NTP Developmental and Reproductive Toxicity Technical Report on the Modified One-Generation Study of 2-Ethylhexyl p-Methoxycinnamate (CASRN 5466-77-3) Administered in Feed to Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats with Prenatal, Reproductive Performance, and Subchronic Assessments in F_1 Offspring

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NOTICE

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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 could 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 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 a test article causes developmental toxicity in laboratory animals under the conditions of the study are assumed relevant to humans, unless data are available that demonstrate otherwise. In addition, such positive effects should be assumed to be primary effects, unless clear evidence shows 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 the 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, effect of the change on reproductive function or developmental outcomes, relative sensitivity of endpoints, normal background incidence, and specificity of the effect. For those evaluations that are 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 could be different with increasing dose. For example, histological changes at a lower dose level might 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 could be biologically important.
- Effects seen in many litters might 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 might be greater for a selective effect on the embryo-fetus or pup.
- Concordant effects (syndromic) can strengthen the evidence of developmental toxicity. Single endpoint changes by themselves can 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 endpoint changes by themselves are weaker indicators of effect than concordant effects on multiple, interrelated endpoints.
- 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 increases 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-Ethylhexyl p-Methoxycinnamate (CASRN 5466-77-3) Administered in Feed to Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats with Prenatal, Reproductive Performance, and Subchronic 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-Ethylhexyl p-methoxycinnamate (EHMC), also known as octinoxate and octyl methoxycinnamate, is a common component of sunscreens, cosmetics, and personal care products. Mechanistic screening studies have purported that EHMC, and its metabolites, are capable of activating the estrogen receptor to varying degrees. The objective of this study was to characterize the potential for EHMC to adversely affect any phase of rat development, maturation, and ability to reproduce. The potential for EHMC to induce subchronic toxicity in the F_1 generation, to adversely affect the ability of the F_1 generation to reproduce viable F_2 offspring, and to adversely affect the F_2 embryo-fetal development was assessed in Sprague Dawley (Hsd:Sprague Dawley® SD®) rats administered EHMC in 5K96 feed, a diet low in phytoestrogens, using the National Toxicology Program modified one-generation (MOG) study design. The dietary route of administration was selected to approximate continual exposure in group-housed animals.

Exposure concentration selection for the MOG study was based on a dose range-finding study in which time-mated rats were exposed to 0, 2,250, 5,000, 10,000, or 20,000 ppm EHMC in the diet from gestation day (GD) 6 through lactation day (LD) 28. Dams exposed to 20,000 ppm displayed significantly decreased mean body weights on GD 21 and body weight gain from GD 6 through GD 21. LD 1 through LD 14 feed consumption by the 10,000 ppm group was lower than in the control group. Test article consumption was exposure concentration-proportional. Pups exposed to 20,000 ppm displayed significantly decreased birth weight, lower live litter size, and lower postnatal viability resulting in the group being removed from study on postnatal day (PND) 14. Pup body weights of the 10,000 ppm group were also lower than those in the control group. Therefore, exposure concentrations of 0, 1,000, 3,000, and 6,000 ppm were selected for the subsequent MOG study. EHMC intake for F_0 females in the 2,250, 5,000, 10,000, and 20,000 ppm groups, based on feed consumption and dietary concentrations for GD 6 through GD 21, was approximately 161, 365, 714, and 1,841 mg EHMC/kg body weight/day (mg/kg/day), respectively; from LD 1 through LD 14, EHMC intake was approximately 410, 925, and 1,615 mg/kg/day for the 2,250, 5,000, and 10,000 ppm groups, respectively.

Modified One-Generation Study

F_0 exposure began on GD 6 and was continual. At weaning on PND 28, F_1 offspring were assigned to the reproductive performance (2/sex/litter), prenatal (1/sex/litter), or subchronic cohort (1/sex from 10 litters). 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 EHMC-induced adverse effects (similarity and magnitude thereof) at any phase of growth or development was increased by examining related endpoints and multiple pups within a litter throughout life, across cohorts, and across generations.

EHMC did not induce overt F_0 or F_1 maternal toxicity or affect mating or pregnancy indices. Dam feed consumption and body weights were slightly lower during lactation in the 6,000 ppm group. EHMC exposure at 6,000 ppm was associated with significantly decreased F_1 and F_2 preweaning mean body weights, with an onset at approximately PND 13, consistent with the beginning of pup feed consumption. Significantly decreased F_1 preweaning mean body weights were observed in males and females exposed to 3,000 or 6,000 ppm, whereas only F_2 male and

female preweaning mean body weights of the 6,000 ppm group were significantly decreased relative to their respective control groups. Although mean body weight gains of males (PND 28–105) and females (PND 28– 91) in all EHMC-exposed groups were similar to those of the respective control groups, postweaning F_1 male and female mean body weights of the 6,000 ppm group were significantly decreased by 5%–14% relative to the respective control animals. Both male and female mean body weights of the 3,000 ppm groups were significantly decreased by approximately 5% on PND 28, but by PND 56, their mean body weights were comparable to those of the control groups. Lower F_1 postweaning body weights were not associated with concurrent lower feed consumption. EHMC intake by F_0 females in the 1,000, 3,000, and 6,000 ppm EHMC groups, based on feed consumption and dietary concentrations from GD 6 through GD 21, was approximately 70, 207, and 419 mg/kg/day, respectively; from LD 1 through LD 13, EHMC intake was approximately 161, 475, and 920 mg/kg/day, respectively. EHMC intake by the F_1 generation postweaning (PND 28 through PND 91) in the 1,000, 3,000, and 6,000 ppm groups was approximately 80, 242, and 491 mg/kg/day (males) and 87, 263, and 528 mg/kg/day (females), respectively. EHMC intake by the adult F_1 females in the 1,000, 3,000, and 6,000 ppm groups was approximately 73, 220, and 435 mg/kg/day (GD 0 through GD 21) and 139, 418, and 842 mg/kg/day (LD 1 through LD 13), respectively.

EHMC exposure did not alter anogenital distance or areola/nipple retention. The timing of weaning weight-adjusted vaginal opening (VO) and balanopreputial separation (BPS) was significantly delayed by approximately 2.5 days and 3.5 days, respectively, in the 6,000 ppm group. F_1 rats exposed to 6,000 ppm EHMC displayed more time in estrus.

Reproductive performance (fertility and fecundity) was not affected by EHMC exposure. The numbers of live fetuses and pups were not affected. EHMC exposure was not associated with any effects on fetal weight or the incidences of external, visceral, or skeletal malformations. The 6,000 ppm group did exhibit a higher combined fetal incidence of lumbar 1 rudimentary rib variants (approximately 10% versus 4% in the control group).

In the subchronic cohort, no gross findings, changes in organ weights, or histopathological findings were attributed to EHMC exposure.

Conclusions

Under the conditions of this modified one-generation (MOG) study, there was *no evidence of reproductive toxicity* of 2-ethylhexyl p-methoxycinnamate (EHMC) in Hsd:Sprague Dawley® SD® rats at exposure concentrations of 1,000, 3,000, or 6,000 ppm. Mating and littering were not affected significantly by EHMC exposure.

Under the conditions of this MOG study, there was *equivocal evidence of developmental toxicity* of EHMC in Hsd:Sprague Dawley® SD® rats based on the observed postnatal effects on body weight that showed some indication of recovery by study end, delays in postnatal day 28-adjusted vaginal opening and balanopreputial separation, which could have influenced the apparent transient effects on body weight, and time in estrus was slightly longer in EHMC-exposed females relative to that of the control group. No other signals consistent with alterations in estrogenic, androgenic, or antiandrogenic action were observed. EHMC exposure did not induce any specific fetal malformations.

Synonyms: Octinoxate; ethylhexyl methoxycinnamate; octyl methoxycinnamate; 2-propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester; 2-ethylhexyl 3-(4-methoxyphenyl)prop-2-enoate

Summary of Exposure-related Findings in Rats in the Modified One-Generation Study of 2-Ethylhexyl p-Methoxycinnamate

	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
F_0 Generation				
Maternal Parameters				
Number mated	26	26	26	26
Number pregnant (%)	22 (84.6)	24 (92.3)	19 (73.1)	22 (84.6)
Number not pregnant (%)	4 (15.4)	2 (7.7)	7 (26.9)	4 (15.4)
Number littered (%)	22 (100.0)	24 (100.0)	19 (100.0)	22 (100.0)
Clinical Observations	None	None	None	None
Mean Body Weight[a,b]				
Body weight: GD 21	359.6 ± 4.4	370 ± 3.9	360.8 ± 4.5	360.2 ± 4.6
Body weight: LD 28	283.1 ± 3.3	283.5 ± 2.9	280.4 ± 3.1	282.7 ± 2.3
Necropsy Observations	None	None	None	None
F_1 Generation (Preweaning)[b]				
Clinical Observations	None	None	None	None
Live Litter Size				
PND 0	10.8 ± 0.7	13.0 ± 0.4*	11.7 ± 0.4	11.1 ± 0.7
PND 4 (prestandardization)	10.7 ± 0.7	12.9 ± 0.4*	11.5 ± 0.4	10.9 ± 0.7
PND 4 (poststandardization)	8.9 ± 0.4	9.9 ± 0.1	9.8 ± 0.2	9.1 ± 0.4
PND 28	8.9 ± 0.4	9.7 ± 0.1	9.7 ± 0.2	8.9 ± 0.4
Male Pup Mean Body Weight				
PND 1	6.96 ± 0.08	7.01 ± 0.09	7.05 ± 0.08	7.17 ± 0.11
PND 28	82.66 ± 1.00**	82.13 ± 1.07	78.92 ± 0.94*	71.92 ± 0.90**
Female Pup Mean Body Weight				
PND 1	6.65 ± 0.07	6.64 ± 0.08	6.63 ± 0.07	6.69 ± 0.09
PND 28	75.37 ± 1.11**	73.63 ± 1.03	69.81 ± 1.03**	64.17 ± 0.87**
F_1 Generation (Postweaning)				
Mean Body Weight[a,b]				
Male body weight: PND 28	82.0 ± 1.5**	78.8 ± 1.2	76.3 ± 0.9*	71.9 ± 1.5**
Male body weight: PND 91	396.6 ± 6.6**	392.0 ± 4.2	387.1 ± 3.9	376.3 ± 4.0**
Female body weight: PND 28	75.4 ± 1.8**	70.8 ± 1.1	67.4 ± 1.0**	64.5 ± 1.5**
Female body weight: PND 91	253.0 ± 4.2**	244.5 ± 3.7	241.3 ± 3.0	236.4 ± 2.9**
F_1 and F_2 Generations				
Endocrine Endpoints, Developmental Landmarks, and Pubertal Endpoints[b]				
Vaginal opening (F_1)				
Adjusted mean day of vaginal opening (litter mean)[c]	34.4 ± 0.3**	35.1 ± 0.2	35.7 ± 0.3*	36.5 ± 0.3**
Body weight at acquisition[a]	106.7 ± 2.0	107.3 ± 1.3	107.1 ± 1.4	107.7 ± 2.4
Balanopreputial separation (F_1)				

	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
Adjusted mean day of balanopreputial separation (litter mean)[c]	45.6 ± 0.3**	45.6 ± 0.6	45.2 ± 0.3	47.8 ± 0.5**
Body weight at acquisition[a]	207.9 ± 3.5	203.5 ± 4.0	199.2 ± 1.9	214.1 ± 3.4
Prenatal Cohort				
Mating and Fertility Performance				
Number of mating pairs	21	23	19	22
Mated females/paired (%)	90.5	91.3	94.7	90.9
Pregnant females/mated (%)	100.0	85.7	83.3	80.0
Mean Body Weight[a,b]				
Body weight gain: GD 0–21	168.0 ± 3.5	147.8 ± 8.4*	170.9 ± 3.0	151.9 ± 5.5
Uterine Content Data[b]				
Mean number of corpora lutea/female	17.74 ± 0.73	16.22 ± 0.55	18.71 ± 0.61	17.50 ± 0.74
Implantations/female	15.21 ± 0.68	13.11 ± 1.19	15.75 ± 0.51	14.19 ± 0.88
Live fetuses/litter	14.89 ± 0.65	13.47 ± 1.11	15.25 ± 0.54	13.63 ± 0.93
Fetal Findings				
External findings	None	None	None	None
Visceral findings	None	None	None	None
Skeletal findings[d]				
Lumbar, 1, unilateral or bilateral, rudimentary – [V]				
Fetuses	12 (4.24)	8 (3.79)	7 (3.83)	22 (10.09)
Litters	5 (26.32)	5 (29.41)	2 (16.67)	7 (43.75)
Lumbar, 1, bilateral, rudimentary – [V]				
Fetuses	4 (1.41)	4 (1.90)	4 (2.19)	8 (3.67)
Litters	2 (10.53)	3 (17.65)	2 (16.67)	5 (31.25)
Lumbar, 1, left, rudimentary – [V]				
Fetuses	0 (0.00)[#]	4 (1.90)	0 (0.00)	8 (3.67)
Litters	0 (0.00)	4 (23.53)	0 (0.00)	4 (25.00)
Lumbar, 1, right, rudimentary – [V]				
Fetuses	8 (2.83)	0 (0.00)	3 (1.64)	6 (2.75)
Litters	5 (26.32)	0 (0.00)	1 (8.33)	4 (25.00)
Reproductive Performance Cohort				
Mating and Fertility Performance				
Number of mating pairs	36	46	35	37
Mated females/paired (%)	94.4	89.1	91.4	91.9
Littered females/mated (%)	76.5	82.9	77.4	76.5

	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
Mean Body Weight[a,b]				
Body weight: GD 21	431.0 ± 10.2	416.5 ± 6.2	419.2 ± 7.9	402.2 ± 6.9
Body weight: LD 28	318.7 ± 5.8*	311.4 ± 4.5	304.2 ± 5.3	302.6 ± 3.4
Live Litter Size[b]				
PND 0	14.1 ± 0.8	13.0 ± 0.7	15.0 ± 0.6	13.1 ± 0.8
PND 4 (prestandardization)	13.5 ± 0.9	13.1 ± 0.7	14.0 ± 0.8	12.5 ± 0.8
PND 4 (poststandardization)	9.4 ± 0.5	9.4 ± 0.3	9.6 ± 0.4	9.3 ± 0.4
PND 28	7.4 ± 0.7	8.2 ± 0.5	8.0 ± 0.6	8.4 ± 0.6
Male Pup Mean Body Weight[b]				
PND 1	6.88 ± 0.09	6.78 ± 0.14	6.63 ± 0.09	6.68 ± 0.08
PND 28	78.45 ± 2.28**	78.20 ± 1.68	73.29 ± 2.05	67.29 ± 1.32**
Female Pup Mean Body Weight[b]				
PND 1	6.50 ± 0.14	6.43 ± 0.10	6.33 ± 0.08	6.43 ± 0.10
PND 28	71.21 ± 2.07**	71.79 ± 1.65	67.82 ± 1.84	63.62 ± 1.31**
Adult Necropsies				
Clinical Pathology				
Subchronic cohort	None	None	None	None
Gross Necropsy Findings				
All cohorts	None	None	None	None
Organ Weights				
All cohorts	None	None	None	None
Histopathological Findings				
All cohorts	None	None	None	None
Andrology	None	None	None	None
Vaginal Cytology	None	↑ Estrus stage length	↑ Estrus stage length	↑ Estrus stage length
Level of Evidence of Reproductive Toxicity: No Evidence				
Level of Evidence of Developmental Toxicity: Equivocal 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 $p \leq 0.05$; **$p \leq 0.01$.
[#]Statistically significant at $p \leq 0.05$ in litter-based analysis of fetuses.
GD = gestation day; LD = lactation day; PND = postnatal day; [V] = variation.
[a]Body weight results given in grams.
[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 (%).

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-ethylhexyl p-methoxycinnamate (EHMC) 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), metabolism and disposition following oral gavage and dermal exposure, and characterization of the potential effects of continuous EHMC exposure over multiple generations using the NTP modified one-generation study design. In this study, exposure to EHMC 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; 1 pup/sex from 10 litters was allocated to the subchronic cohort; and 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, the potential effects on parturition and early growth of the F_2 generation were assessed in the reproductive performance cohort, and the potential effects on adult F_1 organ systems were evaluated in the subchronic cohort. Apical indicators sensitive to endocrine modulation were measured.

Introduction

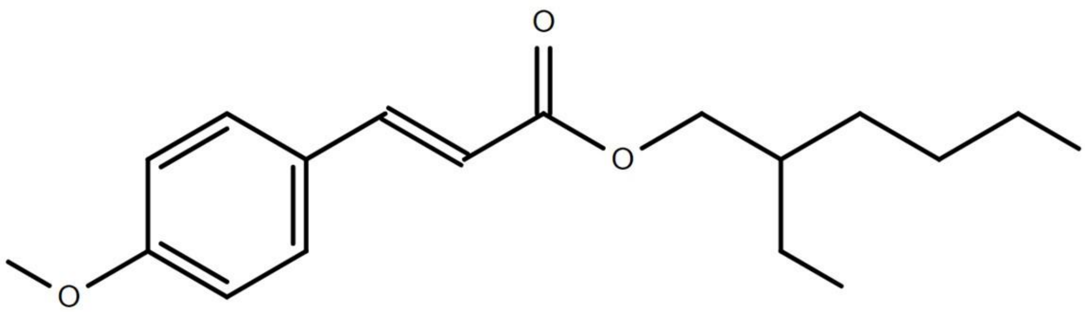


Figure 1. 2-Ethylhexyl p-Methoxycinnamate (CASRN 5466-77-3; Chemical Formula: $C_{18}H_{26}O_3$; Molecular Weight: 290.40)

Image generated with ChemSpider[1]
Synonyms: Octinoxate; ethylhexyl methoxycinnamate; octyl methoxycinnamate; 2-propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester; 2-ethylhexyl 3-(4-methoxyphenyl)prop-2-enoate.

Chemical and Physical Properties

2-Ethylhexyl p-methoxycinnamate (EHMC; CASRN 5466-77-3) is a mixture of *cis*- and *trans*-isomers, with the *trans*-isomer (CASRN 83834-59-7) predominating. EHMC, also called octinoxate or octyl methoxycinnamate, is a colorless to light-yellow viscous liquid that is relatively insoluble in water (0.04 mg/L at 24ºC, pH 7.1) and is readily soluble in most organic solvents.[2; 3] EHMC absorbs ultraviolet (UV) A (320–400 nm) and UVB (290–320 nm) light and is photostable.[4; 5]

Production, Use, and Human Exposure

EHMC is synthesized by an insertion reaction of ketene with p-methoxybenzaldehyde or from enzymatic esterification of methoxycinnamic acid.[6; 7]

EHMC at concentrations ≤7.5% is used in sunscreens and other personal care products to protect the wearer from solar erythema (21 CFR § 352.10). Per the Environmental Working Group's Skin Deep® Database,[8] EHMC is found in approximately 750 sunscreens, lip balms, and moisturizers. EHMC (or its metabolites) has been detected in amounts as high as 19 ng/mL in human urine.[9]

Regulatory Status

The U.S. Food and Drug Administration has approved use of up to 7.5% (w/w) EHMC in sunscreen, either alone or in combination formulations. Section 8(a) of the Toxic Substances Control Act requires manufacturers of this chemical to report preliminary assessment information concerned with production, exposure, and use to the U.S. Environmental Protection Agency.[10]

Absorption, Distribution, Metabolism, and Excretion

Experimental Animals

Absorption, distribution, metabolism, and excretion (ADME) data for EHMC in animals are limited. The National Toxicology Program (NTP) investigated the ADME of EHMC in Sprague Dawley (Hsd:Sprague Dawley® SD®) rats and B6C3F1/N mice after single gavage administration (8, 80, or 800 mg EHMC/kg body weight [mg/kg]), intravenous administration (8 mg/kg), or dermal application (0.8, 8, or 80 mg/kg, representing, respectively, 0.1%, 1%, or 10% of the formulation concentration) of [^{14}C]EHMC.[11] After gavage administration in male (8, 80, or 800 mg/kg) and female (8 mg/kg) rats, [^{14}C]EHMC was highly absorbed (≥78%) and excreted mainly in urine (76%–82%), with approximately 2%–8% excreted in feces and approximately 1%–7% excreted as expired carbon dioxide (CO_2) by 72 hours following administration. Very little (<1%) of the administered dose remained in tissues.

After a single gavage administration of 8 mg/kg [^{14}C]EHMC in male and female mice, 57%–73%, 15%–25%, and 2%–3% of the administered dose was recovered by 72 hours postadministration in urine, feces, and as exhaled CO_2, respectively. While the pattern of disposition of EHMC in mice was similar to that in rats, the higher amount of the dose recovered in feces in mice compared to rats is likely due to contamination of feces with urine as has been observed in other mice disposition studies. The disposition of [^{14}C]EHMC after intravenous administration was similar to that following gavage administration.[11]

Absorption of [^{14}C]EHMC was high after a single dermal application, using ethanol or acetone as a vehicle, to a covered dose site. In male and female rats after a single application of 8 mg/kg [^{14}C]EHMC, approximately 34%–42% of the applied dose was absorbed. In male (0.8, 8, or 80 mg/kg) and female (8 mg/kg) mice following a single dermal application of [^{14}C]EHMC, approximately 36%–62% of the applied dose was absorbed. Using a lotion vehicle (olive oil:emulsifying wax:water 15:15:70 [v/w/v]), most of the applied dose was unabsorbed in rats; only 11% of the dose was absorbed with approximately 4% remaining at the dose site skin. The pattern of disposition and metabolism of [^{14}C]EHMC following dermal application in rats and mice was similar to that after gavage administration.[11]

Numerous metabolites were detected in urine, including the purported developmental toxicants 2-ethylhexanol and 2-ethylhexanoic acid (Figure 2); parent EHMC was not detected under the conditions used in these assessments.[11] Huang et al.[9] also reported five metabolites of EHMC in urine and plasma following single gavage administration of 200 or 1,000 mg/kg EHMC in male Sprague Dawley rats. EHMC was cleared rapidly in rat and mouse hepatocytes with estimated half-lives of ≤3 minutes.[11]

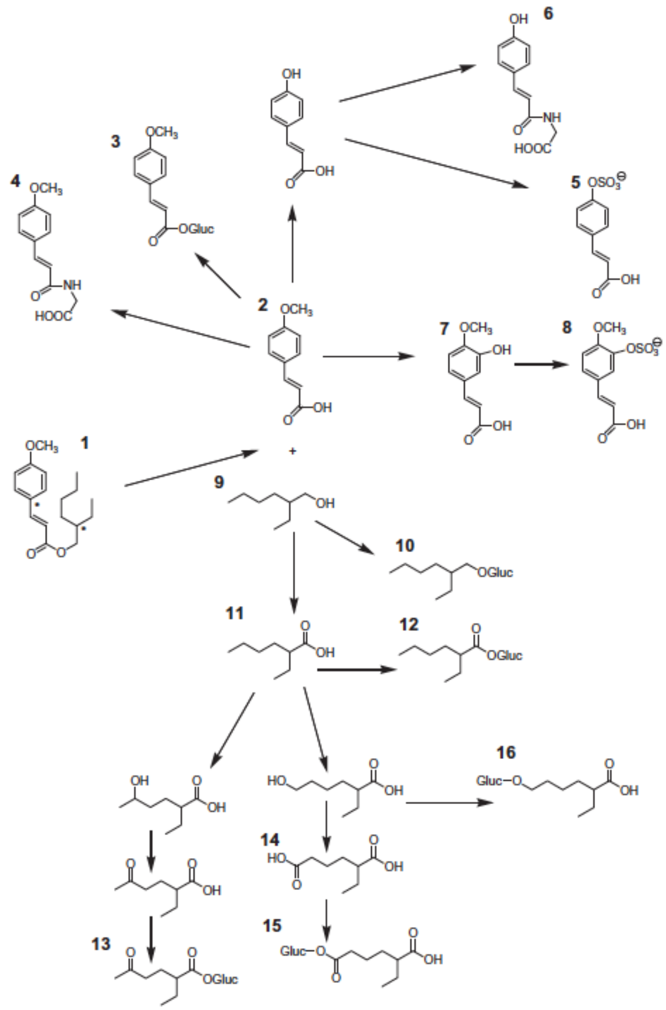


Figure 2. Metabolism of 2-Ethylhexyl p-Methoxycinnamate in Rodents

(1) 2-Ethylhexyl p-methoxycinnamate; (2) p-methoxycinnamate; (3) p-methoxycinnamate glucuronide; (4) p-methoxycinnamate glycine; (5) hydroxycinnamate sulfate; (6) hydroxycinnamate glycine; (7) hydroxy methoxycinnamate; (8) hydroxy methoxycinnamate sulfate; (9) ethylhexanol; (10) ethylhexanol glucuronide; (11) 2-ethylhexanoic acid; (12) 2-ethylhexanoic acid glucuronide; (13) 2-ethyl-5-ketohexanoic acid glucuronide; (14) 2-ethyladipate; (15) ethyladipate glucuronide; (16) hydroxyethylhexanoic acid glucuronide.[11]

Humans

Following a whole-body application of 2 mg/cm^2 of basic cream formulation containing 10% w/w EHMC to 32 human volunteers, EHMC was detected in plasma and urine.[12] Several in vitro investigations of dermal absorption of EHMC in isolated skin preparations have reported uptake of EHMC.[13; 14] Klimová et al.[15] estimated systemic human exposures of up to 1,032 μg/kg/day from in vitro uptake studies of oil-water EHMC sunscreen emulsion applications to pig-ear skin. In another in vitro study investigating the absorption of EHMC through pig skin, considerably greater amounts of the dose were absorbed when EHMC was applied in an emulsion rather than when the material was applied in a microencapsulated formulation.[16] EHMC absorption was approximately 50% lower after in vitro application to human skin encapsulated in solid lipid nanoparticles than after application in an oil-water emulsion.[17] A study of children aged 6 to 18 from a suburban district of Shanghai identified EHMC, 4-methoxycinnamic acid, and 4-methoxyacetophenone present in urine at approximately 19, 41, and 27 ng/mL, respectively.[9] EHMC was cleared in human hepatocytes more slowly than in rodents with an estimated half-life of ≤48 minutes.[11]

Developmental and Reproductive Toxicity

Models of Endocrine Activity

EHMC has been reported to have weak in vitro estrogenic activity, to induce estrogen receptor (ER) transactivation, and to stimulate ER-dependent MCF-7 cell proliferation (median effective concentration [EC_{50}] = 2.37 μM).[18-20] Schlumpf et al.[18] reported that EHMC induced a uterotrophic response in immature rats (median effective dose [ED_{50}] = 934 mg/kg/day). Other investigators observed uterotrophy in ovariectomized adult rats administered 1 g/kg, along with "estrogen" consistent increases in uterine C3, pituitary truncated estrogen receptor product-1 (TERP-1), and liver insulin-like growth factor 1 (IGF1) expression.[21] EHMC did not repress androgen receptor (AR)-mediated transition in AR CALUX® (Chemically Activated LUciferase eXpression) cells, but resulted in repression of transcription of human progesterone receptor (PR) in PR CALUX cells (median inhibition concentration [IC_{50}] = 0.5 μM).[20]

Experimental Animals

Some animal studies suggest possible effects on reproduction. F_1 male Wistar Han rats exposed perinatally to EHMC displayed lower sperm counts and lower ventral prostate weights than control males.[22]

In a two-generation dietary study (0, 150, 450, or 1,000 mg/kg/day), after a 14-week premating period, no EHMC-related effects on mating performance or fertility were observed. F_0 and F_1 female Wistar rats exposed to 1,000 mg/kg/day displayed reductions in the numbers of implantation sites and apparent litter size, which were attributed to maternal toxicity.[23] F_1-exposed males displayed a slight reduction in cauda sperm concentration. EHMC exposure was associated with lower postnatal body weight gain in pups. F_1 and F_2 generations exposed to 1,000 mg/kg/day displayed delays in vaginal opening, balanopreputial separation, and lower body weights on day of attainment.[23; 24]

Limited data do not suggest developmental abnormalities in experimental animals exposed to EHMC. In a guideline rabbit study (stock not defined), does administered EHMC at 0, 80, 200, or 500 mg/kg/day by gavage during fetal organogenesis (days not defined; dose level justification not presented) exhibited a slight decrease in maternal weight. Fetal weight was only slightly lower in the 500 mg/kg/day group, and "no fetal abnormalities" were reported (details limited).[25] In a guideline rat study (strain not defined), mated rats were administered 0–1,000 mg/kg/day EHMC from gestation days (GDs) 6–14, consistent with a pilot study (presumed gavage), and a subset was allowed to litter and rear their offspring. The percentage of resorptions in the 1,000 mg/kg/day dose group was higher than in all other groups but was attributed to unexpected low numbers of resorptions observed in those groups. No other findings were noted.[25]

