additional information on ingredients, vitamins, and minerals in the 5K96 rat diet can be found online.[117]

Table B-1. Nutrient Composition of 5K96 Rat Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by Weight)	21.04 ± 0.6188	19.9–21.7	7
Crude Fat (% by Weight)	4.23 ± 0.1604	4.0–4.4	7
Crude Fiber (% by Weight)	3.21 ± 0.2260	2.95–3.63	7
Ash (% by Weight)	6.73 ± 0.3696	6.13–7.20	7
Vitamins			
Vitamin A (IU/kg)	18,714 ± 2,918	14,800–22,600	7
Thiamine (ppm)[a]	16.86 ± 1.753	14.2–19.8	7
Minerals			
Calcium (%)	1.273 ± 0.1316	1.18–1.56	7
Phosphorus (%)	0.963 ± 0.0668	0.886–1.09	7

[a]As hydrochloride.

Table B-2. Contaminant Levels in 5K96 Rat Ration

Contaminant	Mean ± Standard Deviation	Range	Number of Samples
Arsenic (ppm)	0.3366 ± 0.0501	0.267–0.398	7
Cadmium (ppm)	0.041 ± 0.0041	0.0327–0.0457	7
Lead (ppm)	0.2393 ± 0.0122	0.224–0.263	7
Mercury (ppm)	0.0106 ± 0.0010	0.01–0.0126	7
Selenium (ppm)	0.4451 ± 0.0421	0.404–0.53	7
Aflatoxins (ppb)[a]	<2.0	–	7
Nitrate Nitrogen (ppm)[b]	14.73 ± 10.95	1.69–24.6	7
Nitrite Nitrogen (ppm)[a,b]	<1.0	–	7
BHA (ppm)[c]	0.743 ± 0.4392	0.1–1.0	7
BHT (ppm)[c]	0.793 ± 0.4903	0.1–1.35	7
Aerobic Plate Count (CFU/g)[d]	1,275 ± 2,712	10–6,800	7
Coliform (MPN/g)	<3.0	–	7
Escherichia coli (MPN/g)	<10.0	–	7
Enterobacteriaceae (MPN/g)	<3.0	–	7
Total Nitrosamines (ppb)[e]	9.9 ± 8.4	0–24.8	7
N-N-dimethylamine (ppb)[e]	6.6 ± 6.9	0–20.3	7
N-N-pyrrolidine (ppb)[e]	3.3 ± 2.5	0–7.5	7
Pesticides (ppm)			
α-BHC[a]	–	–	7
β-BHC[a]	–	–	7

Contaminant	Mean ± Standard Deviation	Range	Number of Samples
γ-BHC[a]	–	–	7
δ-BHC[a]	–	–	7
Heptachlor[a]	–	–	7
Aldrin[a]	–	–	7
Heptachlor Epoxide[a]	–	–	7
DDE[a]	–	–	7
DDD[a]	–	–	7
DDT[a]	–	–	7
HCB[a]	–	–	7
Mirex[a]	–	–	7
Methoxychlor[a]	–	–	7
Dieldrin[a]	–	–	7
Endrin[a]	–	–	7
Telodrin[a]	–	–	7
Chlordane[a]	–	–	7
Toxaphene[a]	–	–	7
Estimated PCBs[a]	–	–	7
Ronnel[a]	–	–	7
Ethion[a]	–	–	7
Trithion[a]	–	–	7
Diazinon[a]	–	–	7
Methyl Chlorpyrifos	0 ± 0.02	0.02	7
Methyl Parathion[a]	–	–	7
Ethyl Parathion[a]	–	–	7
Malathion	0 ± 0.02	0.02	7
Endosulfan I[a]	–	–	7
Endosulfan II[a]	–	–	7
Endosulfane Sulfate[a]	–	–	7

All samples were irradiated. BHA = butylated hydroxyanisole; BHT = butylated hydroxytoluene; CFU = colony-forming units; MPN = most probable number; BHC = hexachlorocyclohexane or benzene hexachloride; DDE = dichlorodiphenyldichloroethylene; DDD = dichlorodiphenyldichloroethane; DDT = dichlorodiphenyltrichloroethane; HCB = hexachlorobenzene; PCB = polychlorinated biphenyl.

[a]All values were below the detection limit. The detection limit is given as the mean.

[b]Sources of contamination include alfalfa, grains, and fish meal.

[c]Sources of contamination include soy oil and fish meal.

[d]Preirradiation values given.

[e]All values were corrected for percent recovery.

Appendix C. Sentinel Animal Program

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Tables

Table C-1. Methods and Results for Sentinel Animal Testing in Female Rats.....C-2

C.1. Methods

Rodents used in the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that might affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicological evaluation of test compounds. Under this program, the disease state of the rodents is monitored via sera or feces from extra (sentinel) or exposed animals in the study rooms. The sentinel animals and the study animals are subject to identical environmental conditions. Furthermore, the sentinel animals come from the same production source and weanling groups as the animals used for the studies of test compounds.

For this modified one-generation study, blood samples were collected from each sentinel animal and allowed to clot, and the serum was separated. All samples were processed appropriately with serology testing performed by IDEXX BioAnalytics (formerly Rodent Animal Diagnostic Laboratory [RADIL], University of Missouri), Columbia, MO, for determination of the presence of pathogens.

The laboratory methods and agents for which testing was performed are tabulated below; the times at which samples were collected during the studies are also listed (Table C-1).