Endocrine Disruptor Screening Panel Studies

NTP sponsored mammalian Endocrine Disruptor Screening Panel (EDSP) Tier 1 studies[26] in which EHMC at maximal feasible doses did not interact with ER isolated from rat uteri (100 μM), induce ER transcriptional activation in HeLa-9903 cells (0.01 μM), or induce a uterotrophic response (1 g/kg) in young ovariectomized Sprague Dawley Crl:CD® IGS rats. EHMC at maximal feasible doses was categorized as a nonbinder of AR isolated from rat prostate (100 μM), did not induce transcriptional activation, and had no apparent inhibitory effect on dihydrotestosterone-induced AR transcriptional activity in MDA-kb2 cells (32 μM). In the Hershberger assay, EHMC (1 g/kg) had no effect on androgen-dependent organ weights in the absence of androgenic action. In the presence of testosterone propionate, EHMC did not attenuate the expected androgen-mediated increase in organ weights, demonstrating that EHMC does not exhibit antiandrogenic activity in vivo at the doses assessed. EHMC was classified as a noninhibitor of aromatase activity (100 μM) and was negative in the H295R human adrenocarcinoma cell steroidogenesis assay at the highest concentration that could be evaluated (0.1 μM).[26]

Humans

In a study using human sperm, EHMC was shown to induce Ca^{2+} signaling (EC_{50} = 1.9 μM), which is normally associated with the progesterone-induced acrosomal reaction via the Catsper channel (sperm-specific, Ca^{2+}-permeable, pH-sensitive, and weakly voltage-dependent ion channel). The signal was not sufficient to significantly induce the acrosomal reaction or to affect sperm penetration or viability.[27; 28]

General Toxicity

Experimental Animals

Acute and subchronic toxicity appears to be low. The acute oral median lethal dose (LD_{50}) of EHMC is >8 g/kg for mice and >5 g/kg for rats.[29] In a 13-week study using Füllinsdorf Albino SPF rats (with recovery group) at dietary concentrations of 0, 200, 450, or 1,000 mg/kg/day, the 1,000 mg/kg/day group displayed a transient increase in kidney weight, which was attributed to the physiological response to increased EHMC eliminatory activity.[25] This exposed group also displayed lower glycogen levels and a higher iron concentration in Kupfer cells. Two animals in this exposed group exhibited minimal centrilobular necrosis with infiltration (a finding also observed in control rats but with less severity). High-exposure concentration females exhibited transiently increased glutamate dehydrogenase levels. The no-observed-adverse-effect level (NOAEL) was established at 450 mg/kg/day. A 13-week dermal study in Sprague Dawley rats at doses up to 555 mg/kg/day did not reveal any adverse responses, other than an increase in liver weight at the highest dose without concurrent adverse histopathological findings. The sponsor suggested the NOAEL to be 555 mg/kg but given the effect on liver weight observed at this dose, the European Commission's Scientific Committee on Cosmetology rationalized the NOAEL as the next lower dose (227 mg/kg/day).[25]

Humans

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

Immunotoxicity

Experimental Animals

Limited available data do not indicate immunotoxicity of EHMC. EHMC did not induce irritation upon instillation in the rabbit conjunctival sac[30] or after topical application on guinea pigs for 16 days.[31] When EHMC was applied daily for 16 days to guinea pigs, and the animals were challenged 3 days after the last application, there were no signs of sensitization.[25]

Humans

Studies examining the potential for EHMC to induce allergic contact dermatitis are limited. When patients that previously presented with an eczematous reaction in areas likely exposed to sunlight were subjected to photopatch tests using a standard series of sunscreens, EHMC induced a low relative photoallergenic response (1/26 positives; 1/82 subjects) and did not induce contact dermatitis.[32]These findings are consistent with a study conducted in Singapore[33] and a retrospective analysis of photoallergic and allergic contact results from patients using one of 11 UV filters.[34]

Topical application of EHMC for 24 and 48 hours was not associated with irritation of the skin.[25] A Draize repeated insult patch with a 2% formulation of EHMC (vehicle not stated) did not result in sensitization. A formulation of 7.5% EHMC in petroleum jelly that was topically applied and occluded for 48 hours and repeated 11 times, followed by a challenge application 14 days after the last application, was not associated with any adverse reactions. Similar results were observed with a 10% formulation of EHMC in dimethylphthalate.[25]

Study Rationale

EHMC was nominated by the National Cancer Institute and recommended for comprehensive toxicological characterization, including carcinogenicity and developmental toxicity studies, and for characterization of photodecomposition products. The nomination was based on EHMC's extensive use, widespread consumer exposure in sunscreens, and reported estrogenic and reproductive effects. This study is part of a larger NTP effort examining whether UV filters are associated with toxicity in in vitro and in vivo models that inform potential human hazard.[35] Given the purported effects on hormonally responsive endpoints, NTP characterized the estrogenic, androgenic, and antiandrogenic potential of EHMC in in vitro and short-term in vivo EDSP studies.[26] To characterize potential EHMC-induced effects on fertility, fecundity, and subchronic toxicity, the toxicological potential of EHMC was assessed in the rat modified one-generation study design. This design was chosen to increase the likelihood of identifying adverse responses over interrelated endpoints. The design includes assessment of F_1 general toxicity and histological examinations that could identify early proliferative lesions.

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 (Table 1; Figure 4). 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) and continues through weaning which occurred 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 examination 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.[36]

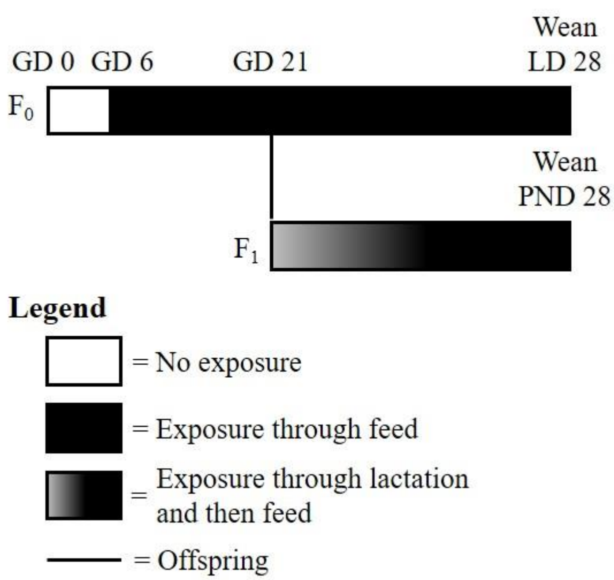


Figure 3. Design of a Dose Range-finding Study

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.

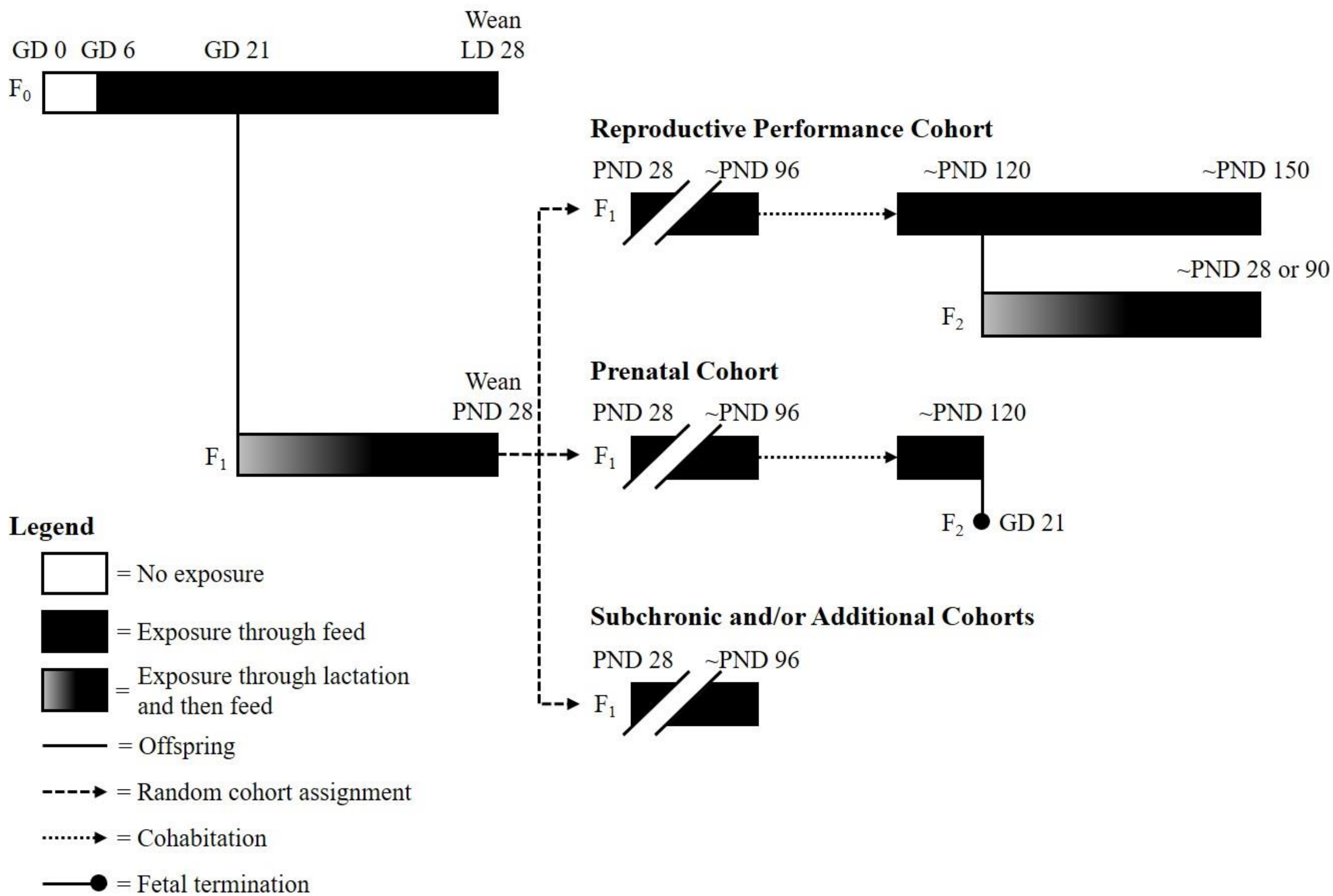


Figure 4. Design of a Modified One-Generation Rat Study

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 day (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, but might not adversely affect survival or health,[37] consistent with descriptions by Makris et al.[38] 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-Ethylhexyl p-methoxycinnamate (EHMC) was obtained from Acros Organics (Fair Lawn, NJ) in a single lot (A0293319). Identity, purity, and stability analyses were conducted by the analytical chemistry lab at MRIGlobal (Kansas City, MO) (Appendix A). Reports on analyses performed in support of the EHMC study are on file at the National Institute of Environmental Health Sciences (NIEHS).

EHMC is a clear, colorless liquid. The identity of lot A0293319 was evaluated using Fourier Transform infrared (FT-IR) spectroscopy, ^{1}H nuclear magnetic resonance (NMR) spectroscopy, ^{13}C NMR spectroscopy, and gas chromatography (GC) with mass spectrometry (MS) (Table A-1).

The FT-IR, ^{1}H NMR, and ^{13}C NMR spectra (Appendix A) were consistent with the structure of EHMC and reference spectra for the *trans*-isomer in the National Institute of Advanced Industrial Science and Technology Spectral Database (No. 19199). The GC/MS spectra corresponded with the National Institute of Standards and Technology Mass Spectral Library reference for EHMC.

Elemental analysis was consistent with the composition of EHMC. Karl Fisher titration indicated a water content of <0.1%. The purity of lot A0293319 determined using GC with flame ionization detection (FID) with two different column chemistries was 99.17% and 98.99% (Table A-1). Both methods identified three impurities having an area ≥0.05%. The purity of lot A0293319 was determined to be >98%.

Accelerated stability studies confirmed that the bulk lot A0293319 was stable when protected from light and stored for 2 weeks at approximately 5°C, 25°C, 60°C, or −20°C. Upon receipt by the analytical laboratory, the 150 kg drum of lot A0293319 was homogenized and transferred to 1-gallon narrow-mouthed amber glass bottles sealed with Teflon-lined lids. Periodic reanalysis of the bulk chemical performed during and after the studies showed no degradation.

Preparation and Analysis of Dose Formulations

Dose formulations of EHMC in LabDiet 5K96 Verified Casein Diet 10 IF feed were prepared following the protocols outlined in Table A-2. Dose formulations of 1,000, 3,000, and 6,000 ppm were used for the modified one-generation study. Formulations were stored at approximately 5°C and were considered stable for 35 days.

The method of preparation was validated for concentration ranges of 400–25,000 ppm.

Prior to study start, the stability and homogeneity of the dose formulations were determined using GC/FID. Stability of the 1,000 ppm formulation was confirmed for 35 days at refrigerated temperatures (5°C). A 7-day simulated dose study of the 1,000 ppm formulation was conducted to determine stability in animal room conditions. Formulations mixed with rodent urine and feces were stable for up to 4 days at a concentration of 1,000 ppm. Homogeneity of the dose formulations was confirmed at 1,000, 2,250, and 20,000 ppm.

Analyses of preadministration and postadministration dose formulations were conducted throughout the study. Postadministration samples were collected from the animal room at the end of the first exposure period. All samples were within 10% of the target concentration (Table A-3).

Animal Source

Female Sprague Dawley (Hsd:Sprague Dawley® SD®) rats were obtained from Envigo (formerly Harlan Laboratories, Inc, Indianapolis, IN and Dublin, VA) for use in the dose range-finding and MOG studies. Sexually mature (12 to 14 weeks old) females were time-mated overnight at the vendor and were received on GD 1 or GD 2 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), 10 female sentinel animals were evaluated in the dose range-finding study. Twenty female sentinel and 10 F_1 male animals were evaluated in the MOG study. 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, 2,250, 5,000, 10,000, or 20,000 ppm of EHMC on GD 6 through LD 28. Feed and water were available ad libitum. Information on feed composition and contaminants is provided in Appendix B. The high exposure concentration of 20,000 ppm was estimated to result in a daily “limit” oral dose of at least 1 g EHMC/kg body weight/day. Half-dose spacing was used to identify a maximally tolerated dose that the dam could tolerate and so the MOG study could be populated with a sufficient number of offspring.

Eight time-mated females were allocated to each exposure group. Six additional time-mated females were allocated to the control, 2,250, 10,000, and 20,000 ppm EHMC 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 severely decreasing litter size and resulting in an insufficient number of pups available for postnatal assessments and cohort-specific endpoints. Further details of animal maintenance and study design are given in Table 2.

Modified One-Generation Study with Prenatal, Reproductive Performance, and Subchronic Cohorts

Time-mated F_0 female rats, 26 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, 1,000, 3,000 or 6,000 ppm EHMC ad libitum on GD 6. The exposure concentration of 6,000 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 EHMC 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 10 pups (5/sex/litter, when possible) on PND 4. At weaning on PND 28, F_1 offspring were randomly assigned to a reproductive performance (1/sex/litter), prenatal (1/sex/litter), subchronic (1/sex from 10 litters), or biological sampling cohort (1/sex/litter). Information on feed composition and contaminants is provided in Appendix B. Additional details of animal maintenance and study design are given in Table 2.

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 26 (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 subsequently 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).[39]

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 ≤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 was 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 with 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 in each litter were examined for soft tissue alterations under a stereomicroscope.[40; 41] The heads were removed from approximately half of the fetuses in each litter, fixed in Bouin’s solution, and subsequently examined by freehand sectioning.[42] 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.[43; 44] External, visceral, and skeletal fetal findings were recorded as developmental variations or malformations. After positive evidence of mating, male sires were euthanized and 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 10 pups (5/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 21–24 weeks of age. F_2 offspring were given a gross necropsy. F_1 sires were euthanized and 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 approximately −80°C for subsequent determination of epididymal sperm concentration from the left cauda epididymis. The left testis was frozen at approximately −80°C for subsequent determination of homogenization-resistant spermatid head counts for calculations of daily sperm production and efficiency of daily sperm production.[45] 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).

Subchronic Cohort

General toxicity was assessed in one male and one female from 10 random litters (within an exposure concentration) and all exposure groups. F_1 males and females were euthanized and necropsied on PND 110 to PND 112 and PND 111 to PND 113, respectively. The animals were anesthetized with carbon dioxide and euthanized by exsanguination. Blood was collected by cardiac puncture. Blood for hematology was collected into a tripotassium ethylenediaminetetraacetic acid (K_3EDTA)-treated tube and analyzed on an Advia 120 hematology analyzer (Erlangen, Germany). Blood for clinical chemistry analyses was collected into a serum separator tube and the serum harvested and analyzed on an Olympus 640e clinical chemistry analyzer (Center Valley, PA). The samples for clinical pathology analyses were stored at approximately 4°C until transferred to Antech® GLP (Morrisville, NC) on the same day as necropsy for the clinical pathology analyses. The parameters measured are listed in Table 3.

In addition, approximately 200 µL of whole blood was collected into a K_3EDTA-treated tube for micronucleus determination. The micronucleus samples were stored at approximately 4°C until transferred to the designated NTP laboratory (Integrated Laboratory Systems, LLC, Durham, NC) on the same day as the necropsy.

Biological Sampling Cohort

On PND 28 and PND 56 (5/sex/time point/exposure group), plasma, kidneys, liver, epididymides, testes, and ovaries were collected and frozen for potential future analyses. None of the internal dose assessment samples were analyzed because in a preliminary investigation, it was observed that EHMC was not stable under the conditions used for sample collection and storage.

Necropsy and Histopathology

Complete necropsies were performed on adult F_1 male and F_1 females in the subchronic and reproductive performance cohorts, 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 male and F_1 females in the subchronic and reproductive performance cohorts. In the prenatal cohort, organ weights were recorded for the adrenal glands, testes, epididymides, dorsolateral and ventral prostate gland, seminal vesicles with coagulating glands, thyroid gland (fixed), levator ani/bulbocavernosus (LABC) muscle, Cowper's glands, and preputial glands. In the reproductive performance cohort, organ weights were recorded for the adrenal glands, ovaries, testes, epididymides, cauda epididymis, dorsolateral and ventral prostate gland, seminal vesicles with coagulating glands, thyroid gland (fixed), LABC muscle, Cowper's glands, and preputial glands. In the subchronic cohort, organ weights were recorded for the epididymis, heart, kidney, liver, lungs, dorsolateral prostate gland, ventral prostate gland, seminal vesicles with coagulating glands, testes, and thymus.

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. For the F_1 subchronic males and females, all diagnoses from all tissues from six randomly selected animals in the control and 6,000 ppm groups were reviewed. In addition, the dorsal prostate gland, ventral prostate gland, epididymides, and testes were reviewed from all control and 6,000 ppm males in the F_1 subchronic and F_1 reproductive performance cohorts; the ovaries and uterus were reviewed from all control and 6,000 ppm females in the F_1 subchronic and F_1 reproductive performance cohorts.

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[46] and Boorman et al.[47]

Table 2. Experimental Design and Materials and Methods in the Dose Range-finding and Modified One-Generation Studies of 2-Ethylhexyl p-Methoxycinnamate (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., Indianapolis, IN)	Envigo (formerly Harlan Laboratories, Inc., Dublin, VA)
Day of Arrival	
February 14, 2012 (GD 1 or GD 2)	September 25 or 27, 2012 (GD 1 or GD 2)
Average Age on Arrival	
~12 weeks	12–14 weeks
Weight Range at Randomization	
192.8–249.5 g on GD 3	199.8–257.0 g on GD 3
Date of First Exposure	
GD 6 (February 18, 2012)	F_0 females: GD 6 (September 29, 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

Dose Range-finding Study	Modified One-Generation Study
	F_1 rats (biosampling cohort): lifetime exposure through PND 56
	F_1 rats (subchronic cohort): lifetime exposure through PND 110–112 (males) or through PND 111–113 (females)
	F_1 rats (prenatal cohort): lifetime exposure through PND 111–114 (males) or through PND 116–132 (females)
	F_1 rats (reproductive performance cohort): lifetime exposure through PND 161–167 (males) or through PND 151–169 (females)
	F_2 rats (reproductive performance cohort): in utero through PND 28
Date of Last Exposure	
LD 28 (April 4, 2012)	F_0 females: LD 28 (November 15, 2012)
	F_1 rats (biosampling cohort): PND 56 (December 12, 2012)
	F_1 rats (subchronic cohort): PND 110–112 (February 4, 2013) (males) or PND 111–113 (February 5, 2013) (females)
	F_1 rats (prenatal cohort): PND 111–114 (February 7, 2013) (males) or PND 116–132 (February 24, 2013) (females)
	F_1 rats (reproductive performance cohort): PND 161–167 (April 1, 2013) (males) or PND 151–169 (April 3, 2013) (females)
	F_2 rats (reproductive performance cohort): PND 28 (through April 3, 2013)
Necropsy Dates	
Gross necropsies were conducted on F_0 females that did not deliver a litter and F_1 offspring euthanized moribund or found dead.	F_0 females: LD 28 (November 12–15, 2012)
	F_1 rats (biosampling cohort): not performed
	F_1 rats (subchronic cohort): PND 110–112 (February 4, 2013) (males) or PND 111–113 (February 5, 2013) (females)
	F_1 rats (prenatal cohort): PND 111–114 (February 6–7, 2013) (males) or GD 21 (February 11–24, 2013) (females)
	F_1 rats (reproductive performance cohort): PND 161–167 (March 26–April 1, 2013) (males) or PND 28 (March 19–April 2, 2013) (females)
	F_2 rats (reproductive performance cohort): March 19–April 2, 2013
Average Age at Necropsy	
Not performed	F_0 females: ~21 weeks
	F_1 rats (biosampling cohort): not performed

Dose Range-finding Study	Modified One-Generation Study
	F_1 rats (subchronic cohort): 110–112 days (males) or 111–113 days (females)
	F_1 rats (prenatal cohort): 111–114 days (males) or 116–132 days (females)
	F_1 rats (reproductive performance cohort): 161–167 days (males) or 153–169 days (females)
	F_2 rats (reproductive performance cohort): 28 days
Size of F_0 Study Groups	
8–14 time-mated females	26 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, subchronic, and prenatal cohorts): ≤2 (males and females)
	F_1 rats (reproductive performance cohort): ≤2 until PND 91, then housed individually except during cohabitation or when housed with their litters
Diet	
Irradiated certified Advanced Protocol Verified Casein Diet 1 IF 5K96 (PMI Nutrition International, St. Louis, MO), available ad libitum	Same as dose range-finding study
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), changed weekly	Same as dose range-finding study
Cage Filters	
Filter paper (Granville Milling Co., Creedmoor, NC), changed weekly	Same as dose range-finding study

Dose Range-finding Study	Modified One-Generation Study
Racks	
Stainless steel (Lab Products, Inc., Seaford, DE), changed and rotated every 2 weeks during the study	Same as dose range-finding study
Animal Room Environment	
Temperature: 71°F ± 2°F Relative humidity: 49.5% ± 5% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour	Temperature: 72°F ± 3°F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour
Exposure Concentrations	
0, 2,250, 5,000, 10,000, or 20,000 ppm EHMC in feed, available ad libitum	0, 1,000, 3,000, or 6,000 ppm EHMC 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.	Same as dose range-finding 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 10 pups (5/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 (PND 4 pups)	100% carbon dioxide with puncture of the diaphragm (adults and PND 28 pups) or intraperitoneal injection of a solution containing sodium pentobarbital (≤PND 12 pups and fetuses)
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 was recorded. Histopathological analysis of gross lesions was performed if collected.

Dose Range-finding Study	Modified One-Generation Study
Internal Dose Assessment/Additional Tissue Collection	
On GD 18, maternal plasma, amniotic fluid, and fetuses were collected from three pregnant dams/exposure group from the 0, 2,250, and 20,000 ppm groups. On LD 4, maternal plasma was collected from 2 or 3 dams/exposure group from the 0, 2,250, 10,000, and 20,000 ppm groups. On PND 4, pups (3/sex) were collected from 2 or 3 dams/exposure group from the 0, 2,250, and 10,000 ppm groups. On LD 28, maternal plasma was collected from three dams/exposure group from the 0, 2,250, and 10,000 ppm groups. None of the internal dose assessment samples were analyzed because in a preliminary investigation, it was observed that EHMC was not stable under the conditions used for sample collection and 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) for potential EHMC analyses. None of the internal dose assessment samples were analyzed because in a preliminary investigation, it was observed that EHMC was not stable under the conditions used for sample collection and storage.

GD = gestation day; LD = lactation day; PND = postnatal day; EHMC = 2-ethylhexyl p-methoxycinnamate; AGD = anogenital distance.

Table 3. Experimental Design and Materials and Methods in the Modified One-Generation Study of 2-Ethylhexyl p-Methoxycinnamate (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, and 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 [prenatal cohort] or 2 males and 2 females/litter [reproductive performance cohort]) 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), 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 was 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), dorsolateral and ventral prostate, 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 a week of their mating partner. Terminal body weights and the following organ weights were recorded: adrenal glands (paired), ovaries (left and right), 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, testis, epididymis, dorsolateral and ventral prostate gland, seminal vesicles, coagulating glands, LABC muscle, Cowper's glands (paired), 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 approximately −70°C until analysis. **Subchronic Cohort:** F_1 males and females were euthanized on PND 110–112 and PND 111–113, respectively. Blood was collected for hematology, clinical chemistry analyses, and micronucleus determination. The following hematology parameters were analyzed: erythrocyte count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, leukocyte count and differential, reticulocyte count, and platelet count. The following clinical chemistry parameters were analyzed: total protein, albumin, urea nitrogen, creatinine, alanine aminotransferase, sorbitol dehydrogenase, alkaline phosphatase, bile acids, glucose, creatine kinase, cholesterol, and triglycerides. The following organ weights were recorded: epididymides

Modified One-Generation Study
(right and left), heart, kidney (right and left), liver, lungs, dorsolateral prostate gland, ventral prostate gland, seminal vesicles with coagulating glands, testis (right and left), and thymus. In addition to gross lesions, histopathology was performed on the following organs: adrenal glands (paired), bone with marrow, brain, cervix, clitoral glands, epididymides (paired), esophagus, eyes, Harderian glands, heart and aorta, kidneys (paired), large intestine (cecum, colon, and rectum), liver, lungs, lymph nodes (mandibular and mesenteric), mammary glands, nose, ovaries (paired), pancreas, parathyroid glands, pituitary gland, preputial glands, prostate, salivary glands, seminal vesicles with coagulating gland, small intestine (duodenum, jejunum, and ileum), spleen, stomach (forestomach and glandular), testes (paired), thymus, thyroid gland, trachea, urinary bladder, uterus, vagina, and Zymbal's glands.

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 control group. Significance of all trend and pairwise tests is reported at both 0.05 and 0.01 levels.

Analysis of Fetal Malformations and Variations

Incidences of malformations and variations in fetuses were summarized as number of litters affected and as number of fetuses affected. Trend and pairwise analysis 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"[48] 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.[49] 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.,[50] formula $\overline{T}_{RS2}$.

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[51-53] 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,[51] 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.[54] Poly-3 tests used the continuity correction described by Nam.[55]

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[56] for small samples ($n < 20$) and Tukey's outer fences method[57] 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[58] and Williams.[59; 60]

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.[61] 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[62] (as modified by Williams[63]) and Dunn.[64] For these endpoints, the Jonckheere test[65] 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 nonnormally 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[66]). Pairwise comparisons were made by using a modified Wilcoxon test that incorporated litter effects.[67] The Hommel procedure was used to adjust for multiple comparisons.[68]

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.[69] 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 litter mean 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 nonnormality; (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.

A mixed model 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; this approach is adequate when attainment times are approximately normally distributed, and attainment is observed for all animals. Censored observations were not included in mixed models. For multiple comparisons, Dunnett-Hsu adjustments were used for mixed models.

To calculate mean attainment values adjusted for weaning weight, a linear model was fit to attainment day as a function of exposure 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 percent, obtained using the methods of Kaplan-Meier, 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,[70] 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 from 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.[71] 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-06.[72]

Dose Range-finding Study

Maternal Findings

Viability and Clinical Observations

In the dose range-finding study, one female in the 20,000 ppm group was euthanized on lactation day (LD) 15 due to excessive body weight loss and no surviving offspring. In addition, six females were euthanized on LD 4 and one female was euthanized on LD 14 because they had no surviving offspring (other dams were removed from the 0, 2,250, 5,000, and 20,000 ppm groups for scheduled biological sampling collection) (Appendix E). No clinical observations were attributed to 2-ethylhexyl p-methoxycinnamate (EHMC) in any exposure group (Appendix E).

Body Weights and Feed Consumption

On gestation day (GD) 21, the mean body weights of dams exposed to 20,000 ppm EHMC were significantly decreased by 22% relative to the control group (Table 4; Figure 5). This exposed group also displayed a transient loss in mean body weight over the GD 6–9 interval (loss of 3.4 g; control group gained 12.4 g) (Table 4). Maternal mean body weight gain between GD 6 and GD 21 in the 20,000 ppm group was significantly decreased by 68% relative to the control group (Table 4). LD 1 dam mean body weights of the 20,000 ppm group were significantly decreased by 20% relative to the control group (Table 4; Figure 5). Live litter size on postnatal day (PND) 0 was not affected by EHMC exposure (Appendix E); however, pup mean body weight on PND 1 of the 20,000 ppm group was significantly decreased by 39% relative to the control pup mean body weight, which also contributed to the reduction in maternal mean body weight gain observed during gestation (Table 4; Figure 5). During lactation, maternal mean body weights of dams exposed to ≤5,000 ppm were similar to those of the control group (Table 4; Figure 5). From LD 1 through LD 14, mean body weights of dams in the 20,000 ppm group were significantly decreased by 20%–37% compared to the control group mean body weights; the remaining dams in the 20,000 ppm group were removed from the study on LD 14 (Table 4).

Feed consumption by the 20,000 ppm group appeared to be significantly decreased over the GD 9–12 and GD 15–18 intervals (Table 5)—approximately 25% lower than feed consumption by the control group, but suspected feed wastage (dams digging and spilling feed that could not be measured) decreased confidence in the accuracy of the respective EHMC feed consumption data, with actual feed consumption likely less than estimated from measures of feed remaining in the feed dispenser at the time of feed change. The actual feed consumption being lower than what was estimated might have contributed to the lower dam and pup mean body weights of the 20,000 ppm group. Feed consumption by the other EHMC groups was similar to that of the control group (Table 5). EHMC intake for F_0 females in the 2,250, 5,000, 10,000, and 20,000 ppm groups, based on measured feed consumption and dietary concentrations for GD 6–21, was approximately 161, 365, 714, and 1,841 mg EHMC/kg body weight/day (mg/kg/day), respectively. Feed consumption by dams in the 20,000 ppm group between LD 4 and LD 7 was half that of the control group but represented only the two remaining dams with offspring (Table 5). LD 1 through LD 14 feed consumption by the 10,000 ppm group was approximately 83% that of the control group (Table 5), with the mean body weight significantly decreased at LD 4 and LD 14 (Table 4). EHMC intake for F_0 females in the 2,250, 5,000, and 10,000 ppm EHMC groups, based on feed consumption and dietary concentrations for LD 1 through LD 14, was approximately 410, 925, and 1,615 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-Ethylhexyl p-Methoxycinnamate in Feed during Gestation and Lactation (Dose Range-finding Study)

Parameter[a,b]	0 ppm	2,250 ppm	5,000 ppm	10,000 ppm	20,000 ppm
Gestation Day					
6	232.7 ± 4.0 (12)	229.4 ± 2.6 (12)	232.4 ± 3.9 (6)	229.7 ± 3.5 (8)	232.9 ± 3.5 (13)
9	245.0 ± 4.7** (12)	243.1 ± 3.1 (12)	247.5 ± 4.7 (6)	237.9 ± 4.2 (8)	229.5 ± 3.2** (13)
12	259.8 ± 5.0** (12)	258.5 ± 3.0 (12)	262.2 ± 4.7 (6)	251.8 ± 5.1 (8)	231.0 ± 3.0** (13)
15	278.1 ± 5.4** (12)	277.5 ± 3.3 (12)	280.3 ± 5.3 (6)	272.5 ± 6.0 (8)	239.3 ± 3.9** (13)
18	311.6 ± 6.3** (12)	312.6 ± 4.8 (12)	312.0 ± 5.7 (6)	309.4 ± 7.6 (8)	253.4 ± 4.4** (13)
21	339.7 ± 9.3** (9)	344.2 ± 5.6 (9)	350.0 ± 5.8 (6)	343.4 ± 9.0 (7)	265.9 ± 7.2** (10)
Gestation Weight Change					
Gestation Day Interval					
6–21	110.1 ± 8.1** (9)	114.0 ± 5.5 (9)	117.6 ± 3.7 (6)	113.4 ± 6.5 (7)	35.1 ± 8.0** (10)
6–9	12.4 ± 1.3** (12)	13.8 ± 1.3 (12)	15.2 ± 1.7 (6)	8.2 ± 1.2* (8)	−3.4 ± 1.3** (13)
9–12	14.8 ± 0.8** (12)	15.4 ± 0.7 (12)	14.6 ± 1.4 (6)	13.8 ± 1.5 (8)	1.5 ± 1.3** (13)
12–15	18.3 ± 0.9** (12)	19.0 ± 1.0 (12)	18.1 ± 1.7 (6)	20.8 ± 1.9 (8)	8.4 ± 1.5** (13)
15–18	33.5 ± 2.8** (12)	35.0 ± 2.4 (12)	31.7 ± 1.5 (6)	36.9 ± 2.2 (8)	14.0 ± 1.7** (13)
18–21	32.8 ± 3.2* (9)	34.7 ± 3.2 (9)	38.0 ± 1.8 (6)	36.1 ± 2.4 (7)	17.7 ± 4.7* (10)
Lactation Day					
1	254.7 ± 3.7** (8)	249.4 ± 4.5 (9)	255.2 ± 5.4 (6)	247.5 ± 5.4 (8)	203.7 ± 7.9** (8)
4	271.2 ± 5.6** (8)	266.6 ± 3.8 (9)	270.8 ± 4.4 (6)	253.2 ± 6.8* (8)	204.8 ± 6.4** (8)
7	275.8 ± 2.5* (5)	270.3 ± 4.0 (6)	276.3 ± 4.1 (6)	261.2 ± 6.9 (6)	193.5 ± 0.8** (2)
14	279.6 ± 6.3* (5)	287.9 ± 4.8 (6)	289.0 ± 5.7 (6)	248.0 ± 13.0* (6)	176.0 ± 4.2** (2)
21	272.7 ± 9.5 (5)	278.3 ± 5.7 (6)	284.8 ± 6.7 (6)	234.0 ± 14.1* (6)	–[c]
Lactation Weight Change					
Lactation Day Interval					
1–21	17.0 ± 8.8 (5)	26.5 ± 5.6 (6)	29.6 ± 4.9 (6)	−13.4 ± 11.8 (6)	–[c]
1–4	16.5 ± 3.0** (8)	17.1 ± 3.1 (9)	15.5 ± 1.9 (6)	5.6 ± 3.0* (8)	1.1 ± 3.6** (8)
4–7	8.0 ± 2.2 (5)	4.3 ± 7.7 (6)	5.6 ± 6.0 (6)	5.1 ± 2.9 (6)	−6.4 ± 1.9 (2)
7–14	3.8 ± 7.9* (5)	17.6 ± 5.9 (6)	12.7 ± 6.8 (6)	−13.2 ± 7.9 (6)	−17.5 ± 5.0 (2)
14–21	−6.8 ± 11.6 (5)	−9.6 ± 2.5 (6)	−4.1 ± 7.9 (6)	−14.0 ± 9.5 (6)	–[c]

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.
[c]The 20,000 ppm group was removed on lactation day 14 due to excessive body weight loss and no surviving offspring.

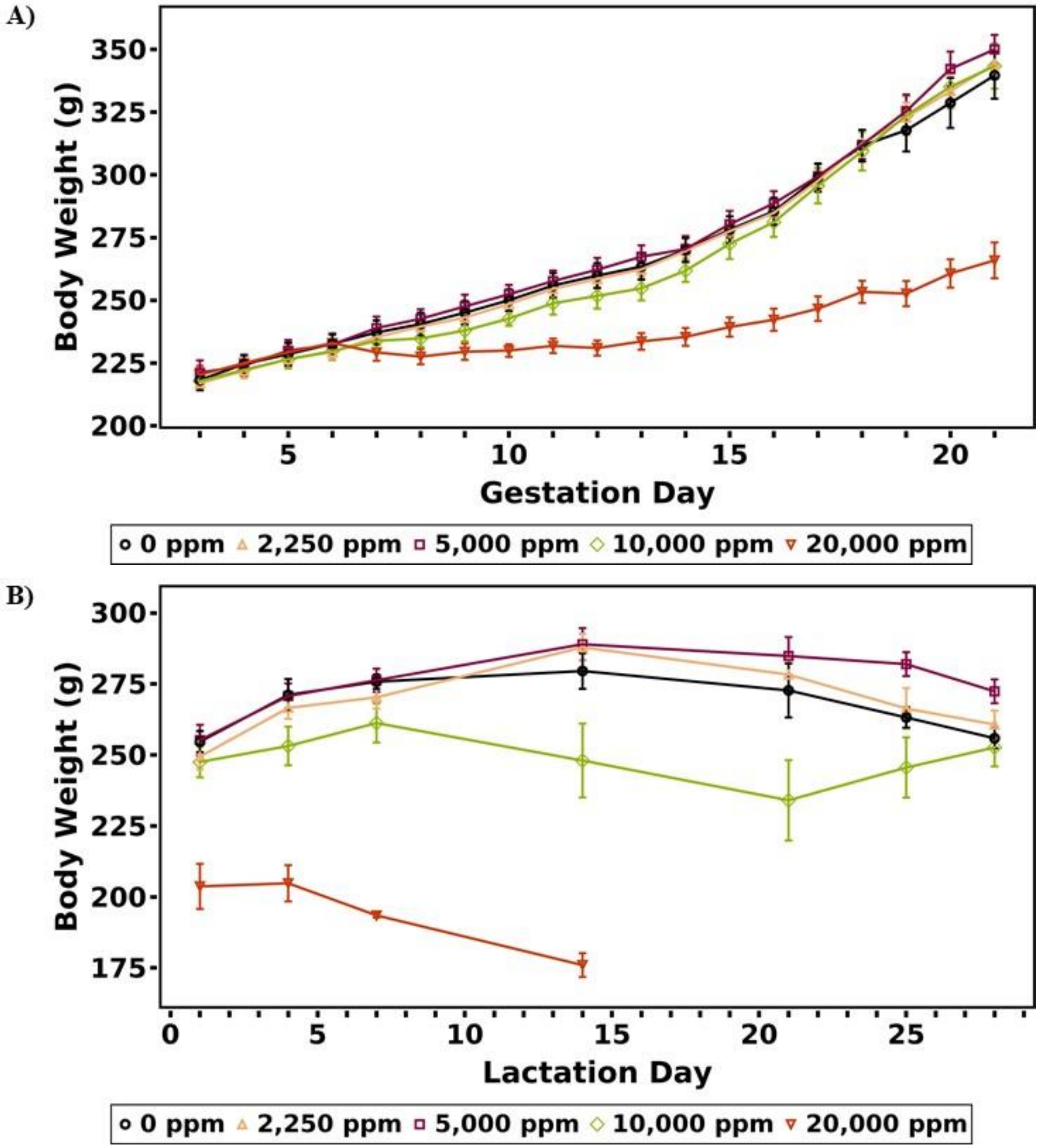


Figure 5. Growth Curves for F_0 Female Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed during Gestation and Lactation (Dose Range-finding Study)

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-Ethylhexyl p-Methoxycinnamate in Feed during Gestation and Lactation (Dose Range-finding Study)

Parameter[a,b]	0 ppm	2,250 ppm	5,000 ppm	10,000 ppm	20,000 ppm
Feed Consumption (g/animal/day)[c]					
Gestation Day Interval					
6–21	19.7 ± 0.8 (9)	19.5 ± 0.4 (8)	20.4 ± 0.7 (6)	19.5 ± 1.0 (8)	21.9 ± 1.5 (10)
3–6	17.8 ± 0.7 (12)	17.3 ± 0.3 (12)	18.0 ± 1.0 (6)	16.7 ± 0.7 (8)	17.3 ± 0.5 (13)
6–9	18.3 ± 0.7 (12)	17.8 ± 0.4 (12)	18.9 ± 0.9 (6)	16.4 ± 1.9 (8)	31.7 ± 2.9* (10)
9–12	19.1 ± 0.6** (12)	18.8 ± 0.5 (12)	19.2 ± 1.0 (6)	17.7 ± 1.1 (8)	13.8 ± 0.5** (13)
12–15	19.6 ± 0.7 (12)	19.8 ± 0.6 (12)	19.9 ± 1.0 (6)	18.9 ± 1.2 (8)	29.5 ± 3.4 (6)
15–18	22.1 ± 0.6** (12)	21.8 ± 0.6 (12)	21.3 ± 0.6 (6)	23.0 ± 1.1 (8)	17.3 ± 0.8** (13)
18–21	21.0 ± 0.9 (9)	22.0 ± 0.8 (8)	22.8 ± 0.6 (6)	21.3 ± 1.0 (8)	27.9 ± 5.9 (5)
Lactation Day Interval					
1–14	49.8 ± 1.7 (5)	48.8 ± 3.3 (6)	50.5 ± 1.0 (6)	41.5 ± 4.7 (5)[d]	–[d,e]
1–4	33.7 ± 2.2 (8)	33.8 ± 2.0 (9)	32.3 ± 1.5 (6)	30.4 ± 2.2 (8)	38.2 ± 9.1 (4)
4–7	43.3 ± 1.2 (5)	43.4 ± 3.9 (6)	43.5 ± 1.1 (6)	40.0 ± 2.3 (6)	19.6 ± 1.1 (2)
7–14	60.3 ± 2.5 (5)	57.6 ± 4.1 (6)	61.2 ± 1.5 (6)	46.9 ± 6.8 (5)	–[e]
Chemical Intake (mg/kg/day)[f,g]					
GD 6–21	0 ± 0.0 (9)	161.1 ± 3.5 (8)	365.2 ± 10.1 (6)	713.5 ± 29.0 (8)	1,841.4 ± 125.7 (10)
LD 1–14	0 ± 0.0 (5)	409.8 ± 31.1 (6)	924.9 ± 14.1 (6)	1,615.0 ± 125.6 (5)[e]	–[d,e]

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).
[c]Statistical analysis performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
[d]Consumption and chemical intake was omitted for animals with no recorded consumption during the LD 7–14 interval.
[e]The 20,000 ppm group was removed on LD 14 due to excessive body weight loss and no surviving offspring.
[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.

Maternal Reproductive Performance

EHMC did not affect the number of animals littering, with the possible exception of the 20,000 ppm group in which only 80% of the dams littered. Litter size on PND 0 was similar across all the exposure groups (Table 6).

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

Parameter[a]	0 ppm	2,250 ppm	5,000 ppm	10,000 ppm	20,000 ppm
Time-mated Females (GD 6)	14[b]	14[b,c]	8	8	14[b]
Females Pregnant (%)	12 (85.7)	12 (85.7)	6 (75.0)	8 (100.0)	13 (92.9)
Females Not Pregnant (%)	2 (14.3)	2 (14.3)	2 (25.0)	0 (0.0)	1 (7.1)
Dams Removed on GD 18[d]	3	3	0	0	3
Dams Not Delivering with Evidence of Pregnancy (%)	1 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)	2 (20.0)
Dams with Litters on PND 0 (%)[e]	8 (88.9)	9 (100.0)	6 (100.0)	8 (100.0)	8 (80.0)
Gestation Length (days)[f,g,h]	22.0 ± 0.0 (8)	22.3 ± 0.2 (9)	22.2 ± 0.2 (6)	21.9 ± 0.1 (8)	22.3 ± 0.2 (8)
Live Litter Size on PND 0[f,h]	11.9 ± 1.0 (8)	11.7 ± 1.0 (9)	11.8 ± 0.5 (6)	13.6 ± 0.8 (8)	11.1 ± 0.9 (8)
PND 1 Pup Weight[h,i,j]	6.90 ± 0.11** 8 (94)	6.78 ± 0.16 9 (104)	7.10 ± 0.25 6 (70)	6.62 ± 0.15 8 (108)	4.19 ± 0.41** 6 (47)
Percent Live Male Pups/Litter[h]	63.07 ± 4.75* (8)	50.25 ± 5.68 (9)	53.14 ± 4.23 (6)	51.09 ± 6.86 (8)	44.22 ± 5.53* (8)

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 study between mating and littering excluded from calculations of % littered females.
[b]Includes six time-mated (pregnant) rats used for biological sample collection for methods development.
[c]Excludes animal removed on GD 5.
[d]Dams removed on GD 18 for biological sample collection.
[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 litters examined (number of pups).
[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

EHMC exposure was associated with fewer live pups per litter in the 20,000 ppm group (approximately four pups per litter) on PND 1 than in the control group; by PND 4, an average of 7.5 pups per litter were alive in the 20,000 ppm group (Table 7). In contrast, average live PND 4 litter size in the control group was 11.8. Live litter size and survival ratios of the other EHMC-exposed groups were similar to those of the control group (Table 7). Over the lactation period (PND 1 through PND 28), there were nine dead/euthanized pups (from three litters) in the 10,000 ppm group and 89 dead/euthanized pups (from eight litters) in the 20,000 ppm group, compared to zero dead/euthanized pups in the control group (Appendix E). In the 5,000 and 2,250 ppm groups, one pup was found dead and two pups (from two litters) were euthanized, respectively (Appendix E).

Male and female pup body weights of the 10,000 and 20,000 ppm groups were significantly decreased (16%–76%) relative to the control groups at most time points (Table 8; Figure 6, Figure 7). Adverse F_1 pup clinical observations in the 10,000 and 20,000 ppm groups were consistent with the effects of EHMC exposure on pup survival (Appendix E). Findings included observations of pups found dead, cannibalized or missing, no milk in the stomach, and emaciated. There were no notable gross findings in the F_1 offspring examined. Necropsy findings for pups found dead on or after PND 1 were limited to absence of milk/food in the stomach (Appendix E).

Table 7. Summary of F_1 Litter Size and Pup Survival Following Perinatal Exposure to 2-Ethylhexyl p-Methoxycinnamate (Dose Range-finding Study)

Postnatal Day	0 ppm	2,250 ppm	5,000 ppm	10,000 ppm	20,000 ppm
No. of Live Pups (Litters)[a]					
0	95 (8)	105 (9)	71 (6)	109 (8)	89 (8)
Total Litter Size[b,c]					
0	12.3 ± 1.0 (8)	12.6 ± 1.4 (9)	11.8 ± 0.5 (6)	13.8 ± 0.9 (8)	12.4 ± 0.8 (8)
Live Litter Size[b,c]					
0	11.9 ± 1.0 (8)	11.7 ± 1.0 (9)	11.8 ±0.5 (6)	13.6 ± 0.8 (8)	11.1 ± 0.9 (8)
1	11.8 ± 0.9 (8)	11.6 ± 1.1 (9)	11.7 ± 0.6 (6)	13.5 ± 0.8 (8)	7.8 ± 1.2 (6)
4[d]	11.8 ± 0.9 (8)	11.6 ± 1.1 (9)	11.7 ±0.6 (6)	13.4 ± 0.8 (8)	7.5 ± 1.5 (2)
7	11.0 ± 0.7 (5)	11.2 ± 1.6 (6)	11.7 ± 0.6 (6)	13.0 ± 0.7 (6)	7.5 ± 1.5 (2)
14	11.0 ± 0.7 (5)	11.0 ± 1.5 (6)	11.7 ± 0.6 (6)	12.0 ± 0.7 (6)	6.0 (1)
21	11.0 ± 0.7 (5)	11.0 ± 1.5 (6)	11.7 ± 0.6 (6)	12.0 ± 0.7 (6)	–[e]
28	11.0 ± 0.7 (5)	11.0 ± 1.5 (6)	11.7 ± 0.6 (6)	12.0 ± 0.7 (6)	–[e]
No. of Dead Pups (Litters)[a]					
0	3 (2)	8 (3)	0 (0)	1 (1)	10 (5)
1–4	1 (1)	1 (1)	1 (1)	2 (2)	74 (7)
5–28	0 (0)	1 (1)	0 (0)	8 (2)	15 (2)
Dead per Litter[b,c]					
0	0.38 ± 0.26 (8)	0.89 ± 0.65 (9)	0.00 ± 0.00 (6)	0.13 ± 0.13 (8)	1.25 ± 0.45 (8)
1–4	0.13 ± 0.13** (8)	0.11 ± 0.11 (9)	0.17 ±0.17 (6)	0.25 ± 0.16 (8)	9.25 ± 1.96** (8)
5–28	0.00 ± 0.00** (5)	0.17 ± 0.17 (6)	0.00 ± 0.00 (6)	1.33 ± 0.88 (6)	7.50 ± 1.50** (2)
1–28	0.00 ± 0.00** (5)	0.33 ± 0.21 (6)	0.17 ± 0.17 (6)	1.50 ± 0.85 (6)	11.13 ± 0.90** (8)
Live Birth Ratio[b,c]					
0	0.97 ± 0.02 (8)	0.95 ± 0.03 (9)	1.00 ± 0.00 (6)	0.99 ± 0.01 (8)	0.89 ± 0.04 (8)
Survival Ratio[b,c]					
0	0.97 ± 0.02 (8)	0.95 ± 0.03 (9)	1.00 ± 0.00 (6)	0.99 ± 0.01 (8)	0.89 ± 0.04 (8)
1–4	0.99 ± 0.01** (8)	0.99 ± 0.01 (9)	0.98 ± 0.02 (6)	0.98 ± 0.01 (8)	0.24 ± 0.16** (8)
5–28	1.00 ± 0.00** (5)	0.99 ± 0.01 (6)	1.00 ± 0.00 (6)	0.91 ± 0.06 (6)	0.00 ± 0.00** (2)
1–28	1.00 ± 0.00** (5)	0.97 ± 0.02 (6)	0.98 ± 0.02 (6)	0.90 ± 0.06 (6)	0.00 ±0.00** (8)

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 $p \leq 0.01$.
[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.
[d]Up to three dams and their litters in the 0, 2,250, 10,000, and 20,000 ppm groups were removed for biological sample collection on postnatal day 4.
[e]The 20,000 ppm group was removed on postnatal day 14 due to pup moribundity and mortality.

Table 8. Summary of F_1 Male and Female Pup Mean Body Weights and Body Weight Gains Following Perinatal Exposure to 2-Ethylhexyl p-Methoxycinnamate (Dose Range-finding Study)[a,b]

Postnatal Day[c]	0 ppm	2,250 ppm	5,000 ppm	10,000 ppm	20,000 ppm
Male					
1	7.00 ± 0.11** 58 (8)[d]	6.83 ± 0.18 50 (9)	7.35 ± 0.28 37 (6)	6.79 ± 0.16 55 (8)	4.42 ± 0.35** 18 (5)
4	10.41 ± 0.14** 58 (8)	10.08 ± 0.29 50 (9)	10.59 ± 0.27 37 (6)	9.48 ± 0.28 54 (8)	4.97 ± 0.71** 14 (4)
7	15.16 ± 0.37** 36 (5)	14.79 ± 0.54 28 (6)	14.58 ± 0.30 37 (6)	12.77 ± 0.64** 34 (6)	6.60 ± 0.46** 5 (2)
14	29.79 ± 0.97** 36 (5)	27.87 ± 0.29 27 (6)	26.08 ± 0.67 37 (6)	20.03 ± 1.60** 32 (6)	7.04 ± 1.06** 5 (2)
21	44.34 ± 1.53** 36 (5)	42.16 ± 1.33 27 (6)	37.84 ± 1.07 37 (6)	24.01 ± 2.81** 32 (6)	–[e]
28	76.84 ± 2.50** 36 (5)	73.13 ± 2.00 27 (6)	67.00 ± 2.26 37 (6)	36.83 ± 4.40** 32 (6)	–[e]
1–28[f]	69.84 ± 2.48** 36 (5)	66.16 ± 2.06 27 (6)	59.67 ± 2.05 37 (6)	30.13 ± 4.27** 32 (6)	–[e]
Female					
1	6.67 ± 0.12** 36 (8)	6.71 ± 0.17 54 (9)	6.77 ± 0.24 33 (6)	6.37 ± 0.17 53 (8)	4.29 ± 0.39** 29 (6)
4	10.01 ± 0.10** 36 (8)	9.96 ± 0.30 54 (9)	9.78 ± 0.29 33 (6)	8.93 ± 0.28* 53 (8)	5.06 ± 0.69** 22 (4)
7	14.96 ± 0.30** 19 (5)	14.88 ± 0.87 39 (6)	13.31 ± 0.46 33 (6)	11.73 ± 0.57** 43 (6)	7.59 ± 0.52** 10 (2)
14	29.22 ± 0.94** 19 (5)	27.75 ± 1.35 39 (6)	24.46 ± 0.76* 33 (6)	18.46 ± 1.26** 40 (6)	7.68 ± 0.76** 10 (2)
21	41.04 ± 2.01** 19 (5)	42.17 ± 2.92 39 (6)	35.47 ± 1.32 33 (6)	21.89 ± 2.09** 40 (6)	–[e]
28	72.90 ± 1.92** 18 (5)	69.33 ± 4.07 39 (6)	60.85 ± 2.50* 33 (6)	33.93 ± 3.29** 40 (6)	–[e]
1–28[f]	66.14 ± 1.80** 18 (5)	62.46 ± 3.93 39 (6)	54.13 ± 2.32* 33 (6)	27.63 ± 3.18** 40 (6)	–[e]
Male and Female					
1	6.90 ± 0.11** 94 (8)	6.78 ± 0.16 104 (9)	7.10 ± 0.25 70 (6)	6.62 ± 0.15 108 (8)	4.19 ± 0.41** 47 (6)
4	10.23 ± 0.11** 94 (8)	9.99 ± 0.28 104 (9)	10.22 ± 0.28 70 (6)	9.21 ± 0.27* 107 (8)	5.01 ± 0.69** 36 (4)
7	15.00 ± 0.33** 55 (5)	14.70 ± 0.60 67 (6)	14.00 ± 0.37 70 (6)	12.25 ± 0.56** 77 (6)	7.06 ± 0.42** 15 (2)
14	29.41 ± 0.91** 55 (5)	27.48 ± 0.72 66 (6)	25.33 ± 0.71* 70 (6)	19.32 ± 1.40** 72 (6)	7.20 ± 0.76** 15 (2)
21	42.64 ± 1.57** 55 (5)	41.81 ± 1.90 66 (6)	36.69 ± 1.18 70 (6)	23.02 ± 2.48** 72 (6)	–[e]

Postnatal Day[c]	0 ppm	2,250 ppm	5,000 ppm	10,000 ppm	20,000 ppm
28	75.36 ± 2.33** 54 (5)	70.38 ± 2.93 66 (6)	64.05 ± 2.30* 70 (6)	35.70 ± 3.93** 72 (6)	–[e]
1–28[f]	68.44 ± 2.25** 54 (5)	63.50 ± 2.87 66 (6)	57.01 ± 2.11* 70 (6)	29.20 ± 3.82** 72 (6)	–[e]

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 (PND) 1.
[d]n = the number of pups examined (number of litters).
[e]The 20,000 ppm group was removed on PND 14 due to pup moribundity and mortality.
[f]Body weight gain (data are presented in grams).