Table C-1. Methods and Results for Sentinel Animal Testing in Female Rats

Modified One-Generation Study				
Collection Time Points	**Quarantine**	**1 Month**	**16 Weeks**	**Study Termination**
Number Examined (Males/Females)[a]	0/5	0/5	0/5	0/5
Method/Test				
Multiplex Fluorescent Immunoassay (MFI)				
Kilham rat virus (KRV)	–	–	–	–
Mycoplasma pulmonis	–	–	–	–
Pneumonia virus of mice (PVM)	–	–	–	–
Rat coronavirus/sialodacryoadenitis virus (RCV/SDA)	–	–	–	–
Rat minute virus (RMV)	–	–	–	–
Rat parvo virus (RPV)	–	–	–	–
Rat theilovirus (RTV)	–	–	–	–
Sendai	–	–	–	–
Toolan's H1	–	–	–	–
Immunofluorescence Assay (IFA)				
Pneumocystis carinii	–	NT	NT	NT
Rat coronavirus/sialodacryoadenitis virus (RCV/SDA)	NT	–	NT	NT

– = negative; NT = not tested.
[a]Age matched nonpregnant females.

C.2. Results

All test results were negative.

Appendix D. Peer-review Report

Note: The peer-review report will appear in a future draft of this report.

Appendix E. Supplemental Data

Tables with supplemental data can be found here: https://doi.org/10.22427/NTP-DATA-DART-05.

E.1. Dose Range-finding Study – Rats

E.1.1. Data Tables

I01 – Animal Removal Summary

I02 – Animal Removals

I03 – Growth Curve

I03C – Growth Curve

I04 – Mean Body Weights and Survival

I04G – Mean Body Weight Gain

I05 – Clinical Observations Summary

I05P – Pup Clinical Observations Summary

I06 – Mean Feed Consumption

I08 – Mean Test Compound Consumption

R01 – Multigeneration Cross Reference

R02 – Reproductive Performance Summary

R03 – Litter Data Summary

R19 – Pup Bodyweight Summary

R19C – Pup Growth Curves

R19G – Pup Bodyweight Gain Summary

R20 – Pup Necropsy Summary

E.1.2. Individual Animal Data

Individual Animal Body Weight Data

Individual Animal Clinical Observations Data

Individual Animal Consumption Data

Individual Animal Gross Pathology Data

Individual Animal Litter Data

Individual Animal Pup Body Weight Data

Individual Animal Pup Clinical Observations Data

Individual Animal Pup Necropsy Data

Individual Animal Removal Reasons Data

Individual Animal Reproductive Performance Data

E.2. Modified One-Generation Study – Rats

E.2.1. Data Tables

F1 – All Cohorts Vaginal Cytology Plots

F1 – All Cohorts Vaginal Cytology Summary

I01 – Animal Removal Summary

I02 – Animal Removals

I03 – Growth Curve

I03C – Growth Curve

I04 – Mean Body Weights

I04G – Mean Body Weight Gain

I05 – Clinical Observations Summary

I05P – Pup Clinical Observations Summary

I06 – Mean Feed Consumption

I08 – Mean Test Compound Consumption

PA02R – Neoplastic Lesion Summary with Percent and Litter Incidence

PA03R – Non-Neoplastic Lesion Summary with Percent and Litter Incidence

PA05R – Incidence Rates of Neoplastic Lesions with Litter Incidence Systemic Lesions Abridged

PA06R – Organ Weights Summary

PA08R – Statistical Analysis of Neoplastic Lesions with Litter Incidence

PA10R – Statistical Analysis of Non-Neoplastic Lesions and Litter Incidence

PA14 – Redline Individual Histopathology Data

PA18R – Non-Neoplastic Lesion Summary with Severity Grade and Litter Incidence

PA46R – Gross Pathology Summary with Litter Incidence
R01 – Multigeneration Cross Reference
R02 – Reproductive Performance Summary
R03 – Litter Data Summary
R04 – Anogenital Distance Summary
R06 – Andrology Summary
R09 – Uterine Content Summary
R10 – Fetal Defects
R11 – Fetal Defect Summary
R13 – Fetal Defect Cross Reference Summary
R14 – Developmental Markers Summary
R14C – Time to Attainment Curves for Testicular Descent
R16 – Pubertal Markers Summary
R16C – Time to Attainment Curves for Pubertal Markers
R19 – Pup Bodyweight Summary
R19C – Pup Growth Curves
R19G – Pup Bodyweight Gain Summary
R20 – Pup Necropsy Summary
Vaginal Cytology Markov Model

E.2.2. Individual Animal Data

F1 – Fertility Cohort Vaginal Cytology Plots
F1 – Prenatal Cohort Vaginal Cytology Plots
Individual Animal Andrology Data
Individual Animal Body Weight Data
Individual Animal Clinical Observations Data
Individual Animal Consumption Data
Individual Animal Developmental Markers Data
Individual Animal Gross Pathology Data

Individual Animal Histopathology Data
Individual Animal Litter Data
Individual Animal Organ Weight Data
Individual Animal Pup Body Weight Data
Individual Animal Pup Clinical Observations Data
Individual Animal Pup Necropsy Data
Individual Animal Removal Reasons Data
Individual Animal Reproductive Performance Data
Individual Animal Teratology Dam Data
Individual Animal Teratology Fetal Weight Data
Individual Animal Teratology Implant Findings Data