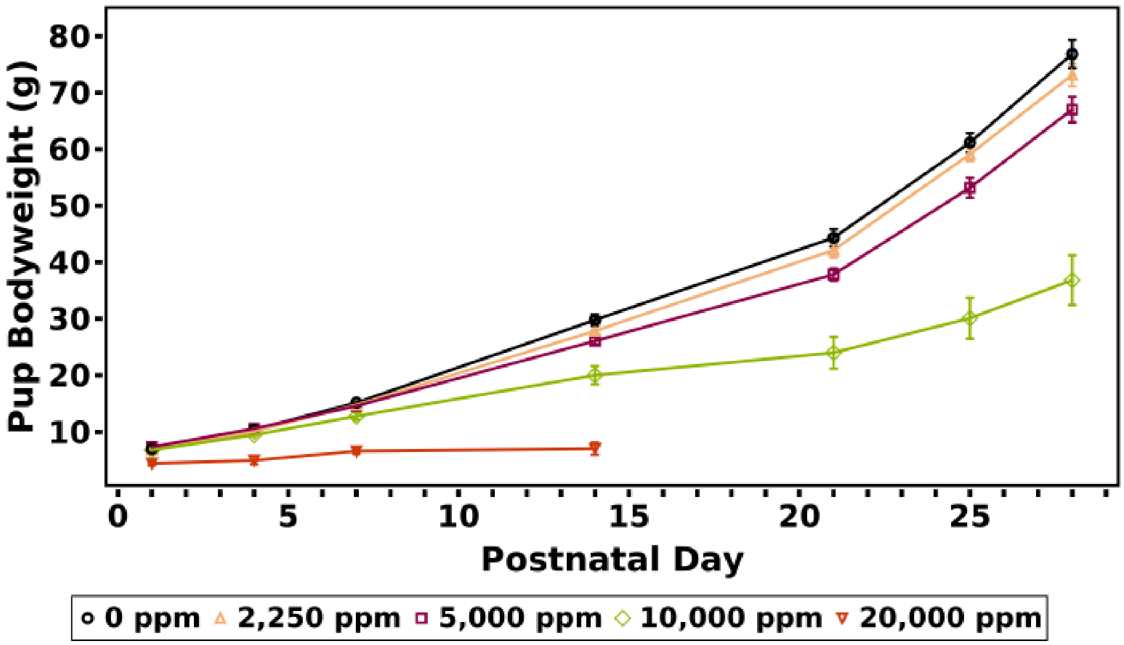


Figure 6. Lactation Growth Curves for F_1 Male Pups Following Perinatal Exposure to 2-Ethylhexyl p-Methoxycinnamate (Dose Range-finding Study)

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

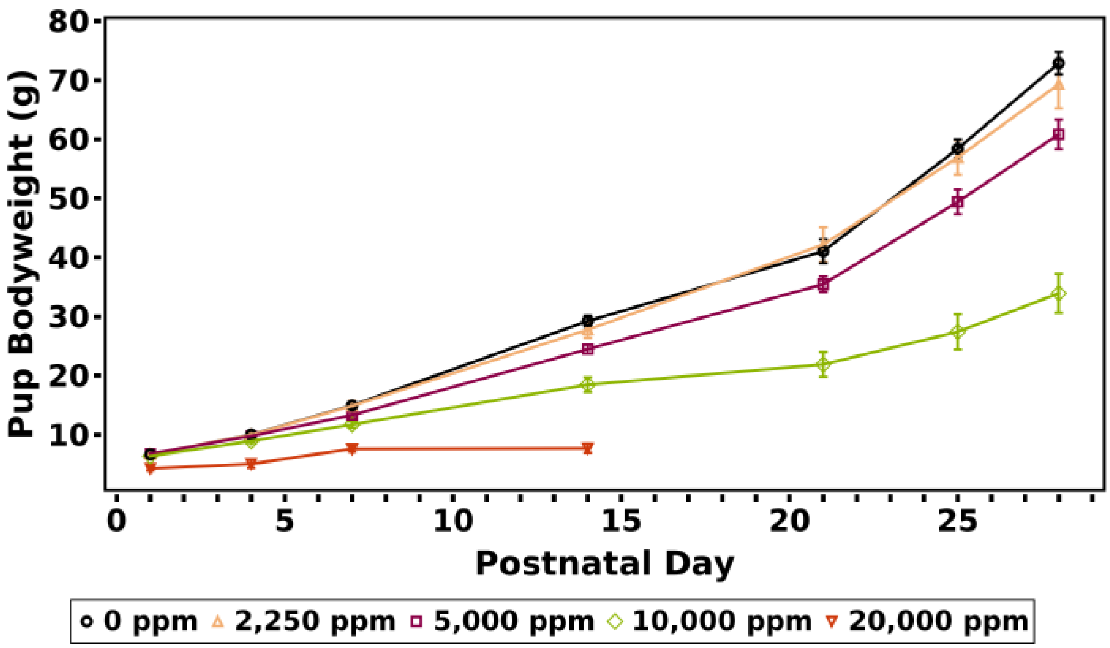


Figure 7. Lactation Growth Curves for F_1 Female Pups Following Perinatal Exposure to 2-Ethylhexyl p-Methoxycinnamate (Dose Range-finding Study)

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-Ethylhexyl p-Methoxycinnamate

Selection of 6,000 ppm as the highest exposure concentration for the modified one-generation (MOG) study was based on excessively lower pup mean body weight observed at the 10,000 ppm exposure concentration for the dose range-finding study. Compared to the control group on PND 28, relative pup body weights of dams exposed to 5,000 ppm were lower for both females (17%, significant) and males (13%), approximating the targeted 10% reduction to ensure a challenge recognizing the limited sample size (Table 8). Exposure concentration spacing for the MOG study (1,000, 3,000, and 6,000 ppm) was selected to achieve an ideal no-observed-adverse-effect level and to avoid excessive exposure overlap due to higher feed consumption during pregnancy and lactation.

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.

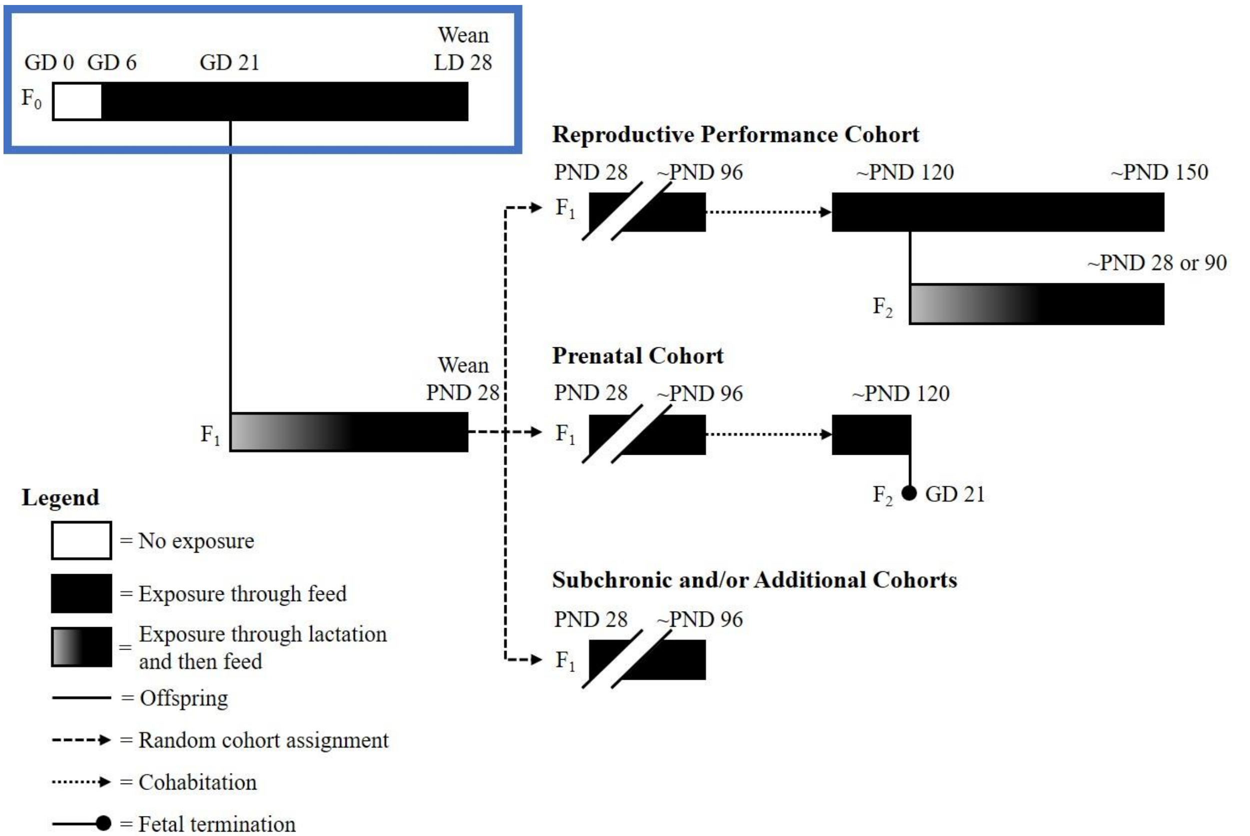


Figure 8. Design of the Modified One-Generation Study—F_0 Generation

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

F_0 Viability and Clinical Observations

EHMC exposure did not affect viability of the F_0 females (Appendix E). One female in the 6,000 ppm group was removed on study day 8 with exophthalmos and a head tilt; histopathology revealed retinal atrophy. Due to the timing of the lesion, and given this was an isolated case, the observation was not considered related to EHMC exposure. No clinical observations were attributed to EHMC exposure (Appendix E).

F_0 Gestation Body Weights and Feed Consumption

F_0 females exposed to EHMC displayed similar mean body weights and body weight gains throughout gestation as the control group (Table 9; Figure 9). EHMC exposure did not adversely affect feed consumption during gestation (Table 10). EHMC intake based on feed consumption and dietary concentrations during gestation (F_0 [Table 10] and both F_1 cohorts [Appendix E]) was similar to postweaning intake by both sexes (Appendix E), with intake ranging from 70 to 87, 207 to 263, and 419 to 528 mg/kg/day by the 1,000, 3,000 and 6,000 ppm groups, respectively. EHMC intake was similar during the early lactational period of both generations and was approximately twofold greater than it was during the other periods (Appendix E).

Table 9. Summary of Mean Body Weights and Body Weight Gains of F_0 Female Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed during Gestation

Parameter[a,b]	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
Gestation Day				
6	241.9 ± 2.5 (22)	242.0 ± 2.5 (24)	239.6 ± 2.7 (19)	239.5 ± 3.2 (22)
9	254.5 ± 2.6 (22)	254.2 ± 2.2 (24)	252.1 ± 2.8 (19)	251.8 ± 2.4 (22)
12	269.1 ± 2.8 (22)	269.9 ± 2.2 (24)	266.1 ± 2.8 (19)	267.9 ± 2.3 (22)
15	287.1 ± 3.0 (22)	288.9 ± 2.4 (24)	284.3 ± 3.2 (19)	286.0 ± 2.7 (22)
18	322.3 ± 3.4 (22)	329.1 ± 2.9 (24)	321.7 ± 3.7 (19)	322.8 ± 3.8 (22)
21	359.6 ± 4.4 (22)	370.0 ± 3.9 (24)	360.8 ± 4.5 (19)	360.2 ± 4.6 (22)
Gestation Weight Change				
Gestation Day Interval				
6–21	117.7 ± 3.5 (22)	128.0 ± 3.2 (24)	121.2 ± 3.3 (19)	120.7 ± 2.7 (22)
3–6	13.7 ± 1.4 (22)	13.6 ± 1.8 (24)	11.1 ± 1.2 (19)	11.9 ± 2.1 (22)
6–9	12.6 ± 0.7 (22)	12.2 ± 0.8 (24)	12.6 ± 0.6 (19)	12.3 ± 1.4 (22)
9–12	14.6 ± 0.7 (22)	15.7 ± 0.7 (24)	13.9 ± 0.7 (19)	16.0 ± 0.7 (22)
12–15	18.0 ± 0.9 (22)	19.0 ± 0.6 (24)	18.3 ± 0.9 (19)	18.1 ± 0.8 (22)
15–18	35.3 ± 1.3 (22)	40.2 ± 1.2* (24)	37.3 ± 1.1 (19)	36.8 ± 1.4 (22)
18–21	37.2 ± 1.5 (22)	40.9 ± 1.6 (24)	39.1 ± 1.9 (19)	37.4 ± 1.3 (22)

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

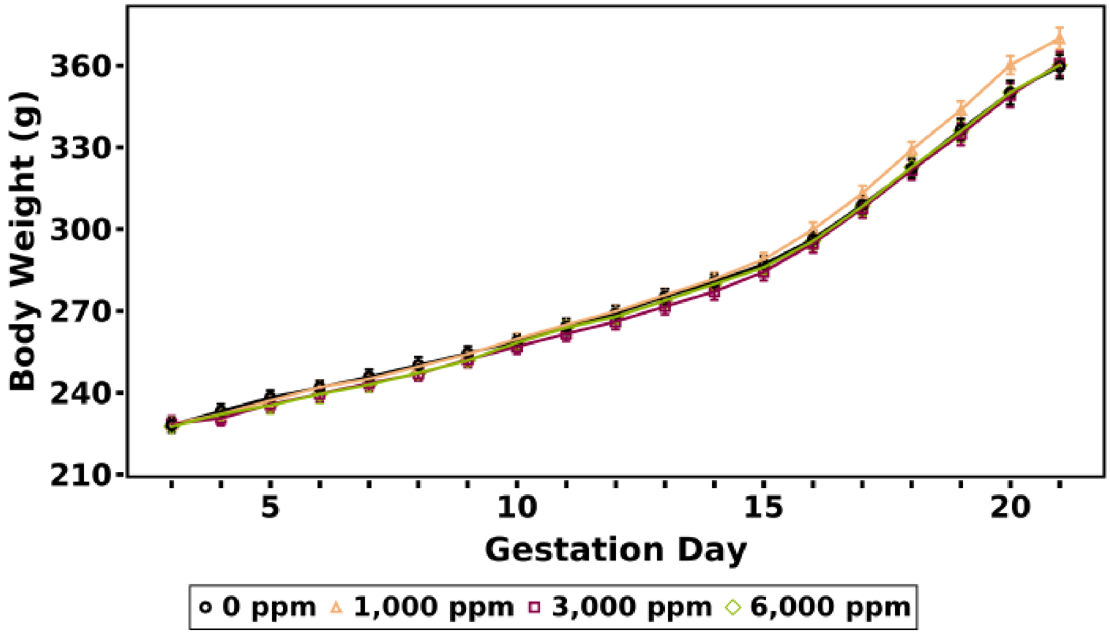


Figure 9. Growth Curves for F_0 Female Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed during Gestation

Information for statistical significance in maternal weights is provided in Table 9.

Table 10. Summary of Feed and Test Article Consumption of F_0 Female Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed during Gestation

Gestation Day Interval[a]	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
Feed Consumption (g/animal/day)[b]				
6–21	20.2 ± 0.4 (22)	20.2 ± 0.2 (24)	19.7 ± 0.3 (19)	19.9 ± 0.3 (21)
3–6	17.8 ± 0.4 (22)	17.2 ± 0.3 (24)	17.2 ± 0.4 (19)	17.6 ± 0.3 (21)
6–9	18.4 ± 0.5** (22)	18.2 ± 0.2 (24)	17.6 ± 0.4 (19)	16.8 ± 0.4** (21)
9–12	18.9 ± 0.5 (22)	18.8 ± 0.2 (24)	17.8 ± 0.5 (19)	18.9 ± 0.3 (21)
12–15	19.8 ± 0.5 (22)	19.8 ± 0.2 (24)	19.1 ± 0.5 (19)	19.6 ± 0.4 (21)
15–18	21.8 ± 0.5 (22)	22.5 ± 0.4 (24)	22.1 ± 0.3 (19)	22.3 ± 0.5 (22)
18–21	22 ± 0.6 (22)	21.8 ± 0.5 (24)	21.9 ± 0.4 (19)	22.2 ± 0.4 (22)
Chemical Intake (mg/kg/day)[c,d]				
6–21	0.0 ± 0.0 (22)	69.6 ± 0.6 (24)	207.2 ± 3.4 (19)	418.7 ± 6.9 (21)

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 $p \leq 0.01$.
[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]Statistical analysis performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
[c]Chemical intake calculated as: ([exposure concentration × feed consumption]/[average body weight of day range]).
[d]No statistical analysis performed on the chemical intake data.

Maternal Reproductive Performance

Across all exposure groups, 17 of 104 time-mated rats were not pregnant (Table 11; Appendix E). There was no effect of EHMC exposure on the proportion of dams that produced viable litters, or on gestation length (Table 11). PND 0 litter size was slightly, but significantly, increased in the 1,000 ppm group relative to the control group, which was likely the result of the control group litter size being slightly lower than expected (Table 11). Litter sizes among all other groups were similar. There was no effect of EHMC exposure on PND 1 pup weight or sex ratio (Table 11).

Table 11. Summary of the Reproductive Performance of F_0 Female Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed during Gestation

Parameter[a]	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
Time-mated Females (GD 6)	26	26	26	26
Females Pregnant (%)	22 (84.6)	24 (92.3)	19 (73.1)	22 (84.6)
Females Not Pregnant (%)	4 (15.4)	2 (7.7)	7 (26.9)	4 (15.4)
Dams with Litters on PND 0 (%)[b]	22 (100.0)	24 (100.0)	19 (100.0)	22 (100.0)
Gestation Length (days)[c,d,e]	22.0 ± 0.0 (22)	22.0 ± 0.0 (24)	21.9 ± 0.1 (19)	22.1 ± 0.1 (22)
Live Litter Size on PND 0[c,e]	10.8 ± 0.7 (22)	13.0 ± 0.4* (24)	11.7 ± 0.4 (19)	11.1 ± 0.7 (22)
PND 1 Pup Weight[e,f,g]	6.90 ± 0.07 235 (22)	6.89 ± 0.08 311 (24)	6.91 ± 0.07 221 (19)	7.01 ± 0.09 244 (22)
Percent Live Male Pups/Litter[c,e]	57.11 ± 3.34 (22)	48.94 ± 2.94 (24)	49.98 ± 2.55 (19)	49.80 ± 3.27 (22)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p \leq 0.05$.
GD = gestation day; PND = postnatal day.
[a]Animals removed from the study between mating and littering were excluded from calculations of % littered females.
[b]Percentage is the number of littered females/pregnant females. Statistical analysis performed by the Cochran-Armitage (trend) and Fisher's exact (pairwise) tests.
[c]Statistical analysis performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
[d]Gestation length was calculated for time-mated females that delivered a litter.
[e]Data are displayed as mean ± standard error (n).
[f]n = the number of pups examined (number of litters).
[g]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.

Lactation Body Weights and Feed Consumption

F_0 females exposed to EHMC displayed similar mean body weights and body weight gains throughout most of lactation (Table 12; Figure 10). On LD 10 and LD 13 the mean body weights of the 6,000 ppm group were slightly but significantly decreased and were lower on LD 16 (approximately 3%, negative trend) relative to the control group and were preceded by slightly but significantly decreased (7%, negative trend) feed consumption over the LD 7–10 interval (Appendix E). These lower weights, although small in magnitude, occurred concomitantly with lower pup weights (Appendix E).

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

Lactation Day	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
Body Weight (g)[b]				
1	267.9 ± 4.4 (22)	265.4 ± 3.9 (24)	265.2 ± 3.2 (19)	262.4 ± 3.6 (22)
10	303.4 ± 3.3** (21)	300.7 ± 2.8 (24)	295.5 ± 3.4 (19)	293.1 ± 2.3* (22)
13	306.6 ± 3.1* (21)	303.4 ± 2.5 (24)	301.5 ± 3.2 (19)	295.6 ± 2.7* (22)
16	305.4 ± 3.4* (21)	305.4 ± 2.5 (24)	304.6 ± 3.0 (19)	296.6 ± 2.6 (22)
28	283.1 ± 3.3 (21)	283.5 ± 2.9 (24)	280.4 ± 3.1 (19)	282.7 ± 2.3 (22)
Body Weight Gain (g)[b]				
1–28	15.2 ± 3.0 (21)	18.1 ± 3.2 (24)	15.2 ± 2.7 (19)	20.3 ± 2.5 (22)
Feed Consumption[c]				
1–13 (g/animal/day)	45.2 ± 1.3* (21)	46.2 ± 0.7 (24)	44.9 ± 0.7 (19)	43.3 ± 1.1 (21)
1–13 (g/kg/day)	156.4 ± 4.6 (21)	161.2 ± 2.7 (24)	158.3 ± 2.7 (19)	153.4 ± 4.0 (21)
Chemical Intake (mg/kg/day)[d,e]				
1–13	0 ± 0.0 (21)	161.2 ± 2.7 (24)	474.8 ± 8.2 (19)	920.2 ± 24.2 (21)

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 displayed as mean ± standard error (n), where n = the number of dams.
[b]Statistical analysis performed by the Jonckheere (trend) and Williams or Dunnett (pairwise) tests.
[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.

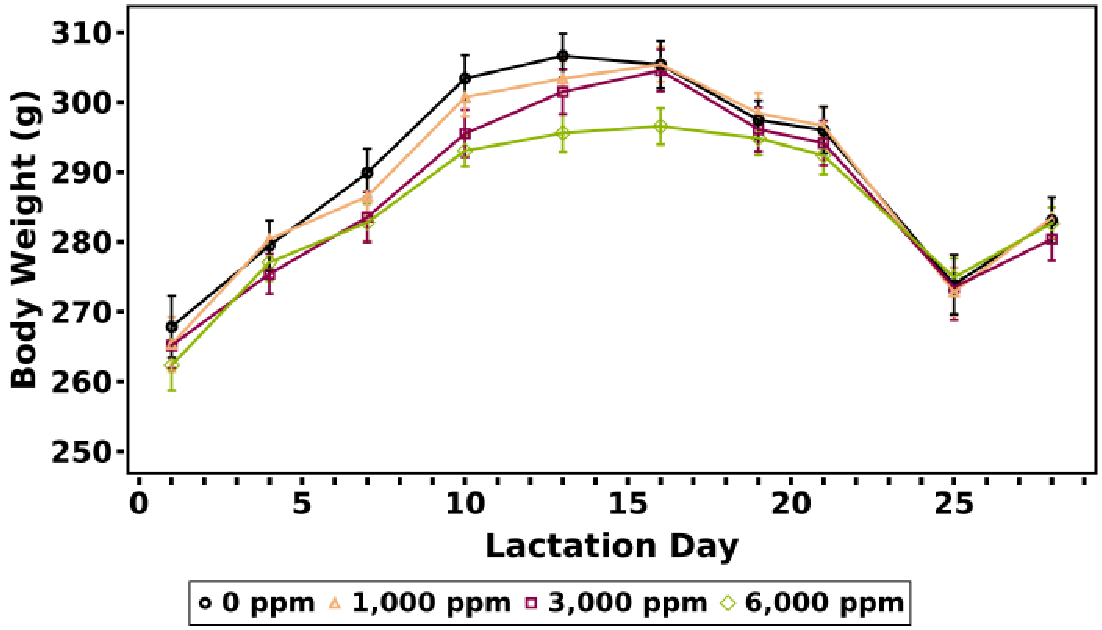


Figure 10. Growth Curves for F_0 Female Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed during Lactation

Information for statistical significance in maternal weights is provided in Table 12.

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 11Figure 11. Viability, clinical observations, and mean body weight results are presented below.

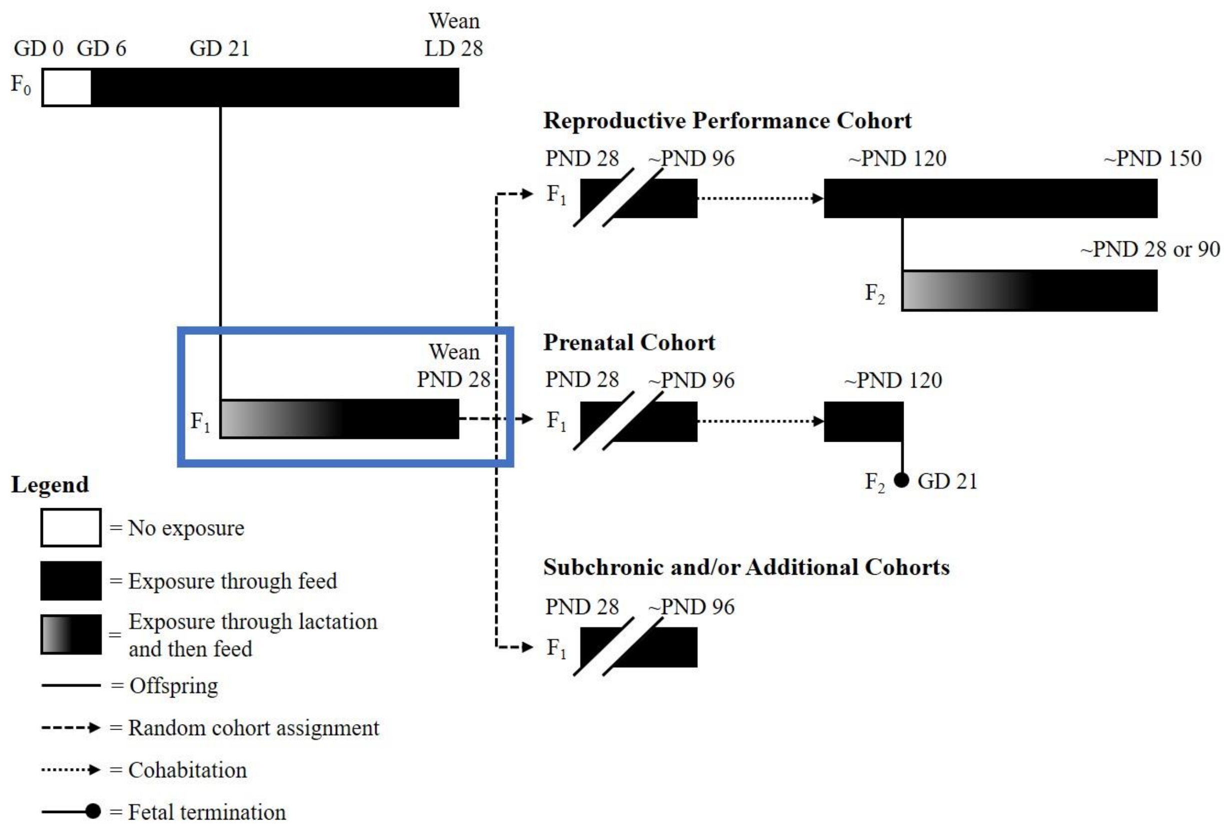


Figure 11. Design of the Modified One-Generation Study—F_1 Generation: Preweaning

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

F_1 Viability and Clinical Observations

Clinical observations noted for individual pups from all groups, including the control group, typically were indicative of an individual pup not thriving and included being cold to the touch and no milk in the stomach (Appendix E). Dams in the 1,000 ppm group had significantly increased total and live litter sizes on PND 0–4 relative to the control group (approximately two pups) (Table 13). Given the small magnitude of response and absence of an exposure concentration-response trend, it was not considered related to EHMC exposure. Given the larger PND 0 litter size in the 1,000 ppm group, litter size for that group was slightly larger for the first week of lactation. There was no observed effect of EHMC on pup survival (Table 13).

Table 13. Summary of F_1 Litter Size and Pup Survival Following Perinatal Exposure to 2-Ethylhexyl p-Methoxycinnamate

Postnatal Day	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
No. of Live Pups (Litters)[a]				
0	246 (22)	317 (24)	230 (19)	249 (22)
Total Litter Size[b,c]				
0	11.2 ± 0.7 (22)	13.2 ± 0.4* (24)	12.1 ± 0.4 (19)	11.3 ± 0.7 (22)
Live Litter Size[b,c]				
0	10.8 ± 0.7 (22)	13.0 ± 0.4* (24)	11.7 ± 0.4 (19)	11.1 ± 0.7 (22)
1	10.7 ± 0.7 (22)	13.0 ± 0.4* (24)	11.6 ± 0.4 (19)	11.1 ± 0.7 (22)
4 (prestandardization)	10.7 ± 0.7 (21)	12.9 ± 0.4* (24)	11.5 ± 0.4 (19)	10.9 ± 0.7 (22)
4 (poststandardization)	8.9 ± 0.4 (21)	9.9 ± 0.1 (24)	9.8 ± 0.2 (19)	9.1 ± 0.4 (22)
13	8.9 ± 0.4 (21)	9.7 ± 0.1 (24)	9.8 ± 0.2 (19)	8.9 ± 0.4 (22)
21	8.9 ± 0.4 (21)	9.7 ± 0.1 (24)	9.8 ± 0.2 (19)	8.9 ± 0.4 (22)
28	8.9 ± 0.4 (21)	9.7 ± 0.1 (24)	9.7 ± 0.2 (19)	8.9 ± 0.4 (22)
No. of Dead Pups (Litters)[a]				
0	9 (6)	5 (5)	8 (6)	4 (3)
1–4	12 (4)	3 (3)	3 (3)	5 (5)
5–28	1 (1)	4 (3)	1 (1)	5 (4)
Dead per Litter[b,c]				
0	0.41 ± 0.16 (22)	0.21 ± 0.08 (24)	0.42 ± 0.18 (19)	0.18 ± 0.11 (22)
1–4	0.55 ± 0.37 (22)	0.13 ± 0.07 (24)	0.16 ± 0.09 (19)	0.23 ± 0.09 (22)
5–28	0.05 ± 0.05 (21)	0.17 ± 0.10 (24)	0.05 ± 0.05 (19)	0.23 ± 0.11 (22)
Survival Ratio[b,c]				
0	0.96 ± 0.02 (22)	0.98 ± 0.01 (24)	0.97 ± 0.01 (19)	0.98 ± 0.01 (22)
1–4	0.94 ± 0.05 (22)	0.99 ± 0.01 (24)	0.99 ± 0.01 (19)	0.98 ± 0.01 (22)
5–28	0.99 ± 0.01 (21)	0.98 ± 0.01 (24)	0.99 ± 0.01 (19)	0.97 ± 0.01 (22)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p \leq 0.05$.
[a]n = the number of pups examined (number of litters).
[b]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).
[c]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.

F_1 Body Weights

Male Pups

An exposure concentration- and time-related reduction in male pup mean body weights per litter was observed in the groups exposed to 3,000 or 6,000 ppm EHMC relative to the control group (Table 14; Figure 12). On PND 28, male pup mean body weights per litter in the 3,000 and 6,000 ppm groups significantly decreased by 5% and 13%, respectively, relative to the control group. After the PND 4–7 interval, mean body weight gains in all subsequent intervals were significantly decreased in the 6,000 ppm group compared to the control group (Table 14; Appendix E). Mean body weight gains over the PND 13–16 interval were also significantly decreased in the 3,000 ppm group relative to the control group (Appendix E). Over the poststandardization PND 4–28 interval, male pups in the 3,000 and 6,000 ppm groups displayed significant decreases of 6% and 15%, respectively, relative to the mean body weight gains of the control group (Table 14).

Female Pups

An exposure concentration- and time-related reduction in female pup mean body weights per litter was observed in the groups exposed to 3,000 or 6,000 ppm EHMC relative to the control group (Table 14; Figure 13). On PND 28, female pup mean body weights per litter in the 3,000 and 6,000 ppm groups significantly decreased by 7% and 15%, respectively, relative to the control group. Except for the PND 21–25 interval, mean body weight gains of female pups were significantly decreased in the 6,000 ppm group compared to the control group, starting at the PND 7–10 interval (Appendix E). Mean body weight gains were also significantly decreased in the 3,000 ppm group compared to the control group for the PND 7–10, 10–13, and 13–16 intervals (Appendix E). Over the poststandardization PND 4–28 interval, female pups exposed to 3,000 or 6,000 ppm displayed mean body weight gains that significantly decreased by 8% and 17%, respectively, relative to the control group (Table 14).

Table 14. Summary of F_1 Male and Female Pup Mean Body Weights and Body Weight Gains Following Perinatal Exposure to 2-Ethylhexyl p-Methoxycinnamate[a,b]

Postnatal Day	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
Male				
1	6.96 ± 0.08 131 (22)[c]	7.01 ± 0.09 153 (24)	7.05 ± 0.08 110 (19)	7.17 ± 0.11 120 (22)
4[d]	10.14 ± 0.18 126 (21)	10.07 ± 0.15 151 (24)	10.23 ± 0.16 109 (19)	10.25 ± 0.17 118 (22)
7	15.39 ± 0.30 100 (21)	14.96 ± 0.30 115 (24)	15.38 ± 0.27 91 (19)	14.94 ± 0.23 97 (22)
13	28.78 ± 0.45** 100 (21)	27.90 ± 0.42 115 (24)	27.66 ± 0.58 91 (19)	25.94 ± 0.34** 95 (22)
28	82.66 ± 1.00** 100 (21)	82.13 ± 1.07 114 (24)	78.92 ± 0.94* 91 (19)	71.92 ± 0.90** 95 (22)
4-28[e]	72.46 ± 0.87** 100 (21)	72.08 ± 0.97 114 (24)	68.36 ± 0.85** 91 (19)	61.60 ± 0.88** 95 (22)
Female				
1	6.65 ± 0.07 104 (21)	6.64 ± 0.08 158 (24)	6.63 ± 0.07 111 (19)	6.69 ± 0.09 124 (22)
4[d]	9.41 ± 0.32 101 (21)	9.33 ± 0.14 158 (24)	9.38 ± 0.13 110 (19)	9.38 ± 0.16 122 (22)
7	14.39 ± 0.35 86 (20)	13.75 ± 0.31 122 (24)	13.95 ± 0.29 95 (19)	13.60 ± 0.23 102 (22)
13	27.15 ± 0.47** 86 (20)	25.99 ± 0.41 119 (24)	24.95 ± 0.48** 95 (19)	23.82 ± 0.32** 101 (22)
28	75.37 ± 1.11** 86 (20)	73.63 ± 1.03 119 (24)	69.81 ± 1.03** 95 (19)	64.17 ± 0.87** 101 (22)
4-28[e]	65.75 ± 0.98** 86 (20)	64.31 ± 0.96 119 (24)	60.22 ± 0.95** 95 (19)	54.87 ± 0.81** 101 (22)

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. 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 weights are presented in grams.
[c]n = the number of pups examined (number of litters).
[d]PND 4 weights are prestandardization.
[e]Body weight gain (data are presented in grams).

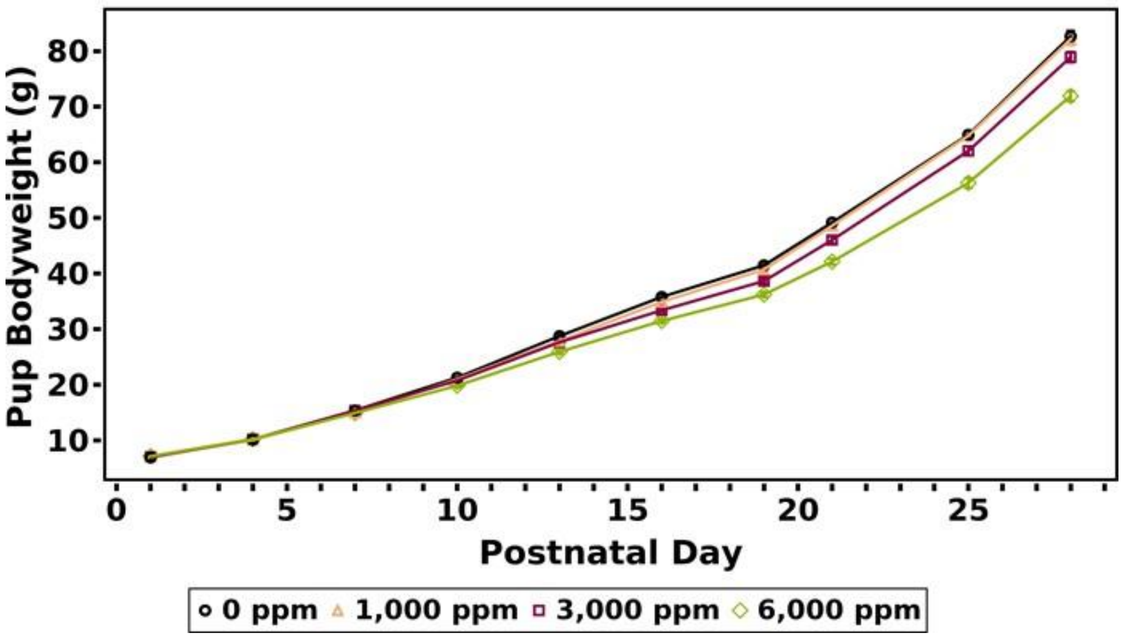


Figure 12. Lactation Growth Curves for F_1 Male Pups Following Perinatal Exposure to 2-Ethylhexyl p-Methoxycinnamate

Information for statistical significance in male pup weights is provided in Table 14.

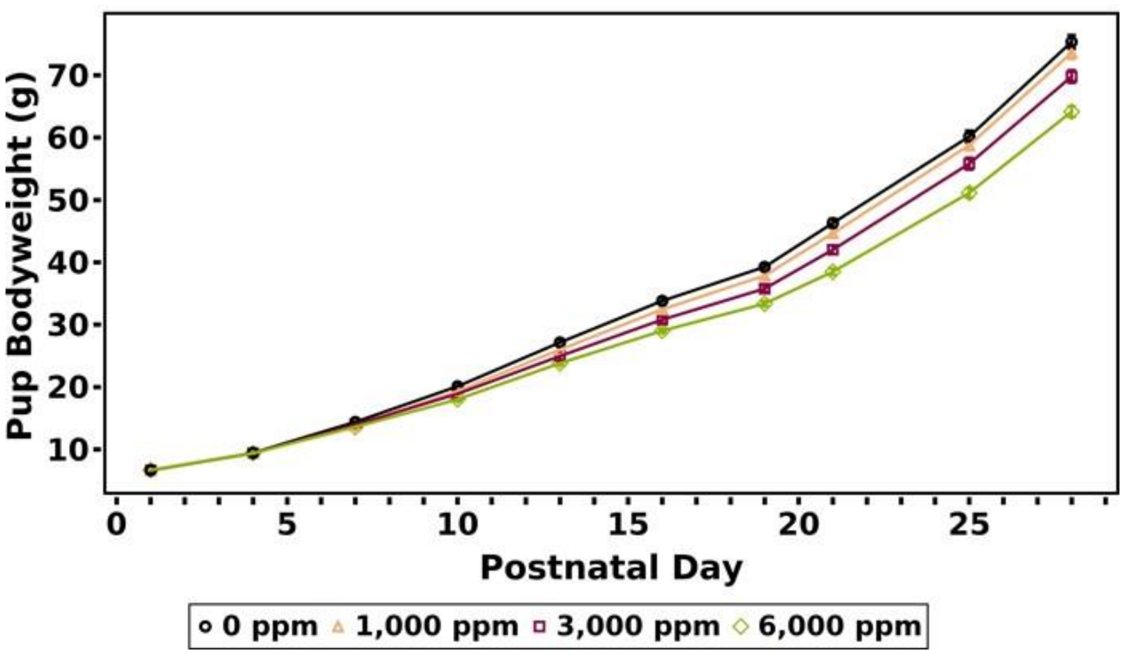


Figure 13. Lactation Growth Curves for F_1 Female Pups Following Perinatal Exposure to 2-Ethylhexyl p-Methoxycinnamate

Information for statistical significance in female pup weights is provided in Table 14.

F_0 Necropsy

F_0 females were necropsied on LD 28 following pup weaning, when the F_0 females were 15–21 weeks of age. No gross or histological findings were associated with exposure to EHMC (Appendix E). The only finding observed was retinal atrophy in one animal. Given the singular occurrence, it was not attributed to EHMC exposure (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.

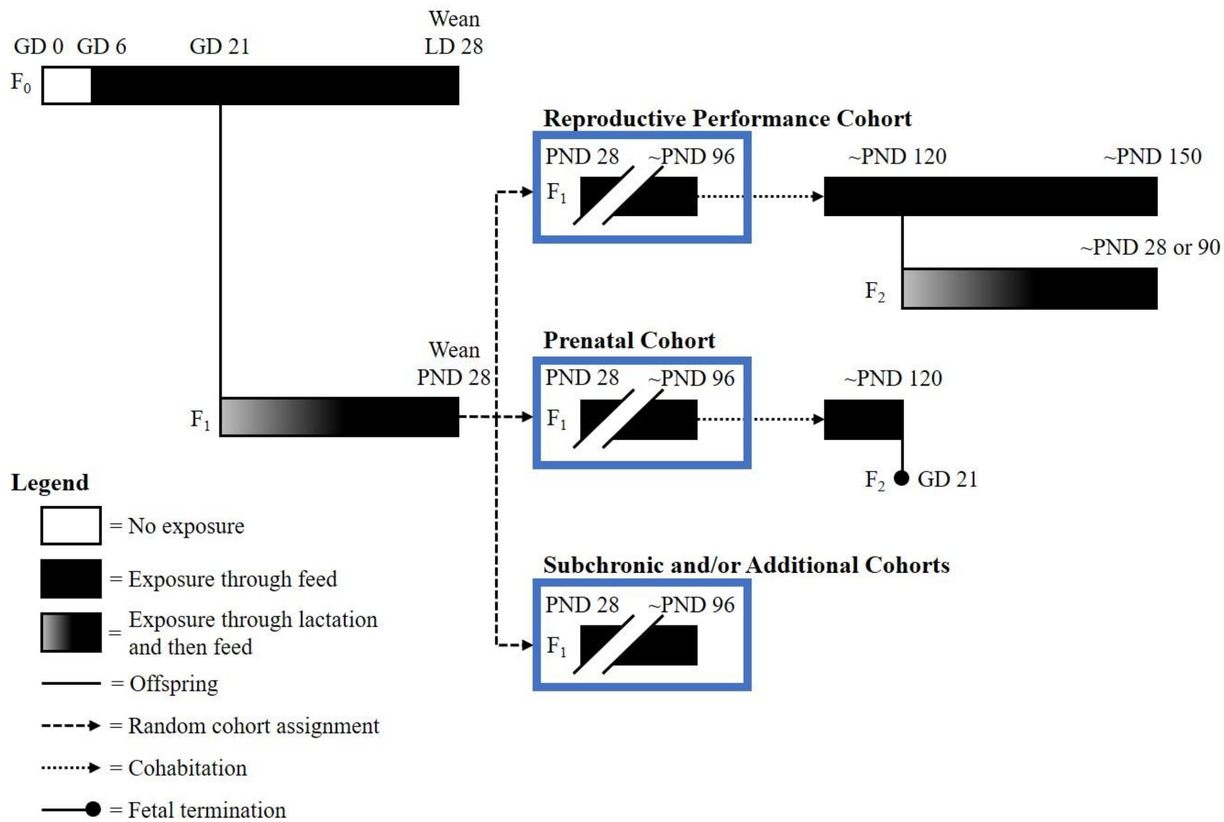


Figure 14. Design of the Modified One-Generation Study—F_1 Generation: Postweaning

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

F_1 Viability and Clinical Observations

EHMC exposure did not alter viability in the F_1 generation. Clinical observations were noted in all groups, including the control groups, on a sporadic basis. No clinical observations showed an increase in incidence or severity in association with exposure to EHMC (Appendix E).

F_1 Body Weights and Feed Consumption

Males (Postweaning)

The mean body weights of males in the 6,000 ppm group between PND 28 and PND 105 significantly decreased (5%–12%) relative to the control group (Table 15; Figure 15). In the 3,000 ppm group, mean body weights on PND 28 significantly decreased by approximately 7%, relative to the control group, and the PND 35–42 weight gain interval significantly decreased relative to the control group; however, for most of the rest of the study, mean body weights and body weight gains of the 3,000 ppm group did not differ significantly from the control group (Table 15; Appendix E).

Feed consumption (g/animal/day) over the entire postweaning period was not affected by EHMC exposure (Table 15). Significant decreases in absolute feed consumption were observed in the 6,000 ppm group after PND 70 (Appendix E). Relative feed consumption (g/kg/day) over the entire postweaning period were significantly increased in the 6,000 ppm group relative to the control group. Through PND 63, relative feed consumption was significantly increased due to the lower body weights of the animals.(Appendix E). EHMC intake for F_1 males, based on feed consumption and dietary concentrations for PND 28 through PND 91, was approximately 80, 242, and 491 mg/kg/day at 1,000, 3,000, and 6,000 ppm EHMC, respectively (Table 15).

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-Ethylhexyl p-Methoxycinnamate in Feed

Postnatal Day[a]	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
Body Weight (g)[b,c]				
28	82.0 ± 1.5** 72 (21)	78.8 ± 1.2 84 (24)	76.3 ± 0.9* 69 (19)	71.9 ± 1.5** 74 (22)
91	396.6 ± 6.6** 67 (21)	392.0 ± 4.2 79 (24)	387.1 ± 3.9 64 (19)	376.3 ± 4.0** 69 (22)
105	418.3 ± 6.9** 67 (21)	411.5 ± 4.2 79 (24)	408.4 ± 4.0 64 (19)	396.4 ± 4.4** 69 (22)
Body Weight Gain (g)[b,c]				
28–105	336.4 ± 5.6* 67 (21)	332.6 ± 4.0 79 (24)	332.3 ± 3.5 64 (19)	324.5 ± 3.7 69 (22)
Postweaning Feed Consumption[d,e]				
28–91 (g/animal/day)	21.4 ± 0.3* (30)	21.4 ± 0.2 (35)	21.2 ± 0.3 (31)	20.7 ± 0.3 (32)
28–91 (g/kg/day)	79.1 ± 0.7** (30)	79.9 ±0.7 (35)	80.8 ±0.8 (31)	81.9 ± 0.9** (32)
Chemical Intake (mg/kg/day)[f,g]				
28–91	0.0 ± 0.0 (30)	79.9 ± 0.7 (35)	242.3 ± 2.3 (31)	491.4 ± 5.3 (32)

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 displayed as mean ± standard error (n).
[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 comparisons.
[c]n = number of pups examined (number of litters).
[d]Statistical analysis performed using the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
[e]n = number of cages.
[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.

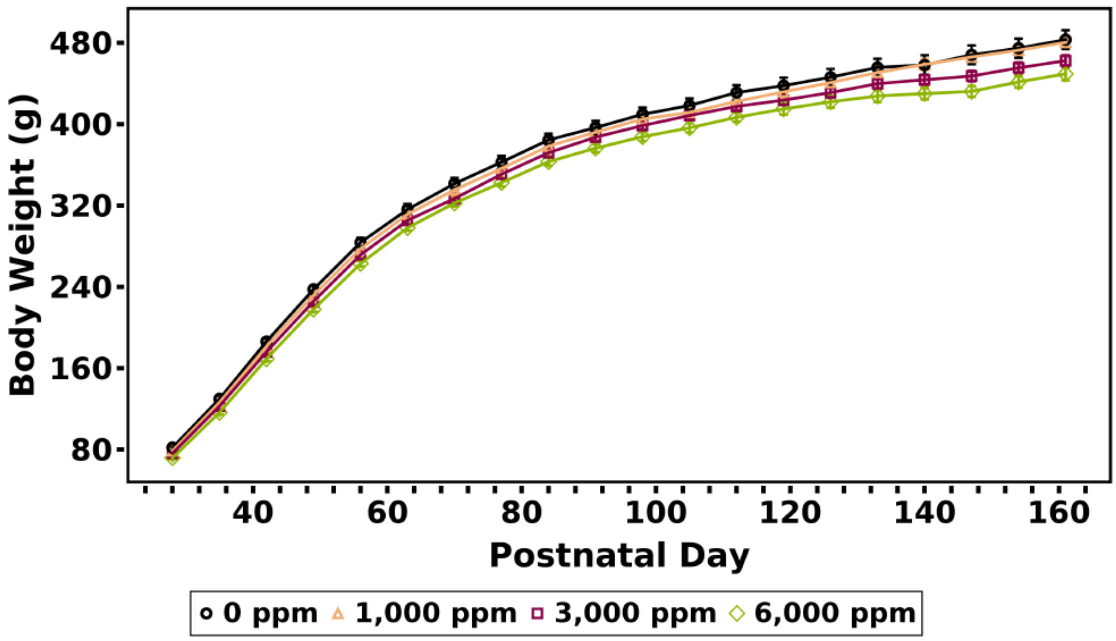


Figure 15. Postweaning Growth Curves for All F_1 Male Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed

Information for statistical significance in F_1 male rat weights is provided in Table 15.

Females (Postweaning)

Throughout the postweaning exposure period, mean body weights of females exposed to 6,000 ppm EHMC were significantly decreased (7%–14%) relative to the control group (Table 16; Figure 16); by PND 91, female mean body weights of the 6,000 ppm group were significantly decreased by 7% relative to the control group, indicating adaptation or a compensatory response. Female mean body weights of the 3,000 ppm group were significantly decreased (6%–11%) relative to the control group until PND 56, after which the mean body weights were <5% lower than the control group (Appendix E). The mean body weights of females in the 1,000 ppm group were similar to those of the control group. Mean body weight gains of all groups of exposed females during the PND 28–91 interval were similar to those of the control group (Table 16).

In general, EHMC-exposed female rats displayed similar feed consumption values compared to the control group over the postweaning period (Table 16; Appendix E). In the 6,000 ppm group, absolute feed consumption significantly decreased during the PND 28–35 and PND 70–77 intervals, but there was no significant difference compared to the control group in the overall absolute feed consumption (g/animal/day) during the postweaning period (PND 28–91). Relative feed consumption (g/kg/day) significantly increased relative to the control group during some intervals by all of the exposed groups of females (Table 16; Appendix E). EHMC intake for F_1 females, based on feed consumption and dietary concentrations for PND 28 through PND 91, was approximately 87, 263, and 528 mg/kg/day at 1,000, 3,000, and 6,000 ppm EHMC, respectively (Table 16).

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-Ethylhexyl p-Methoxycinnamate in Feed

Postnatal Day[a]	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
Body Weight (g)[b,c]				
28	75.4 ± 1.8** 80 (20)	70.8 ± 1.1 94 (24)	67.4 ± 1.0** 79 (19)	64.5 ± 1.5** 85 (22)
91	253.0 ± 4.2** 67 (20)	244.5 ± 3.7 79 (24)	241.3 ± 3.0 64 (19)	236.4 ± 2.9** 69 (22)
Body Weight Gain (g)[b,c]				
28–91	177.4 ± 3.4 67 (20)	173.7 ± 2.9 79 (24)	174.2 ± 2.6 64 (19)	171.8 ± 3.1 69 (22)
Postweaning Feed Consumption[d,e]				
28–91 (g/animal/day)	15.5 ± 0.2* (31)	15.6 ± 0.2 (36)	15.3 ± 0.2 (31)	14.9 ± 0.1 (31)
28–91 (g/kg/day)	84.5 ± 0.7* (31)	87.0 ± 0.9 (36)	87.5 ± 0.9* (31)	88.0 ± 1.2* (31)
Chemical Intake (mg/kg/day)[f,g]				
28–91	0.0 ± 0.0 (31)	87.0 ± 0.9 (36)	262.6 ± 2.7 (31)	528.1 ± 7.0 (31)

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 displayed as mean ± standard error (n).
[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 comparisons.
[c]n = number of pups examined (number of litters).
[d]Statistical analysis performed using the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
[e]n = number of cages.
[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.

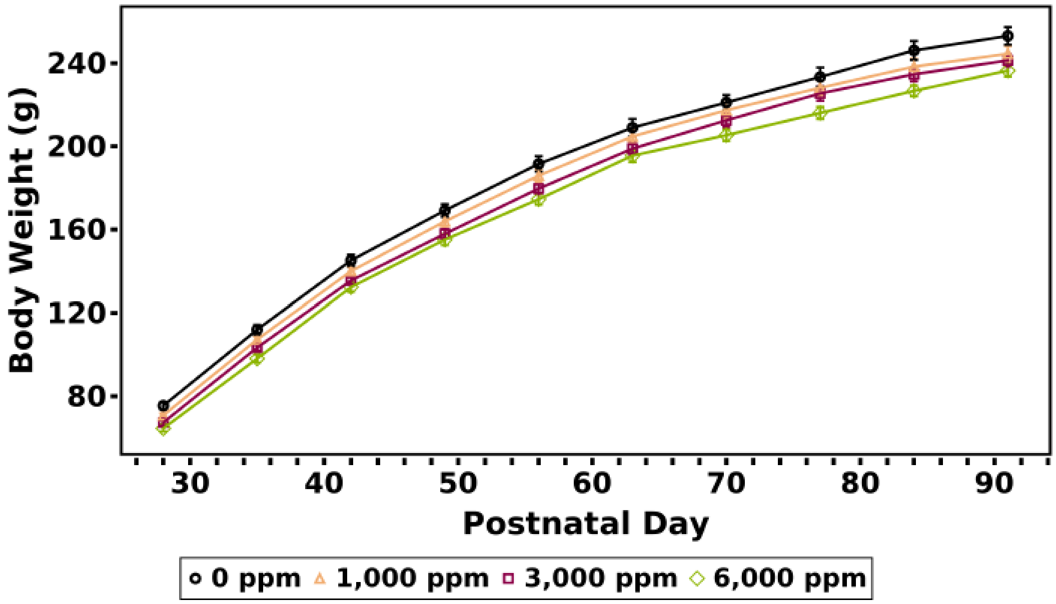


Figure 16. Postweaning Growth Curves for All F_1 Female Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed

Information for statistical significance in F_1 female rat weights is provided in Table 16.

Developmental Endpoints

Anogenital Distance

F_1 male, F_2 male, and F_1 female offspring exposed to EHMC did not display any alterations in PND 1 mean body weight-adjusted anogenital distance (AGD) (Table 17). F_2 female offspring exposed to 6,000 ppm displayed a slightly shorter (6%) AGD compared to the control group; however, this was likely the result of the F_2 control group displaying slightly larger AGD than expected. All other AGDs across exposure groups and generations were similar to each other. Given this minimal magnitude, direction of change, and absence of pairwise statistical significance, this finding was not considered related to EHMC exposure.

Table 17. Summary of Anogenital Distance of F_1 and F_2 Male and Female Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed

Parameter[a]	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
Anogenital Distance (PND 1)				
Male F_1				
No. examined[b]	131 (22)	153 (24)	110 (19)	120 (22)
Adjusted AGD (mm)[c,d]	2.17 ± 0.03	2.19 ± 0.02	2.23 ± 0.03	2.19 ± 0.02
Male F_2				
No. examined	165 (25)	208 (33)	167 (24)	159 (25)
Adjusted AGD (mm)	2.34 ± 0.05	2.31 ± 0.04	2.31 ± 0.07	2.23 ± 0.05

Parameter[a]	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
Female F_1				
No. examined	104 (21)	158 (24)	111 (19)	124 (22)
Adjusted AGD (mm)	1.08 ± 0.03	1.07 ± 0.02	1.11 ± 0.03	1.12 ± 0.02
Female F_2				
No. examined	194 (26)	214 (33)	185 (24)	171 (25)
Adjusted AGD (mm)	1.18 ± 0.03*	1.17 ± 0.02	1.13 ± 0.03	1.11 ± 0.02

Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$.
PND = postnatal day; AGD = anogenital distance.
[a]Data are displayed as mean ± standard error. Animals found dead, cannibalized, or missing (presumed dead) were excluded from analysis. For F_1 and F_2 pups, data are displayed as the mean of litter values ± standard error of litter values (n = number of litters produced by F_0 dams). For F_2 pups, n is dependent on the number of litters produced by the F_0 generation where up to two nonindependent F_1 offspring/sex/litter were selected to produce F_2 pups through nonsibling mating.
[b]No. examined = number of pups examined (number of litters represented).
[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 pairwise comparisons.
[d]Adjusted AGD calculated using the formula: adjusted AGD = raw AGD – (slope*[body weight for that animal – overall body weight mean]), where the slope is the regression slope of AGD versus body weight.

Areolae/Nipple Retention

F_1 male offspring exposed to EHMC exhibited singular occurrences of areolae/nipple retention, which was not observed in the F_2 male offspring (Appendix E).

Testicular Descent

Exposure to EHMC did not affect testicular descent in F_1 or F_2 male offspring (Appendix E).

Vaginal Opening

Females exposed to 3,000 or 6,000 ppm exhibited significant delays in the mean day of attaining vaginal opening (VO) (approximately 1.5 and 2.5 days, respectively) (Table 18). Mean body weights on day of attainment of the EHMC-exposed groups were similar to those of the control group. When weaning body weight was used to adjust day of VO attainment, the delays remained significant (Table 18). The adjusted individual and litter cumulative response graphs display an apparent shift to the right as a function of increasing exposure concentration (Figure 17; Appendix E).

Table 18. Summary of Vaginal Opening of F_1 Female Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed

Parameter[a]	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
No. Examined[b]	80 (20)	94 (24)	79 (19)	84 (22)
No. Not Attaining[c]	0	0	0	0
Day of VO				
Litter mean[d,e]	34.1 ± 0.3**	35 ± 0.2	35.8 ± 0.4**	36.8 ± 0.3**
Adjusted litter mean[d,e,f]	34.4 ± 0.3**	35.1 ± 0.2	35.7 ± 0.3*	36.5 ± 0.3**
Mean Body Weight at Acquisition (g)[g]	106.7 ± 2.0	107.3 ± 1.3	107.1 ± 1.4	107.7 ± 2.4
Mean Body Weight at Weaning (g)[g]	77.5 ± 1.8**	73.0 ± 1.1	69.4 ± 1.0**	66.1 ± 1.6**

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 $p \leq 0.05$; **$p \leq 0.01$.
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]No. Examined = the number of pups examined (number of litters).
[c]No. Not Attaining = number of pups that survived to the end of the observation period without attaining VO.
[d]Summary statistics and mixed model results are presented for animals that attained during the observation period.
[e]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]Adjusted based on body weight at weaning.
[g]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.

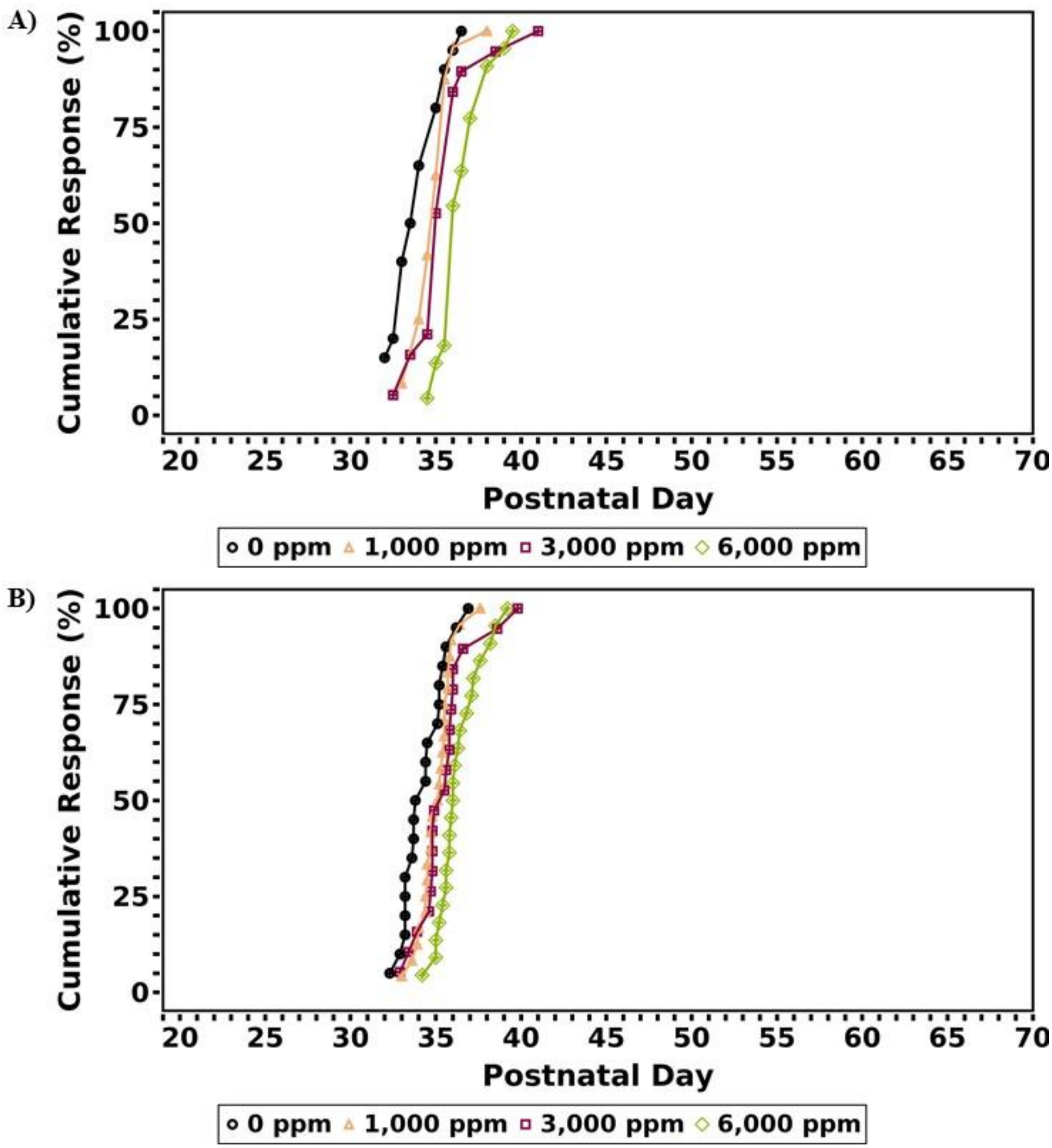


Figure 17. Time to Vaginal Opening of F_1 Female Offspring Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed

(A) Litter response and (B) litter response adjusted for body weight at weaning.

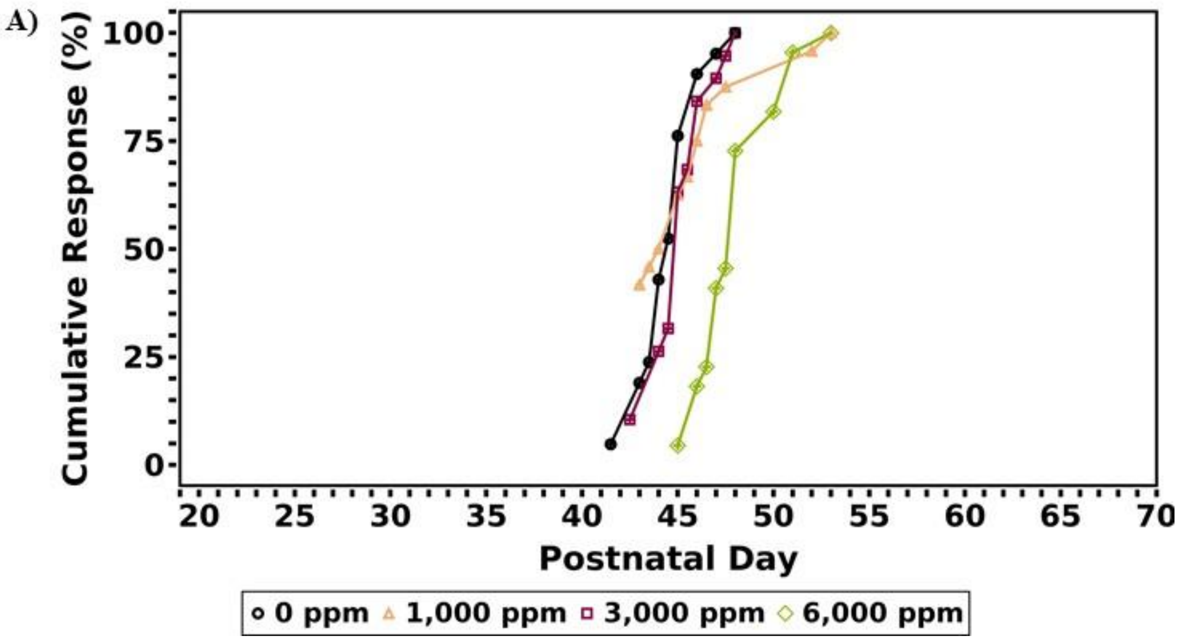
Balanopreputial Separation

Male rats in the 6,000 ppm group displayed a significant delay (approximately 3.5 days) in the mean day of attaining balanopreputial separation (BPS) when analyzed as litter means (Table 19). When graphically expressed as a cumulative litter response the 6,000 ppm group shifted to the right (Figure 18; Appendix E). Mean body weights on day of attainment were similar, and when litter means were adjusted using the corresponding body weight on day of weaning, this delay was slightly shortened—but remained significant—relative to the control group (Table 19; Figure 18). The cumulative litter mean and individual PND 28-adjusted responses for the 6,000 ppm group still display the shift to the right (Figure 18; Appendix E).

Table 19. Summary of Balanopreputial Separation of F_1 Male Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed

Parameter[a]	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
No. Examined[b]	72 (21)	84 (24)	69 (19)	74 (22)
No. Not Attaining[c]	0	0	0	0
Day of BPS				
Litter mean[d,e]	44.9 ± 0.3**	45.4 ± 0.6	45.3 ± 0.4	48.4 ± 0.6**
Adjusted litter mean[d,e,f]	45.6 ± 0.3**	45.6 ± 0.6	45.2 ± 0.3	47.8 ± 0.5**
Mean Body Weight at Acquisition (g)[g]	207.9 ± 3.5	203.5 ± 4.0	199.2 ± 1.9	214.1 ± 3.4
Mean Body Weight at Weaning (g)[g]	84.5 ± 1.6**	80.9 ± 1.2	78.2 ± 0.9**	73.6 ± 1.5**

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 $p \leq 0.01$.
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]No. Examined = number of pups examined (number of litters).
[c]No. Not Attaining = number of pups that survived to the end of the observation period without attaining BPS.
[d]Summary statistics and mixed model results are presented for animals that attained during the observation period.
[e]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]Adjusted based on body weight at weaning.
[g]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.



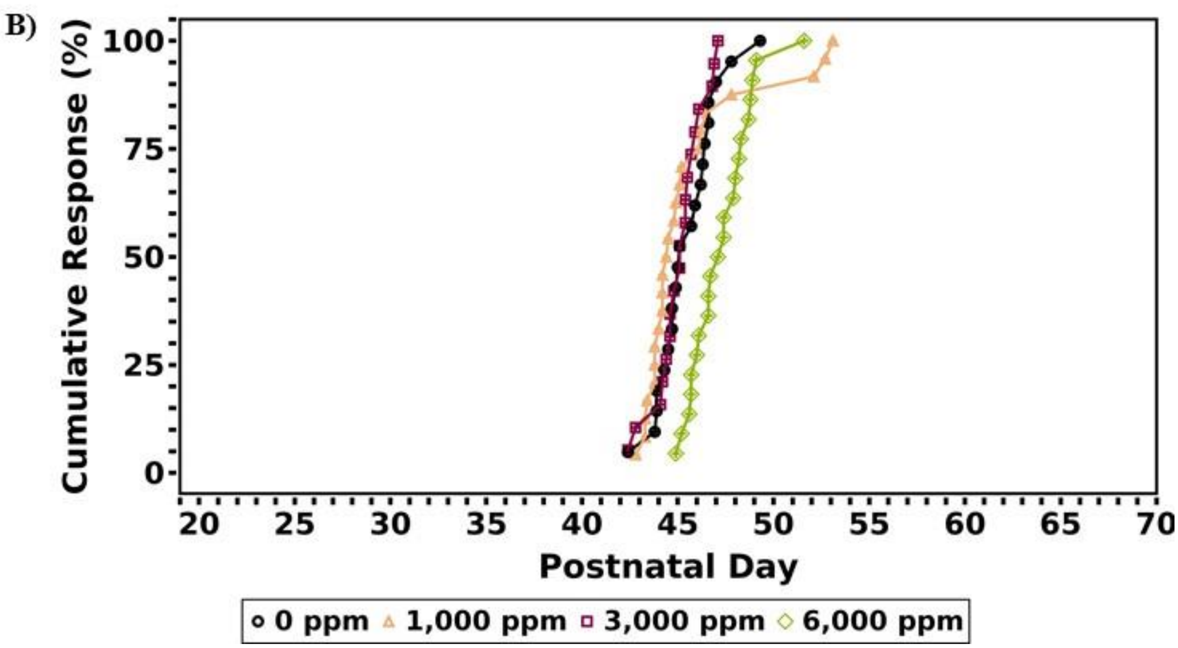


Figure 18. Time to Balanopreputial Separation of F_1 Male Offspring Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed

(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.

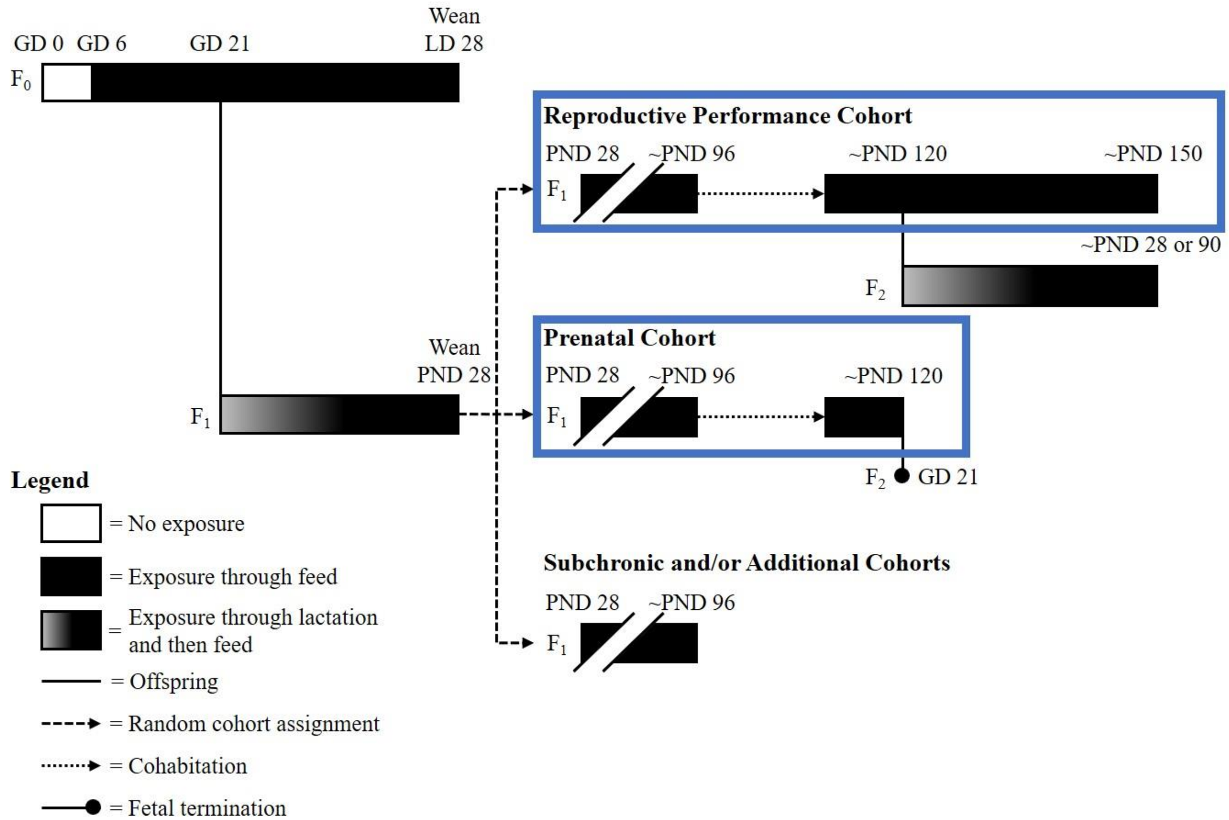


Figure 19. Design of the Modified One-Generation Study—Prenatal and Reproductive Performance Cohorts

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

Viability and Clinical Observations

There were no EHMC-related clinical observations, and no morbidity or mortality, in the prenatal and reproductive performance cohorts (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.

Vaginal Cytology

The collective analysis of F_1 female vaginal cytology indicated that EHMC exposure did not affect the number of rats that were cycling (Table 20; Figure 20). However, rats in the 6,000 ppm group spent more time in estrus compared to the control group (approximately 28% of the days versus approximately 20%, respectively). Analysis of estrous cyclicity utilizing the continuous-time Markov model demonstrated a slight but significant increase in estrus stage length in all EHMC-exposed groups compared to the control (Table 20; Figure 20; Appendix E).

Table 20. Markov Model Estimates of Estrous Stage Length and 95% Confidence Intervals for All F_1 Female Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed

	0 ppm		1,000 ppm		3,000 ppm		6,000 ppm	
Stage[a]	Stage Length (Days)	95% CI	Stage Length (Days)	95% CI	Stage Length (Days)	95% CI	Stage Length (Days)	95% CI
Diestrus	3.7	(3.3, 4.3)	3.0*	(2.7, 3.3)	3.3	(2.9, 3.8)	2.9**	(2.5, 3.2)
Proestrus	0.4	(0.3, 0.4)	0.4	(0.4, 0.5)	0.3	(0.2, 0.4)	0.3	(0.2, 0.4)
Estrus	1.1	(1.0, 1.2)	1.3**	(1.2, 1.4)	1.3**	(1.2, 1.4)	1.3**	(1.2, 1.4)
Metestrus	0.2	–[b]	0.2	–	0.2	–	0.2	–

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p < 0.05$; ** $p < 0.01$.
CI = confidence interval.
[a]Pairwise tests are performed using a permutation null hypothesis testing method and have been adjusted for multiple comparisons using a Hommel correction within each stage.
[b]Due to a very low number of observations of metestrus, stage lengths were estimated using a profile likelihood approach. As a result, confidence intervals are not available for the metestrus stage length estimate.

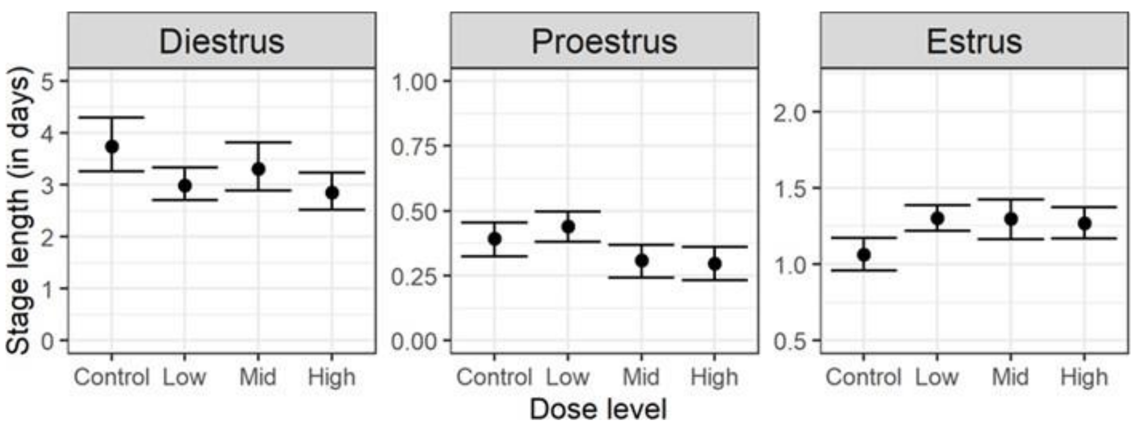


Figure 20. Markov Model Estimates of Estrous Stage Length and 95% Confidence Intervals for All F_1 Female Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed

Fertility

The precoital interval and number of females that mated (i.e., those that were sperm-positive, littered, or had implantation sites) were similar across the EHMC-exposed groups and the control group (Table 21. Summary of Mating and Fertility Performance of F_1 Male and Female Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in FeedTable 21). The number of pregnant females was also similar among groups, indicating that F_1 male and female fertility was not affected by EHMC exposure at the concentrations examined. Responses observed were consistent between the cohorts.

F_1 Reproductive Performance Cohort Andrology

There were no EHMC-related effects on motile sperm, progressively motile sperm, testis spermatid head, cauda epididymal sperm counts, or cauda epididymal sperm concentration in the prenatal and reproductive performance cohorts (Appendix E). Males in the 6,000 ppm group displayed slightly higher cauda epididymis weights (6%, positive trend), but epididymis and testis weights were similar to those of the control group. These findings were not associated with histopathological changes or significant changes in reproductive performance (Appendix E).

Table 21. Summary of Mating and Fertility Performance of F_1 Male and Female Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed

Parameter	0 ppm		1,000 ppm		3,000 ppm		6,000 ppm	
	RPC	PC	RPC	PC	RPC	PC	RPC	PC
No. Mating Pairs	36	21	46	23	35	19	37	22
No. Mated	34	19	41	21	32	18	34	20
No. Females Pregnant	27	19	35	18	27	15	27	16
Percent of Mated Females/Paired[a]	94.4	90.5	89.1	91.3	91.4	94.7	91.9	90.9
Precoital Interval[b,c]	4.9 ± 0.7 (19)	4.3 ± 0.9 (19)	5.1 ± 0.6 (22)	4.9 ± 1.0 (21)	4.8 ± 0.7 (19)	2.9 ± 0.6 (15)	4.6 ± 0.6 (20)	5.4 ± 0.9 (20)

RPC = reproductive performance cohort; PC = prenatal cohort.
[a]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.
[b]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.
[c]Precoital interval in days is calculated for sperm-positive females; data are displayed as mean ± standard error (n).

Gestation Body Weights

As previously reported, in the F1 Body Weights and Feed Consumption

section, females in the 3,000 ppm group had significantly decreased mean body weights at postweaning (PND 28), but their body weights recovered by sexual maturity (PND 91) and were similar to those of the control group (Table 16). In contrast, at sexual maturity before mating (PND 91), mean body weights of females in the 6,000 ppm group were significantly decreased by approximately 7% relative to the control group (Table 16). GD 0 mean body weights of the reproductive performance cohort were also slightly lower (5%, negative trend) (Table 22). This response on GD 0 was not observed in the prenatal cohort, likely due to the smaller number of animals and litters represented. Females in the 6,000 ppm group in the reproductive performance cohort displayed slightly lower (approximately 5%) gestation mean body weights than the control group, often attaining statistical significance (Appendix E). Collectively, these findings suggest the EHMC-related responses observed on gestation mean body weight were consistent between the cohorts; nonetheless, the apparent magnitude of this response is small. Gestational body weight curves of the exposed groups in both cohorts generally paralleled those of the control groups (Figure 21, Figure 22). In both cohorts, GD 0–21 mean body weight gains of the EHMC-exposed groups were similar those of the control groups (Table 22).

Gestation Feed Consumption

Gestational feed consumption (g/animal/day) was significantly decreased in the 6,000 ppm group of the reproductive performance cohort with a negative trend in the prenatal cohort during the GD 0–21 interval. When expressed as a function of body weight (g/kg/day), however, it was similar to that of the control groups (Table 23; Appendix E).

Table 22. Summary of Gestation Mean Body Weight Gains for F_1 Female Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed[a,b]

GD Interval	0 ppm		1,000 ppm		3,000 ppm		6,000 ppm	
	RPC	**PC**	**RPC**	**PC**	**RPC**	**PC**	**RPC**	**PC**
n[c]	16	19	22	18	19	12	18	16
0	260.5 ± 5.7*	247.8 ± 6.4	258.9 ± 4.4	248.1 ± 4.8	253.9 ± 4.6	238.3 ± 4.1	246.5 ± 2.8	246.5 ± 3.3
0–21	170.5 ± 6.2	168.0 ± 3.5	157.6 ± 5.1	147.8 ± 8.4*	165.3 ± 6.5	170.9 ± 3.0	155.7 ± 5.8	151.9 ± 5.5
0–3	18.3 ± 1.6	16.1 ± 1.2	16.3 ± 1.0	14.4 ± 0.8	16.5 ± 1.0	18.5 ± 1.3	17.5 ± 0.7	14.1 ± 0.8
3–6	11.9 ± 1.0	10.8 ± 0.8	11.2 ± 0.7	11.3 ± 0.8	12.1 ± 0.8	12.5 ± 0.8	10.8 ± 0.5	10.8 ± 0.7
6–9	12.5 ± 0.9	12.2 ± 0.6	11.8 ± 0.8	11.3 ± 0.6	11.5 ± 0.9	12.5 ± 0.7	11.1 ± 0.9	10.9 ± 0.7
9–12	14.9 ± 1.0	14.2 ± 0.6	14.1 ± 0.8	13.4 ± 1.0	14.7 ± 0.9	14.8 ± 1.0	14.8 ± 1.0	11.5 ± 0.9
12–15	19.7 ± 1.3	21.5 ± 0.8	17.2 ± 0.9	17.3 ± 1.4*	20.2 ± 1.1	21.6 ± 0.8	16.9 ± 1.0	21.4 ± 1.1
15–18	45.2 ± 1.9	47.0 ± 1.7*	42.3 ± 2.1	39.0 ± 3.2*	42.8 ± 2.6	45.0 ± 1.7	41.3 ± 2.2	39.9 ± 2.0
18–21	48.1 ± 2.2	46.1 ± 1.7	44.7 ± 1.8	41.1 ± 3.0	47.6 ± 2.4	46.0 ± 1.3	43.3 ± 2.1	43.2 ± 2.1

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 $p \leq 0.05$.
GD = gestation day; RPC = reproductive performance cohort; PC = prenatal cohort.
[a]Data are displayed as mean ± standard error. 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 by the Jonckheere (trend) and Williams or Dunnett (pairwise) tests.
[c]n = number of litters.

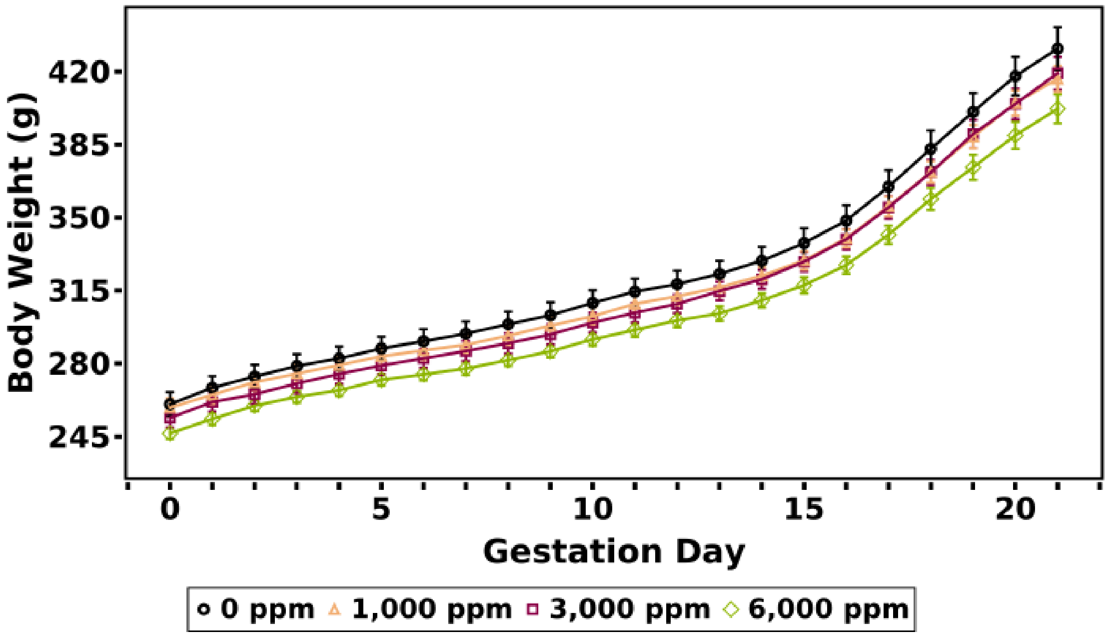


Figure 21. Gestation Growth Curves for F_1 Female Rats in the Reproductive Performance Cohort Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed

Information for statistical significance in F_1 female rat weights is provided in Appendix E.

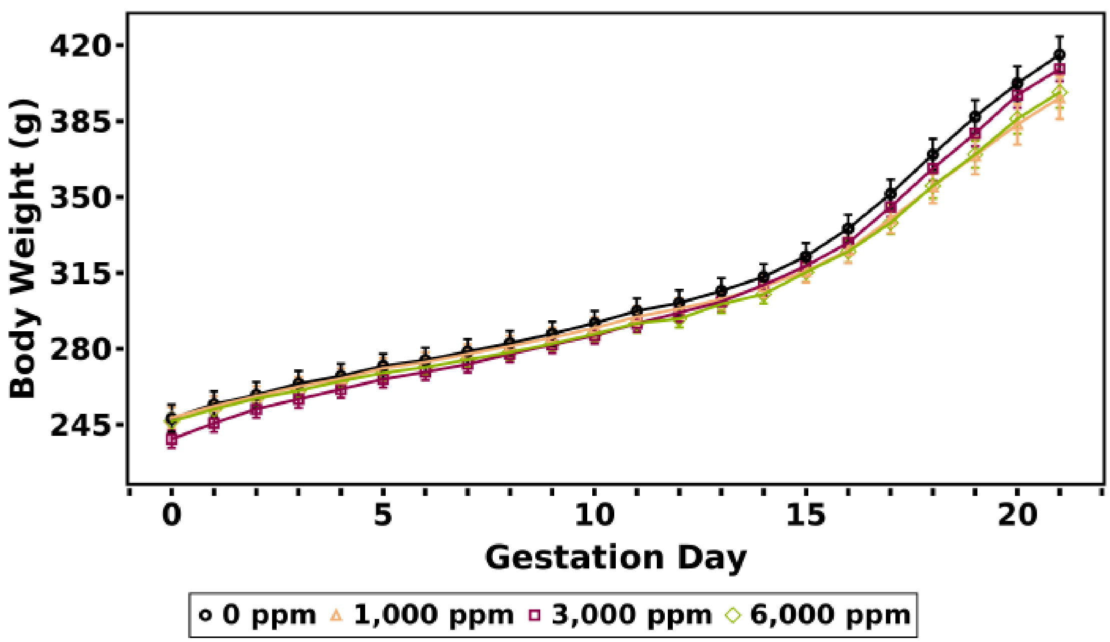


Figure 22. Gestation Growth Curves for F_1 Female Rats in the Prenatal Cohort Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed

Information for statistical significance in F_1 female rat weights is provided in Appendix E.

Table 23. Summary of Gestation Feed and Test Article Consumption for F_1 Female Rats Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed[a]

GD Interval	0 ppm		1,000 ppm		3,000 ppm		6,000 ppm	
	RPC	PC	RPC	PC	RPC	PC	RPC	PC
Feed Consumption (g/animal/day)[b]								
0–21	24.0 ± 0.6 (16)	22.9 ± 0.5* (19)	23.1 ± 0.4 (22)	22.5 ± 0.5 (18)	23.0 ± 0.3 (19)	22.2 ± 0.6 (12)	22.1 ± 0.4* (18)	21.6 ± 0.3 (16)
0–3	21.4 ± 0.6 (16)	19.9 ± 0.8* (19)	20.3 ± 0.5 (21)	20.4 ± 0.6 (18)	20.6 ± 0.5 (19)	19.3 ± 0.6 (12)	19.8 ± 0.3 (18)	18.5 ± 0.3 (16)
3–6	21.8 ± 0.6 (16)	21.0 ± 0.8 (18)[c]	21.3 ± 0.4 (22)	20.7 ± 0.4 (18)	21.8 ± 0.2 (19)	20.2 ± 0.4 (12)	20.4 ± 0.3 (18)	20.0 ± 0.2 (16)
6–9	23.9 ± 0.9* (16)	22.4 ± 0.8* (19)	22.6 ± 0.5 (22)	22.1 ± 0.7 (17)[c]	22.1 ± 0.4 (19)	21.6 ± 0.7 (12)	21.2 ± 0.4* (18)	20.6 ± 0.3 (16)
9–12	22.7 ± 0.5 (16)	21.2 ± 0.5 (19)	22.0 ± 0.4 (22)	21.9 ± 0.6 (18)	22.2 ± 0.2 (19)	20.8 ± 0.5 (12)	21.1 ± 0.4 (18)	20.4 ± 0.3 (16)
12–15	24.2 ± 0.7 (16)	23.7 ± 0.6 (19)	23.0 ± 0.4 (22)	22.7 ± 0.6 (18)	22.9 ± 0.4 (19)	22.3 ± 0.8 (12)	22.1 ± 0.6 (18)	22.3 ± 0.4 (16)
15–18	26.3 ± 0.7 (16)	25.7 ± 0.4 (19)	25.3 ± 0.4 (22)	24.0 ± 0.5** (18)	25 ± 0.4 (19)	25.1 ± 0.6 (12)	24.4 ± 0.5 (18)	24.3 ± 0.4 (16)
18–21	27.7 ± 0.6 (16)	26.7 ± 0.8 (19)	27.0 ± 0.7 (22)	25.3 ± 0.6 (18)	26.9 ± 0.5 (19)	25.9 ± 1.2 (12)	25.6 ± 0.8 (18)	24.8 ± 0.6 (16)
Feed Consumption (g/kg/day)[b]								
0–21	74.2 ± 1.1 (16)	74.8 ± 2.0 (19)	73.2 ± 1.2 (22)	74.4 ± 1.3 (18)	73.5 ± 0.8 (19)	73.3 ± 1.3 (12)	72.5 ± 0.9 (18)	71.7 ± 0.9 (16)
0–3	79.2 ± 2.1 (16)	78.3 ± 3.7 (19)	76.1 ± 1.8 (21)	80.3 ± 2.3 (18)	78.4 ± 1.4 (19)	77.7 ± 1.7 (12)	77.4 ± 1.0 (18)	72.8 ± 1.1 (16)
3–6	76.8 ± 1.8 (16)	77.9 ± 3.7 (18)	76.0 ± 1.3 (22)	77.3 ± 1.3 (18)	78.8 ± 0.8 (19)	76.7 ± 1.5 (12)	75.5 ± 1.0 (18)	75.1 ± 1.1 (16)
6–9	80.4 ± 2.2 (16)	80.2 ± 3.5 (19)	77.7 ± 1.6 (22)	79.2 ± 2.2 (17)	77.0 ± 1.2 (19)	78.2 ± 1.8 (12)	75.6 ± 1.3 (18)	74.6 ± 1.3 (16)
9–12	73.0 ± 1.0 (16)	72.2 ± 1.4 (19)	72.3 ± 1.2 (22)	74.9 ± 1.7 (18)	73.7 ±1.0 (19)	72.0 ± 1.4 (12)	72.0 ± 1.1 (18)	70.9 ± 1.1 (16)
12–15	73.9 ± 1.4 (16)	76.4 ± 2.1 (19)	72.0 ± 1.2 (22)	74.4 ± 2.1 (18)	72.1 ± 1.1 (19)	72.8 ± 2.0 (12)	71.5 ± 1.5 (18)	73.7 ± 1.4 (16)
15–18	73.4 ± 0.9 (16)	74.7 ± 1.0 (19)	72.5 ± 1.3 (22)	71.8 ± 1.1 (18)	71.7 ± 1.1 (19)	74.0 ±1.0 (12)	72.4 ± 0.8 (18)	72.9 ± 1.1 (16)
18–21	68.1 ± 1.7 (16)	68.0 ± 2.0 (19)	68.5 ± 1.8 (22)	67.6 ± 1.5 (18)	68.0 ± 1.3 (19)	66.9 ± 2.8 (12)	67.4 ± 2.0 (18)	65.9 ± 1.5 (16)
Chemical Intake (mg/kg/day)[d,e]								
0–21	0.0 ± 0.0 (16)	0.0 ± 0.0 (19)	73.2 ± 1.2 (22)	74.4 ± 1.3 (18)	220.5 ± 2.5 (19)	220.0 ± 3.9 (12)	435.1 ± 5.7 (18)	430.3 ± 5.4 (16)

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.

Consumption is only reported for pregnant animals.

*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.

GD = gestation day; RPC = reproductive performance cohort; PC = prenatal cohort.

[a]Data are displayed as mean ± standard error (n), where n = number of litters.

[b]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 of the PC performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.

[c]Excludes feed consumption from cages where excess food spillage was observed.

[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.

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 23.

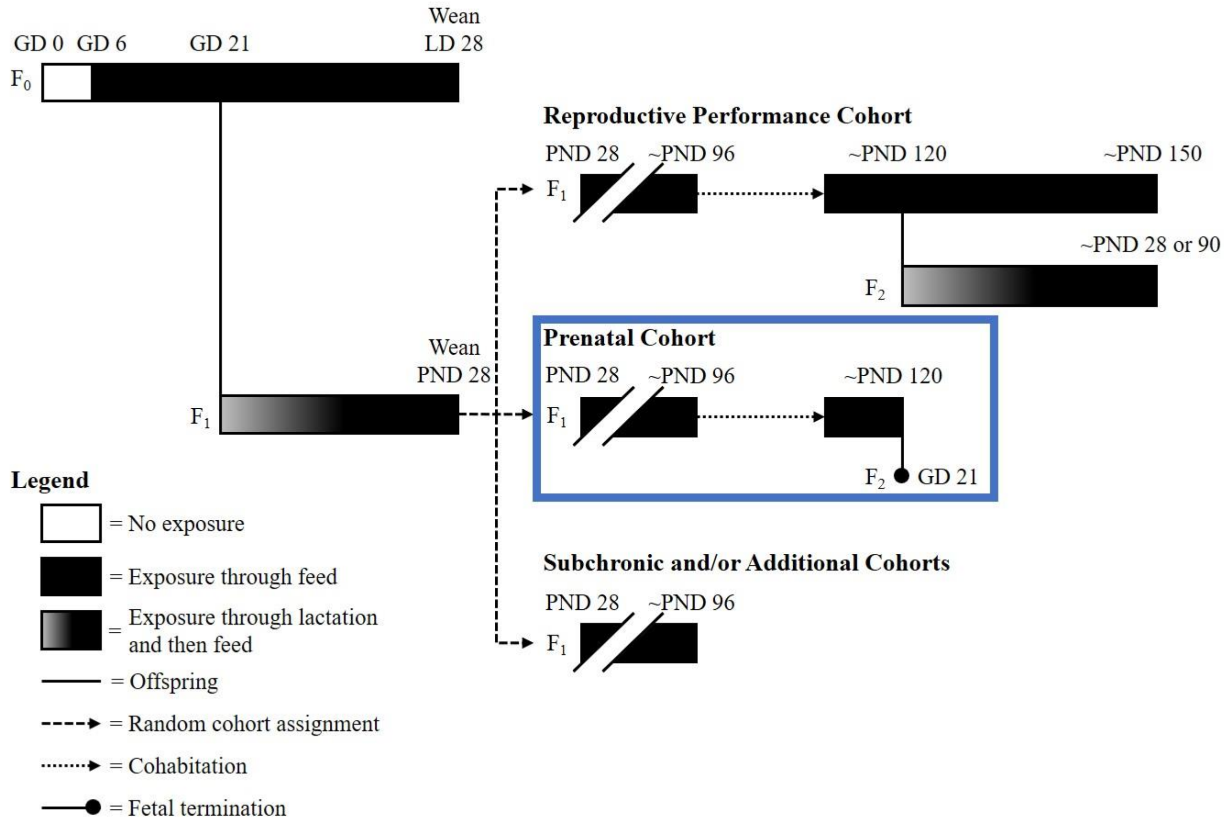


Figure 23. Design of the Modified One-Generation Study—Prenatal Cohort

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

Maternal Reproductive Performance and Uterine Data

In the prenatal cohort, females were between 111 and 113 days of age at the time of laparotomy. There was no effect of EHMC exposure on the number of implants, postimplantation loss, number of live fetuses, sex ratio, fetal weight, or gravid uterine weight (Table 24). Terminal and adjusted terminal mean body weights of the EHMC-exposed groups were similar to the control group.

Table 24. Summary of Uterine Content Data for F_1 Female Rats in the Prenatal Cohort Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed

	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
Pregnancy Summary[a]				
Paired Females	21	23	19	22
Mated Females	19	21	18	20
Pregnant Females[b]	19	18	15	16
Pregnant Females Examined on GD 21	19	18	12	16
Preimplantation Loss[c,d]				
Mean No. of Corpora Lutea/Female	17.74 ± 0.73 (19)	16.22 ± 0.55 (18)	18.71 ± 0.61 (14)	17.50 ± 0.74 (16)
Implantations/Female	15.21 ± 0.68 (19)	13.11 ± 1.19 (18)	15.75 ± 0.51 (12)	14.19 ± 0.88 (16)
Preimplantation Loss (%)	13.60 ± 3.52 (19)	21.73 ± 6.42 (18)	14.11 ± 2.65 (12)	18.54 ± 4.59 (16)
Intrauterine Deaths[d]				
Postimplantation Loss (%)[c]	1.89 ± 0.80 (19)	10.00 ± 6.24 (18)	3.21 ± 1.28 (12)	4.78 ± 1.48 (16)
Total Resorptions per Litter[c]	0.32 ± 0.13 (19)	0.39 ± 0.18 (18)	0.42 ± 0.19 (12)	0.56 ± 0.16 (16)
Early Resorptions per Litter[c]	0.32 ± 0.13 (19)	0.33 ± 0.18 (18)	0.42 ± 0.19 (12)	0.56 ± 0.16 (16)
Late Resorptions per Litter[c]	0.00 ± 0.00 (19)	0.06 ± 0.06 (18)	0.00 ± 0.00 (12)	0.00 ± 0.00 (16)
Dead Fetuses per Litter[c]	0.00 ± 0.00 (19)	0.00 ± 0.00 (18)	0.08 ± 0.08 (12)	0.00 ± 0.00 (16)
No. of Early Resorptions	6	6	5	9
No. of Late Resorptions	0	1	0	0
No. of Whole Litter Resorptions[a]	0	1	0	0
No. of Dead Fetuses	0	0	1	0
Live Fetuses[d]				
No. of Live Fetuses (Litters)	283 (19)	229 (17)	183 (12)	218 (16)
Live Fetuses per Litter[e]	14.89 ± 0.65	13.47 ± 1.11	15.25 ± 0.54	13.63 ± 0.93
Live Male Fetuses per Litter[e]	7.63 ± 0.49	6.47 ± 0.59	6.75 ± 0.57	7.13 ± 0.53
Live Female Fetuses per Litter[e]	7.26 ± 0.55	7.00 ± 0.66	8.50 ± 0.51	6.50 ± 0.58
Live Male Fetuses per Litter (%)[e]	51.75 ± 2.73	48.57 ± 2.11	44.06 ± 3.30	53.49 ± 2.62
Fetal Weight (g)[e,f]				
Fetal Weight per Litter	5.10 ± 0.08	5.07 ± 0.09	4.97 ± 0.06	4.97 ± 0.08
Male Fetal Weight per Litter	5.26 ± 0.08	5.19 ± 0.10	5.07 ± 0.08	5.12 ± 0.09
Female Fetal Weight per Litter	4.94 ± 0.08	4.97 ± 0.08	4.88 ± 0.05	4.80 ± 0.08
Gravid Uterine Weight (g)[e,f]				
Gravid Uterine Weight	105.57 ± 3.94	90.37 ± 8.39[h]	105.90 ± 3.18	95.98 ± 5.59
Terminal Body Weight	414.4 ± 7.8	397.6 ± 9.9[h]	410.4 ± 6.0	397.9 ± 7.3
Adjusted Body Weight[g]	308.85 ± 6.13	307.22 ± 5.12[h]	304.54 ± 4.79	301.96 ± 4.60

GD = gestation day.
[a]Statistical analysis performed by the Cochran-Armitage (trend) and Fisher's exact (pairwise) tests.
[b]Number pregnant included animals that had evidence of pregnancy but were removed from the study before GD 21.
[c]Data are reported per litter as mean ± standard error (number of females) and do not include nonmated, nonpregnant, or unexamined animals or those that did not survive to the end of the study.
[d]Statistical analysis performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.

[e]Data are reported per litter as mean ± standard error and do not include nonpregnant animals or those that did not survive to the end of the study.
[f]Statistical analysis performed by the Jonckheere (trend) and Williams or Dunnett (pairwise) tests.
[g]Body weight adjusted for gravid uterus weight.
[h]Sample size of n=18.

Fetal Findings

Placental Morphology

There was no effect of EHMC exposure on the incidence of placental abnormalities in the prenatal cohort (Appendix E). Fused placentae between two adjacent fetuses were noted for a single litter in the control and 1,000 ppm groups.

External

There was no effect of EHMC exposure on the incidence of fetal external abnormalities in the prenatal cohort (Appendix E). Fetal external abnormalities were limited to a single fetus in the 6,000 ppm group with a right clubbed hindlimb and a single incidence of left clubbed hindlimb in the control group.

Visceral

There was no effect of EHMC exposure on the incidence of fetal visceral abnormalities in the prenatal cohort (Appendix E). Male and female fetuses (combined) exposed to 6,000 ppm displayed a higher incidence of hydronephrosis (malformation; four in one litter) with a positive trend. One animal in the control group had unilateral (right) hydronephrosis, as did one fetus in the 3,000 ppm group. The incidences of dilated renal pelvis (variation), distended ureter (variation), and hydroureter (malformation) in the EHMC-exposed groups were similar to those in the control groups. When the kidney and ureter malformations were combined, no EHMC-related differences in incidence were observed. Similarly, EHMC exposure was not associated with a higher incidence of combined dilated renal pelvis or distended ureter variations.

Head

There was no effect of EHMC exposure on the incidence of fetal head abnormalities in the prenatal cohort (Appendix E).

Skeletal

There was no effect of EHMC exposure on the incidence of fetal skeletal malformations in the prenatal cohort (Appendix E).

Fetuses exposed to 6,000 ppm displayed a slightly higher individual (positive trend) and litter incidence of the variation of left, lumbar 1 rudimentary ribs compared with the control group (Table 25). The incidence of bilateral lumbar 1 rudimentary ribs in the 6,000 ppm group was slightly higher than the control group. When all lumbar 1 rudimentary rib variants were combined, the combined fetal incidence of rudimentary lumbar 1 ribs was higher than control animals (10% versus 4% in the control group) (Table 25).

Table 25. Summary of Select Skeletal Findings in Fetuses Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed

	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
No. Litters Examined	19	17	12	16
No. Fetuses Examined	283	211	183	218
Ribs[a,b]				
Lumbar, 1, Unilateral or Bilateral, Rudimentary – [V][c]				
Fetuses	12 (4.24)	8 (3.79)	7 (3.83)	22 (10.09)
Litters	5 (26.32)	5 (29.41)	2 (16.67)	7 (43.75)
Lumbar, 1, Bilateral, Rudimentary – [V][d]				
Fetuses	4 (1.41)	4 (1.90)	4 (2.19)	8 (3.67)
Litters	2 (10.53)	3 (17.65)	2 (16.67)	5 (31.25)
Lumbar, 1, Left, Rudimentary – [V][e]				
Fetuses	0 (0.00)[#]	4 (1.90)	0 (0.00)	8 (3.67)
Litters	0 (0.00)	4 (23.53)	0 (0.00)	4 (25.00)
Lumbar, 1, Right, Rudimentary – [V][f]				
Fetuses	8 (2.83)	0 (0.00)	3 (1.64)	6 (2.75)
Litters	5 (26.32)	0 (0.00)	1 (8.33)	4 (25.00)

Statistical significance for the vehicle control group indicates a significant trend test.
[#]Statistically significant at $p \leq 0.05$ (litter-based analysis).
[V] = variation.
[a]Upper row denotes number of affected fetuses (%) and lower row the number of affected litters (%).
[b]Statistical analysis for fetal data including litter effects performed using a Rao-Scott modification to the Cochran-Armitage test in which the litter was the random effect for both trend and pairwise analyses.
[c]Historical control incidence: fetuses – 82/1,385 (5.92%), range 0.00% to 13.69%; litters – 29/97 (29.90%), range 0.00% to 65.91%.
[d]Historical control incidence: fetuses – 7/1,385 (0.51%), range 0.00% to 1.41%; litters – 4/97 (4.12%), range 0.00% to 12.50%.
[e]Historical control incidence: fetuses – 5/1,385 (0.36%), range 0.00% to 2.14%; litters – 4/97 (4.12%), range 0.00% to 25.00%.
[f]Historical control incidence: fetuses – 11/1,385 (0.79%), range 0.00% to 2.83%; litters – 8/97 (8.25%), range 0.00% to 26.32%.

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.

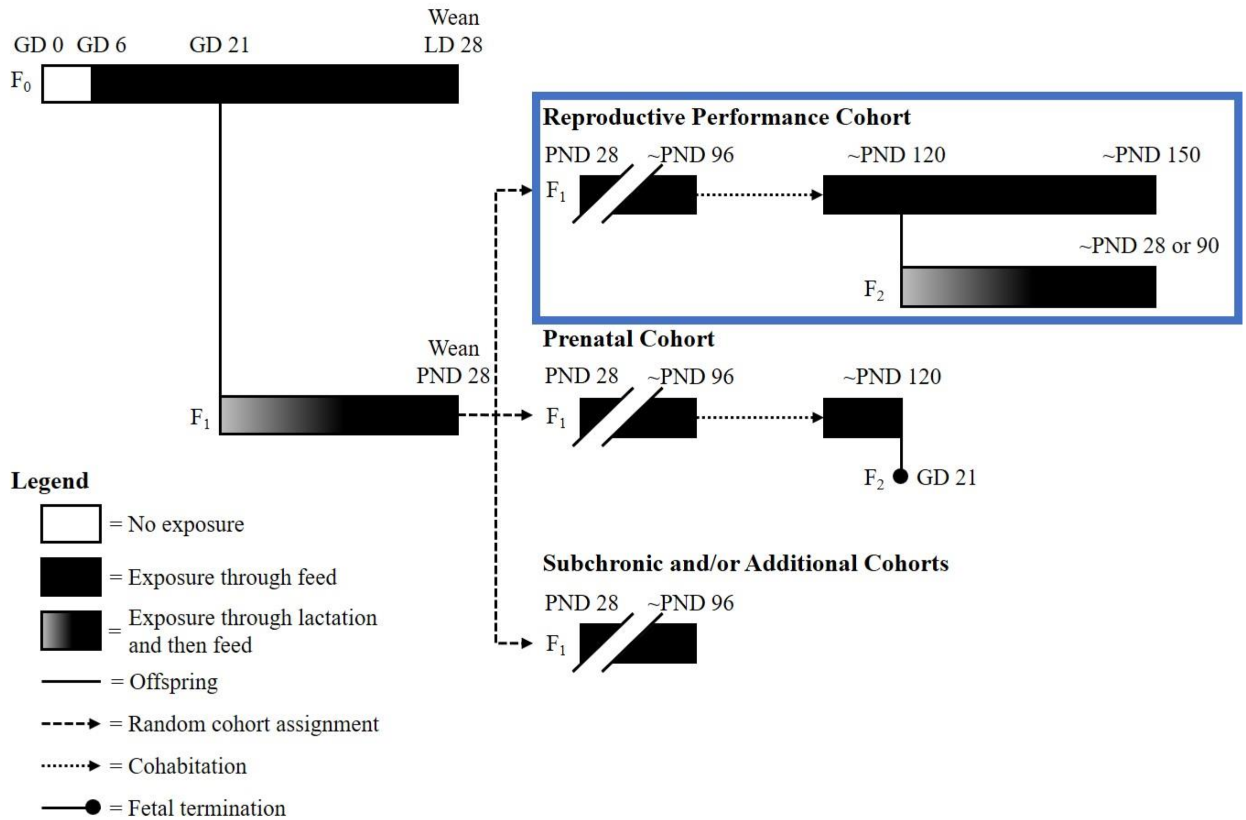


Figure 24. Design of the Modified One-Generation Study—Reproductive Performance Cohort

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

Reproductive Performance and Littering

In the reproductive performance cohort, the time to mating, number of females mated, pregnant, and littering were similar among the EHMC-exposed groups and similar to the control group (Table 26). Although gestation length was generally similar among the EHMC-exposed groups and the control group, gestational length appeared slightly, but significantly, decreased (approximately 7 hours) in the 3,000 ppm group. Given the low confidence in capturing the actual time that mating occurred (time is often recorded the morning when the presence of a vaginal copulation plug or sperm in a vaginal lavage is confirmed), the small magnitude of the response, and absence of an exposure concentration response, the shortened duration of gestation was not considered related to EHMC exposure.

Table 26. Summary of Reproductive Parameters of F_1 Female Rats in the Reproductive Performance Cohort Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed

	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
No. Females Paired	36	46	35	37
No. Females Mated	34	41	32	34
No. Females Littering	26	34	24	26
Percent of Mated Females/Paired[a,b]	94.4	89.1	91.4	91.9
Percent of Littered Females/Paired[a,b]	72.2	73.9	70.6	70.3
Percent of Littered Females/Mated[a,b]	76.5	82.9	77.4	76.5
Precoital Interval (days)[c,d,e]	4.9 ± 0.7 (19)	5.1 ± 0.6 (22)	4.8 ± 0.7 (19)	4.6 ± 0.6 (20)
Gestation Length (days)[c,d,f]	22.5 ± 0.1* (16)	22.7 ± 0.1 (22)	22.2 ± 0.1* (18)	22.3 ± 0.1 (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 $p \leq 0.05$.
[a]Statistical analysis performed using the Rao-Scott Cochran-Armitage test for both trend and pairwise comparisons to adjust for litter effects (unless otherwise noted).
[b]Animals removed from study between mating and littering were excluded from calculations of percent littered females.
[c]Statistical analysis performed using a bootstrapped Jonckheere test for trend and a Datta-Satten modified Wilcoxon test with Hommel adjustment for pairwise comparisons.
[d]Data are displayed as mean ± standard error (n).
[e]Precoital interval calculated for sperm-positive females.
[f]Gestation length calculated for sperm-positive females that delivered a litter.

Lactation Body Weights and Feed Consumption

Consistent with their premating and gestational body weights, F_1 female mean body weights during lactation were significantly decreased in the 6,000 ppm group compared to the control group by 6% and 7% on LD 1 and LD 13, respectively (Table 27; Figure 25). On LD 28, female mean body weights of the 6,000 ppm group were 5% lower than those of the control group. Mean body weight gain between LD 1 and LD 28 of the 6,000 ppm group was higher than that of the control group. In general, feed consumption during lactation by the EHMC-exposed groups was similar to that by the control group (Table 27). EHMC intake during lactation, based on feed consumption and dietary concentrations for LD 1–13, was exposure concentration-proportional and approximately 139, 418, and 842 mg/kg/day at exposure concentrations of 1,000, 3,000, and 6,000 ppm, respectively (Table 27).

Table 27. Summary of Mean Body Weights, Body Weight Gains, and Feed and Test Article Consumption of F_1 Female Rats in the Reproductive Performance Cohort Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed during Lactation

Lactation Day[a]	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
Body Weight (g)[b]				
1	318.0 ± 6.2** (18)	310.2 ± 3.9 (23)	306.0 ± 4.5 (18)	297.5 ±4.0* (18)
13	340.2 ± 5.3** (18)	334.5 ± 4.5 (22)	323.8 ± 4.0 (16)	316.4 ± 2.9** (17)
28	318.7 ± 5.8* (18)	311.4 ± 4.5 (22)	304.2 ± 5.3 (16)	302.6 ± 3.4 (17)
Body Weight Gain (g)[b]				
1–28	0.6 ± 3.2 (18)	2.6 ± 3.0 (22)	2.3 ± 3.1 (16)	8.0 ± 3.2 (17)
Feed Consumption[c]				
1–13 (g/animal/day)	45.3 ± 1.7 (18)	44.6 ± 1.2 (22)	43.8 ± 2.0 (18)	43.1 ± 1.4 (18)
1–13 (g/kg/day)	137.9 ± 5.9 (18)	138.5 ± 3.9 (22)	139.2 ± 6.4 (18)	140.4 ± 5.5 (18)
Chemical Intake (mg/kg/day)[d,e]				
1–13	0 ± 0.0 (18)	138.5 ± 3.9 (22)	417.5 ± 19.2 (18)	842.4 ± 32.8 (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 $p \leq 0.05$; **$p \leq 0.01$.
[a]Data are displayed as mean ± standard error (n), where n = number of litters.
[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 comparisons.
[c]Statistical analysis performed using a bootstrapped Jonckheere test for trend and a Datta-Satten modified Wilcoxon test with Hommel adjustment for pairwise comparisons.
[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.

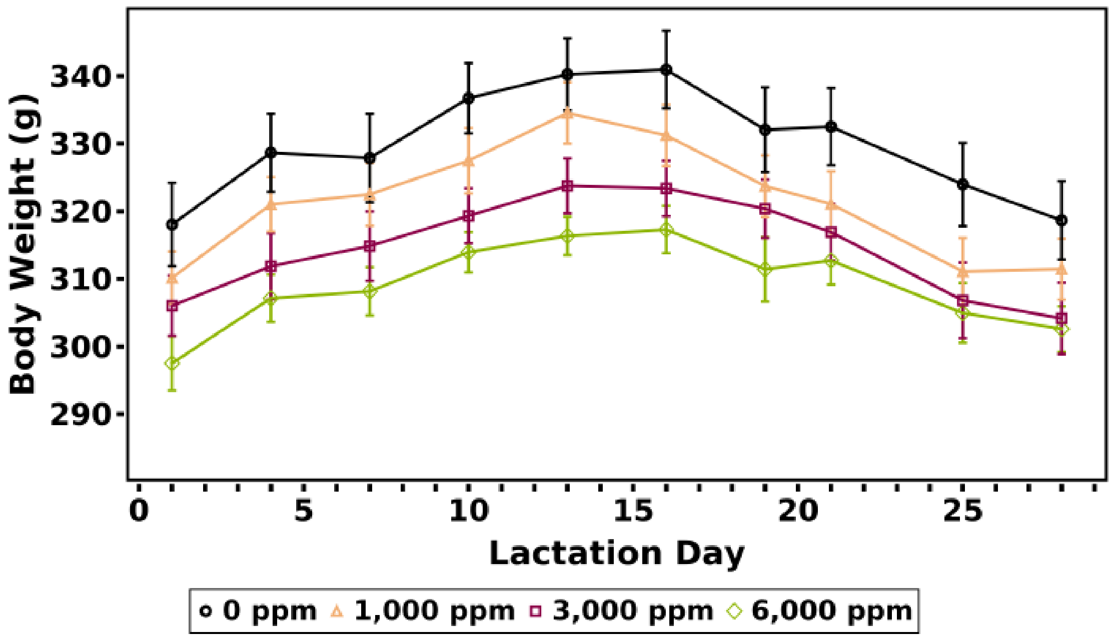


Figure 25. Lactation Growth Curves for F_1 Female Rats in the Reproductive Performance Cohort Exposed to 2-Ethylhexyl p-Methoxycinnamate in Feed

Information for statistical significance in F_1 female rat weights is provided in Table 27.

F_2 Viability and Clinical Observations

Mean total and live litter size of the EHMC-exposed groups from the reproductive performance cohort were similar to the control group, and pup survival was unaffected by EHMC exposure (Table 28). Similar analogous litter parameters were observed in the prenatal cohort.

Clinical observations noted in individual pups in all exposure groups, including the control group, typically were indicative of an individual pup not thriving and included being cold to touch, pale, no milk in the stomach, and bruising. There was no difference in litter size among the groups (Table 28).

Table 28. Summary of F_2 Litter Size and Pup Survival Following Perinatal Exposure to 2-Ethylhexyl p-Methoxycinnamate

Postnatal Day	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
No. of Live Pups (Litters)[a]				
0	396 (26)	464 (34)	382 (24)	363 (26)
Total Litter Size[b,c]				
0	15.3 ± 0.8 (18)	13.6 ± 0.8 (23)	16.1 ± 0.6 (18)	13.7 ± 0.9 (18)
Live Litter Size[b,c]				
0	14.1 ± 0.8 (18)	13.0 ± 0.7 (23)	15.0 ± 0.6 (18)	13.1 ± 0.8 (18)
1	13.8 ± 1.0 (18)	13.0 ± 0.7 (22)	14.7 ± 0.6 (18)	12.8 ± 0.8 (18)
4 (prestandardization)	13.5 ± 0.9 (18)	13.1 ± 0.7 (22)	14.0 ± 0.8 (18)	12.5 ± 0.8 (18)
4 (poststandardization)	9.4 ± 0.5 (18)	9.4 ± 0.3 (22)	9.6 ± 0.4 (18)	9.3 ± 0.4 (18)
7	8.7 ± 0.7 (18)	8.8 ± 0.4 (22)	9.3 ± 0.4 (16)	8.9 ± 0.6 (18)
13	7.4 ± 0.7 (18)	8.2 ± 0.5 (22)	8.0 ± 0.6 (16)	8.4 ± 0.6 (17)
21	7.4 ± 0.7 (18)	8.2 ± 0.5 (22)	8.0 ± 0.6 (16)	8.4 ± 0.6 (17)
28	7.4 ± 0.7 (18)	8.2 ± 0.5 (22)	8.0 ± 0.6 (16)	8.4 ± 0.6 (17)
No. of Dead Pups (Litters)[b,c]				
0	30 (16)	23 (15)	23 (9)	24 (17)
1–4	13 (7)	26 (12)	33 (9)	18 (10)
5–28	44 (16)	43 (10)	52 (16)	38 (11)
Dead per Litter[b,c]				
0	1.21 ± 0.27 (18)	0.59 ± 0.17 (23)	1.06 ± 0.30 (18)	1.00 ± 0.23 (18)
1–4	0.56 ± 0.22 (18)	0.85 ± 0.37 (23)	1.08 ± 0.66 (18)	0.61 ± 0.25 (18)
5–28	2.03 ± 0.60 (18)	1.27 ± 0.46 (22)	2.53 ± 0.67 (18)	1.39 ± 0.57 (18)
Survival Ratio[b,c]				
0	0.91 ± 0.02 (18)	0.96 ± 0.01 (23)	0.94 ± 0.02 (18)	0.90 ± 0.03 (18)
1–4	0.93 ± 0.04 (18)	0.90 ± 0.05 (23)	0.92 ± 0.05 (18)	0.96 ± 0.02 (18)
5–28	0.80 ± 0.06 (18)	0.87 ± 0.05 (22)	0.71 ± 0.08 (18)	0.85 ± 0.06 (18)

[a]n = the number of pups examined (number of F_1 litters).
[b]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.
[c]Statistical analysis performed using the bootstrapped Jonckheere test for trend and a Datta-Satten modified Wilcoxon test with Hommel adjustment for pairwise comparisons. All calculations are based on the last litter observation of the day.

F_2 Body Weights

Male Pups

Male pups exposed to EHMC displayed lower pup mean body weights (litter means) with increasing exposure concentration, and the differences among groups became greater over time (Table 29; Figure 26; Appendix E). On PNDs 4 and 10, male pup mean body weight per litter in the 6,000 ppm group was 8% lower relative to the control group (negative trend). A significant decrease in pup mean body weight was first observed in male offspring on PND 13 (decreased 10% relative to the control group), and on PND 28, pup mean body weights were significantly decreased by 14% relative to the control group. These effects are consistent with what was observed in the F_1 generation.

Female Pups

Female pups exposed to 6,000 ppm also displayed lower pup mean body weights (litter means) compared to the control group (Table 29; Figure 27; Appendix E). A significant decrease in pup mean body weight was also first observed in female offspring on PND 13 (decreased 7% relative to the control group), and on PND 28, pup mean body weights were significantly decreased by 11% relative to the control group. These effects are consistent with what was observed in the F_1 generation.

Table 29. Summary of F_2 Male and Female Pup Mean Body Weights Following Perinatal Exposure to 2-Ethylhexyl p-Methoxycinnamate[a,b]

Postnatal Day	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
Male				
1	6.88 ± 0.09 165 (25)[c]	6.78 ± 0.14 208 (33)	6.63 ± 0.09 167 (24)	6.68 ± 0.08 159 (25)
4	9.61 ± 0.18* 163 (25)	9.38 ± 0.23 206 (32)	8.63 ± 0.27* 160 (24)	8.87 ± 0.22 156 (25)
7	13.52 ± 0.41 102 (25)	13.32 ± 0.41 155 (32)	12.52 ± 0.39 97 (21)	12.87 ± 0.44 109 (24)
10	19.10 ± 0.63* 96 (25)	19.14 ± 0.56 139 (32)	17.45 ± 0.58 91 (21)	17.64 ± 0.49 99 (23)
13	26.35 ± 0.81** 94 (25)	26.07 ± 0.63 135 (32)	24.69 ± 0.67 86 (21)	23.72 ± 0.55** 97 (23)
16	33.77 ± 0.99** 94 (25)	33.16 ± 0.69 133 (32)	31.63 ± 0.86 86 (21)	29.46 ± 0.66** 96 (23)
19	39.68 ± 1.12** 94 (25)	39.07 ± 0.83 135 (32)	37.71 ± 1.00 86 (21)	34.48 ± 0.73** 96 (23)
21	46.17 ± 1.39** 94 (25)	45.73 ± 1.14 135 (32)	43.13 ± 1.20 86 (21)	39.40 ± 0.86** 96 (23)
28	78.45 ± 2.28** 94 (25)	78.20 ± 1.68 135 (32)	73.29 ± 2.05 86 (21)	67.29 ± 1.32** 96 (23)

Postnatal Day	0 ppm	1,000 ppm	3,000 ppm	6,000 ppm
Female				
1	6.50 ± 0.14 194 (26)	6.43 ± 0.10 214 (33)	6.33 ± 0.08 185 (24)	6.43 ± 0.10 171 (25)
4	8.69 ± 0.29 190 (26)	8.70 ± 0.22 211 (32)	8.16 ± 0.28 181 (24)	8.33 ± 0.23 166 (25)
7	12.34 ± 0.50 131 (25)	12.70 ± 0.36 135 (32)	11.67 ± 0.47 104 (21)	12.28 ± 0.47 113 (24)
10	17.66 ± 0.80 116 (25)	18.53 ± 0.51 126 (32)	16.98 ± 0.67 92 (20)	17.33 ± 0.51 103 (23)
13	24.95 ± 0.87** 110 (24)	24.98 ± 0.65 126 (32)	24.13 ± 0.76 85 (20)	23.11 ± 0.54* 102 (23)
16	32.29 ± 0.95** 110 (24)	32.05 ± 0.73 125 (32)	30.68 ± 0.94 85 (20)	28.60 ± 0.61** 102 (23)
19	37.80 ± 1.12** 110 (24)	37.73 ± 0.83 125 (32)	36.48 ± 0.93 85 (20)	33.49 ± 0.66** 102 (23)
21	43.56 ± 1.45** 110 (24)	43.99 ± 1.06 125 (32)	41.29 ± 1.16 85 (20)	38.60 ± 0.85** 102 (23)
28	71.21 ± 2.07** 110 (24)	71.79 ± 1.65 125 (32)	67.82 ± 1.84 85 (20)	63.62 ± 1.31** 102 (23)

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 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]n = number of pups examined (number of F_1 litters).

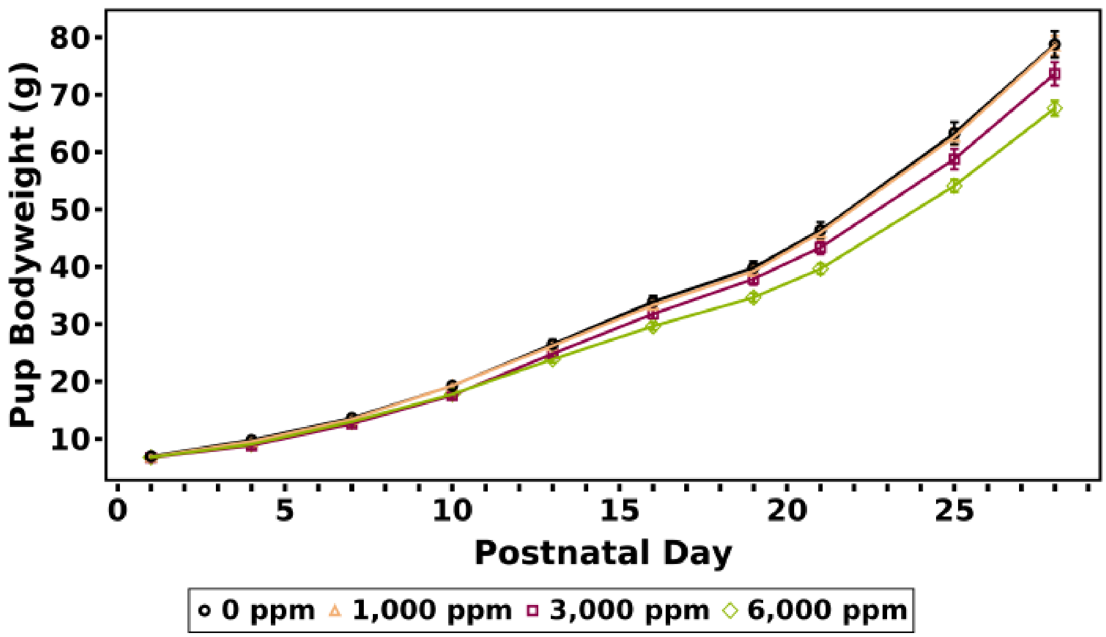


Figure 26. Lactation Growth Curves for F_2 Male Pups Following Perinatal Exposure to 2-Ethylhexyl p-Methoxycinnamate

Information for statistical significance in F_2 male rat weights is provided in Table 29.

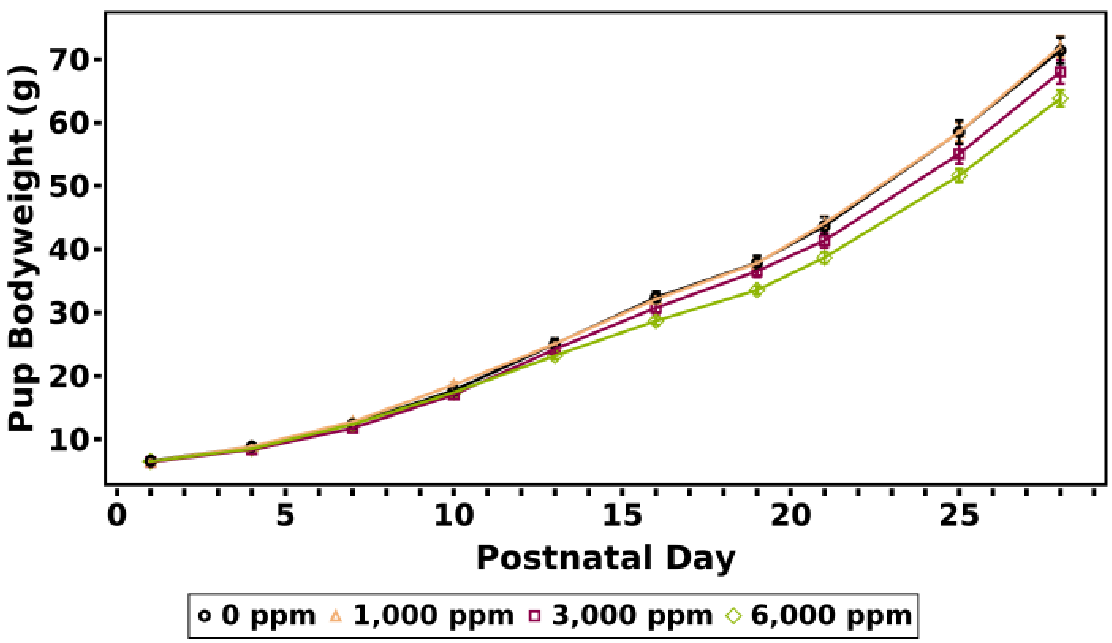


Figure 27. Lactation Growth Curves for F_2 Female Pups Following Perinatal Exposure to 2-Ethylhexyl p-Methoxycinnamate

Information for statistical significance in F_2 female rat weights is provided in Table 29.

Prenatal, Reproductive Performance, and Subchronic Cohorts: Necropsies

F_1 Male Necropsies

F_1 males in the reproductive performance cohort were euthanized following the mating period corresponding to 161–167 days of age. F_1 males in the prenatal and subchronic cohorts were euthanized following completion of prenatal pairing corresponding to 110–114 days of age. Terminal mean body weights of rats exposed to 6,000 ppm were significantly decreased relative to the control groups in both the reproductive performance cohort (7%) and the prenatal cohort (5%) (Appendix E); the lower (4%) terminal mean body weight of the 6,000 ppm subchronic cohort males was not significantly different from that of the control group.

Exposed rats in all adult cohorts did not display any gross pathology findings attributable to EHMC exposure. All exposure groups, including the control group, displayed very low incidences of gross pathology findings, none of which exhibited an exposure concentration-response relationship (Appendix E).

In the subchronic cohort, no changes in the weights of the thymus, heart, lungs, or kidneys were directly attributable to EHMC exposure compared to the subchronic control group. Relative liver weight was slightly higher (positive trend) in the 6,000 ppm group males, which was associated with lower mean body weights (Appendix E). Males in the 6,000 ppm group also displayed significantly decreased absolute and relative ventral prostate gland weights (negative trend and pairwise significance). This response was not observed in either the prenatal or reproductive performance cohorts, which had more animals examined; therefore, lower ventral prostate gland weights were not considered related to EHMC exposure. Rats in the reproductive performance cohort exposed to 3,000 or 6,000 ppm displayed a significant increase (9% and 11%, respectively) in absolute seminal vesicle weights and in relative seminal vesicle weights (14% and 20%, respectively) (Appendix E). Given that sperm parameters and reproductive endpoints measured in the reproductive performance cohort were not affected by EHMC exposure, these decreases in organ weights were not considered toxicologically significant.

F_1 Female Necropsies

F_1 females and F_2 offspring in the reproductive performance cohort were euthanized on PND 28, and the F_1 females were 153–169 days of age at the time of necropsy. Females in the prenatal cohort were between 116–132 days of age at the time of necropsy, and females in the subchronic cohort were 111–113 days of age at necropsy. Terminal/adjusted mean body weights at time of necropsy of the 6,000 ppm group, irrespective of cohort, were <5% lower than those of the control groups (Appendix E). No gross findings were attributed to EHMC exposure in any of the cohorts examined (Appendix E).

F_2 Necropsy

Pups were euthanized on PND 28. No findings were attributed to EHMC exposure (Appendix E). A low incidence of bilateral distended ureter was observed in the 6,000 ppm group (two pups from one litter). Unilateral distended ureter (left) was observed in all groups, including the control group (one in each group). This low incidence in all exposed groups is consistent with what was observed in all exposed groups in the prenatal cohort.

Clinical Pathology

There were significant decreases in alanine aminotransferase (ALT) activity in the 3,000 and 6,000 ppm female rats (Appendix E). The mechanism for the decreased activity is not known but may indicate changes in ALT metabolism; decreases in hepatic enzyme activity have no known toxicological relevance.

Pathology

No histopathological findings in any of the cohorts were considered related to exposure to EHMC.

Discussion

The objective of this study was to characterize the potential for 2-ethylhexyl p-methoxycinnamate (EHMC), a common component of sunscreen and personal care products, to adversely affect any phase of rat development, maturation, or ability to successfully reproduce, and to cause subchronic toxicity in the F_1 generation.

Mechanistic screening studies have indicated that EHMC is capable of transactivation of the estrogen receptor (ER), inducing uterotrophic responses, and attenuating progesterone receptor transactivation.[18-20] Given these reported findings and wide human exposure, the National Toxicology Program (NTP) conducted a study to examine the possible effects of EHMC exposure on developmental and reproductive endpoints and possible subchronic toxicity in the presence of continual EHMC exposure. As disposition is similar following oral and dermal exposures, EHMC exposure via the diet was selected for this study to sustain internal exposure and to avoid variability in internal dose from topical application and subsequent 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. Exposure concentration selection was informed by a dose range-finding study that indicated that 6,000 ppm in the feed would be well-tolerated by the dams and would likely result in approximately 10% lower pup mean body weight. The exposure concentrations of 1,000 and 3,000 ppm were selected to aid in identifying potential exposure concentration-response relationships. This spacing would ideally avoid excessive exposure overlap of the respective ingested doses of mg EHMC/kg body weight/day (mg/kg/day), recognizing that the amount of feed consumed depends on pregnancy state, sex, and age.

In contrast to previously reported in vitro and short-term rat in vivo endocrine disruptor screening studies, EHMC exposure did not appear to induce any substantial effects on androgen receptor (AR)-dependent endpoints. Although F_1 male rats exposed to 6,000 ppm displayed a slight but significant delay in attainment of balanopreputial separation (BPS) (when adjusted for body weight on postnatal day [PND] 28) and F_1 male rats in the subchronic cohort displayed a slight but significant decrease in absolute ventral prostate gland weight, no concomitant effects were observed in anogenital distance or male areolae/nipple retention in F_1 or F_2 male rats. Moreover, similar decreases in ventral prostate gland weight or decreases in any AR-dependent reproductive tissue examined were not observed in either the reproductive performance cohort or the prenatal cohort in which more male animals per exposure group had been examined. Furthermore, there were no malformations in AR-dependent tissues or histopathological findings consistent with alterations in androgen action or apparent effects of EHMC exposure on F_1 male reproductive performance in either mating cohort, indicating a normal functioning male reproductive system. Collectively, the data suggest that the significant decrease in ventral prostate gland weight observed in the subchronic cohort was spurious. The absence of reproductive effects in male Sprague Dawley (Hsd:Sprague Dawley® SD®) rats in the current study are inconsistent with previously reported decreased sperm counts in Wistar Han rats following gestational and lactational EHMC exposure. The different study results could reflect different sensitivities of the two rat strains or the different dosing paradigms, gavage versus dietary. Moreover, the absence of observed EHMC-mediated effects on AR- and ER-dependent processes is consistent with that of the previously reported Endocrine Disruptor Screening

Program studies that demonstrated that EHMC had no apparent effects on AR and ER binding and activation.[26]

Male and female F_1 and F_2 offspring exposed to EHMC displayed mean body weights similar to those of the control groups on PND 0. However, as the lactational period progressed to the point when pups started to eat feed, pups in the 3,000 and 6,000 ppm groups exhibited lower mean body weights and weight gains compared to the control animals. On PND 28, pups in the 6,000 ppm groups weighed approximately 13%–15% less than the control groups; however, by PND 91, the body weights of pups exposed to 6,000 ppm were approximately 5%–7% lower than those of the control groups, demonstrating some reversibility/recovery of the effect on body weight.

F_1 females in the 6,000 ppm group displayed a slight but significant delay of approximately 2 days of litter mean day of vaginal opening (VO) attainment, when adjusted for body weight at weaning (PND 28). Similarly, F_1 male animals in the 6,000 ppm group displayed a comparable 2-day delay of the litter mean day of attaining BPS when adjusted for body weight on PND 28. However, both male and female rats had similar respective body weights on day of attainment, and the magnitude of body weight suppression in the 6,000 ppm group was lessening, indicating "recovery." Intrauterine growth retardation—after ligation of the uterine artery on gestation day (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.[73] Postnatal dietary restriction also has been shown to delay VO with similar body weights at time of VO.[74] 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. Similarly, intrauterine growth retardation as well as postnatal feed restriction, resulting in lower postnatal body weights, have been shown to delay BPS.[73] It is plausible that the similar weights on day of attainment observed in the current study, like VO, have a weight or body mass requirement for attainment of BPS to occur. Nonetheless, given the small magnitude of change, comparable mean body weights on day of attainment, and absence of alterations in AR-mediated endpoints, the observed BPS response is likely secondary to effects on growth rate and not AR-mediated. Although the delay in VO is consistent with those previously reported[22] and occurred in the presence of subtle effects on estrous cyclicity (time in estrus, increase in estrous stage length), the effects were not commensurate with biologically significant alterations in reproductive function or postnatal support of the offspring. Given these apical delays in attainment, concomitant with effects on growth, it was unclear whether these findings were directly attributable to EHMC exposure. Markov model estimates of estrous stage length indicated a slight but significant increase in estrus stage length in all EHMC-exposed groups and respective decrease in diestrus stage length. This did not display an exposure concentration-response relationship nor affect overall cycle length. This apparent finding is likely not due to the lower body weights as feed restriction has been shown to lengthen the estrous cycle.[75] Given this, these discordant minimal responses in cycle length, independent of the delays in VO and BPS, were therefore considered equivocal evidence of developmental toxicity.

The only fetal finding observed that was attributed to EHMC exposure was the higher incidence of rudimental rib, a variation that exceeded the historical control incidence. This common fetal finding, in isolation, is not considered adverse. No delays in ossification were observed, unlike those that have been previously reported.[25] Two of EHMC's known metabolites, 2-ethylhexanol and 2-ethylhexanoic acid, have been shown to have teratogenic potential.[11] Administration of

12.5 mM/kg of 2-ethylhexanol (approximately 1,680 g/kg) to Wistar rats on GD 12 was associated with hydronephrosis and tail and limb malformations. Administration of 2-ethylhexanoic acid at the same mM dose induced a greater response in these endpoints. Cardiovascular defects were also observed.[76]

Exposure of Wistar rats to 2-ethylhexanoic acid from GD 6 through GD 19 via drinking water at exposure concentrations of 100, 300, or 600 mg/kg/day was associated with fetal malformations of clubfoot, absence of fibula, and polydactyly.[77] In contrast, topical application in Fischer 344 rats from GD 6 through GD 15 at exposure concentrations ≤2,520 mg/kg/day was not associated with any teratogenic responses.[78] The absence of malformations in the current study may be the result of metabolites not being produced to an internal concentration that would affect normal fetal development.

EHMC exposure was associated with an increase in liver weight, but this finding was not coupled with any adverse histopathological findings. The weight increase might be a secondary response given that the liver is a major site of EHMC metabolism.

Conclusions

Under the conditions of this modified one-generation (MOG) study, there was *no evidence of reproductive toxicity* of 2-ethylhexyl p-methoxycinnamate (EHMC) in Hsd:Sprague Dawley® SD® rats at exposure concentrations of 1,000, 3,000, or 6,000 ppm. Mating and littering were not affected significantly by EHMC exposure.

Under the conditions of this MOG study, there was *equivocal evidence of developmental toxicity* of EHMC in Hsd:Sprague Dawley® SD® rats based on the observed postnatal effects on body weight that showed some indication of recovery by study end, delays in postnatal day 28-adjusted vaginal opening and balanopreputial separation, which could have influenced the apparent transient effects on body weight, and time in estrus was slightly longer in EHMC-exposed females relative to that of the control group. No other signals consistent with alterations in estrogenic, androgenic, or antiandrogenic action were observed. EHMC exposure did not induce any specific fetal malformations.

References

1. ChemSpider. Structure search. 2021. https://www.chemspider.com/StructureSearch.aspx

2. National Center for Biotechnology Information (NCBI). PubChem Compound Summary for CID 21630, Octyl methoxycinnamate. Bethesda, MD: National Library of Medicine. https://pubchem.ncbi.nlm.nih.gov/compound/21630

3. U.S. Environmental Protection Agency (USEPA). CompTox Database. https://comptox.epa.gov/dashboard

4. Liem DH, Hilderink LTH. U.V. absorbers in sun cosmetics 1978. Int J Cosmet Sci. 1979; 1(6):341-361. doi.org/10.1111/j.1467-2494.1979.tb00228.x

5. Garcia RDA, Maltarollo VG, Honório KM, Trossini GHG. Benchmark studies of UV–vis spectra simulation for cinnamates with UV filter profile. J Mol Model. 2015; 21(6):150. 10.1007/s00894-015-2689-y

6. Lee GS, Widjaja A, Ju YH. Enzymatic synthesis of cinnamic acid derivatives. Biotechnol Lett. 2006; 28(8):581-585. 10.1007/s10529-006-0019-2

7. National Center for Biotechnology Information (NCBI). PubChem Compound Summary for CID 21630, Octyl methoxycinnamate: 9.2.2 Consumer Uses. Bethesda, MD: National Library of Medicine. https://pubchem.ncbi.nlm.nih.gov/compound/Octyl-methoxycinnamate#section=Consumer-Uses

8. Environmental Working Group (EWG). EWG's skin deep: Octinoxate. 2019. https://www.ewg.org/skindeep/ingredient/704203/OCTINOXATE/

9. Huang Y, Law JC-F, Zhao Y, Shi H, Zhang Y, Leung KS-Y. Fate of UV filter ethylhexyl methoxycinnamate in rat model and human urine: Metabolism, exposure and demographic associations. Sci Total Environ. 2019; 686:729-736. https://doi.org/10.1016/j.scitotenv.2019.05.440

10. U.S. Code. 15(Section 2607).

11. Fennell TR, Mathews JM, Snyder RW, Hong Y, Watson SL, Black SR, McIntyre BS, Waidyanatha S. Metabolism and disposition of 2-ethylhexyl-p-methoxycinnamate following oral gavage and dermal exposure in Harlan Sprague Dawley rats and B6C3F1/N mice and in hepatocytes in vitro. Xenobiotica. 2018; 48(11):1142-1156. 10.1080/00498254.2017.1400129

12. Janjua NR, Mogensen B, Andersson A-M, Petersen JH, Henriksen M, Skakkebæk NE, Wulf HC. Systemic absorption of the sunscreens benzophenone-3, octyl-methoxycinnamate, and 3-(4-methyl-benzylidene) camphor after whole-body topical application and reproductive hormone levels in humans. J Invest Dermatol. 2004; 123(1):57-61. https://doi.org/10.1111/j.0022-202X.2004.22725.x

13. Benech-Kieffer F, Wegrich P, Schwarzenbach R, Klecak G, Weber T, Leclaire J, Schaefer H. Percutaneous absorption of sunscreens in vitro: interspecies comparison, skin models and

reproducibility aspects. Skin Pharmacol Appl Skin Physiol. 2000; 13(6):324-335. 10.1159/000029940

14. Hayden CG, Cross SE, Anderson C, Saunders NA, Roberts MS. Sunscreen penetration of human skin and related keratinocyte toxicity after topical application. Skin Pharmacol Physiol. 2005; 18(4):170-174. 10.1159/000085861

15. Klimová Z, Hojerová J, Beránková M. Skin absorption and human exposure estimation of three widely discussed UV filters in sunscreens – in vitro study mimicking real-life consumer habits. Food Chem Toxicol. 2015; 83:237-250. https://doi.org/10.1016/j.fct.2015.06.025

16. Jiménez MM, Pelletier J, Bobin MF, Martini MC. Influence of encapsulation on the in vitro percutaneous absorption of octyl methoxycinnamate. Int J Pharm. 2004; 272(1):45-55. https://doi.org/10.1016/j.ijpharm.2003.11.029

17. Puglia C, Bonina F, Castelli F, Micieli D, Sarpietro MG. Evaluation of percutaneous absorption of the repellent diethyltoluamide and the sunscreen ethylhexyl p-methoxycinnamate-loaded solid lipid nanoparticles: An in-vitro study. Journal of Pharmacy and Pharmacology. 2009; 61(8):1013-1019. 10.1211/jpp.61.08.0004

18. Schlumpf M, Cotton B, Conscience M, Haller V, Steinmann B, Lichtensteiger W. In vitro and in vivo estrogenicity of UV screens. Environ Health Perspect. 2001; 109(3):239-244. 10.1289/ehp.01109239

19. Schlumpf M, Schmid P, Durrer S, Conscience M, Maerkel K, Henseler M, Gruetter M, Herzog I, Reolon S, Ceccatelli R et al. Endocrine activity and developmental toxicity of cosmetic UV filters—an update. Toxicology. 2004; 205(1):113-122. https://doi.org/10.1016/j.tox.2004.06.043

20. Schreurs RHMM, Sonneveld E, Jansen JHJ, Seinen W, van der Burg B. Interaction of polycyclic musks and UV filters with the estrogen receptor (ER), androgen receptor (AR), and progesterone receptor (PR) in reporter gene bioassays. Toxicol Sci. 2005; 83(2):264-272. 10.1093/toxsci/kfi035

21. Klammer H, Schlecht C, Wuttke W, Jarry H. Multi-organic risk assessment of estrogenic properties of octyl-methoxycinnamate in vivo A 5-day sub-acute pharmacodynamic study with ovariectomized rats. Toxicology. 2005; 215(1-2):90-96. 10.1016/j.tox.2005.06.026

22. Axelstad M, Boberg J, Hougaard KS, Christiansen S, Jacobsen PR, Mandrup KR, Nellemann C, Lund SP, Hass U. Effects of pre- and postnatal exposure to the UV-filter octyl methoxycinnamate (OMC) on the reproductive, auditory and neurological development of rat offspring. Toxicol Appl Pharmacol. 2011; 250(3):278-290. 10.1016/j.taap.2010.10.031

23. Schneider S, Deckardt K, Hellwig J, Küttler K, Mellert W, Schulte S, van Ravenzwaay B. Octyl methoxycinnamate: Two generation reproduction toxicity in Wistar rats by dietary administration. Food and Chemical Toxicology. 2005; 43(7):1083-1092. https://doi.org/10.1016/j.fct.2005.02.013

24. European Chemicals Agency (ECHA). Registration dossier: 2-Ethylhexyl trans-4-methoxycinnamate: Toxicity to reproduction: 001 Key | Experimental result. 2021. https://echa.europa.eu/registration-dossier/-/registered-dossier/15876/7/9/2

25. European Commission (EC). Reports of the Scientific Committee on Cosmetology (Ninth Series). 1999. https://ec.europa.eu/health/sites/health/files/scientific_committees/consumer_safety/docs/scc_o_9.pdf

26. National Toxicology Program (NTP). NTP technical report on the toxicology and carcinogenesis studies of 2-hydroxy-4-methoxybenzophenone administered in feed to Sprague Dawley (Hsd:Sprague Dawley SD) rats and B6C3F1/N mice. Research Triangle Park, NC: U.S. Department of Health and Human Services, Public Health Service, National Toxicology Program; 2020. NTP Technical Report No. 597. https://ntp.niehs.nih.gov/publications/reports/tr/500s/tr597/index.html?utm_source=direct&utm_medium=prod&utm_campaign=ntpgolinks&utm_term=tr597abs

27. Rehfeld A, Dissing S, Skakkebæk NE. Chemical UV filters mimic the effect of progesterone on Ca2+ signaling in human sperm cells. Endocrinology. 2016; 157(11):4297-4308. 10.1210/en.2016-1473

28. Rehfeld A, Egeberg DL, Almstrup K, Petersen JH, Dissing S, Skakkebæk NE. EDC IMPACT: Chemical UV filters can affect human sperm function in a progesterone-like manner. Endocrine connections. 2018; 7(1):16-25. 10.1530/EC-17-0156

29. European Chemicals Agency (ECHA). Registration dossier: 2-Ethylhexyl trans-4-methoxycinnamate (CASRN: 83834-59-7): Acute toxicity: Oral: 001 key | Experimental result. 2020. https://echa.europa.eu/registration-dossier/-/registered-dossier/15876/7/3/2

30. European Chemicals Agency (ECHA). Registration dossier: 2-Ethylhexyl trans-4-methoxycinnamate (CASRN 83834-59-7): Eye irritation: 001 key | Experimental result. 2020. https://echa.europa.eu/registration-dossier/-/registered-dossier/15876/7/4/3

31. European Chemicals Agency (ECHA). Registration dossier: 2-Ethylhexyl trans-4-methoxycinnamate (CASRN 83834-59-7): Skin sensitisation: 001 key | Experimental result. 2020. https://echa.europa.eu/pt/registration-dossier/-/registered-dossier/15876/7/5/2

32. Rodriguez E, Valbuena MC, Rey M, Porras de Quintana L. Causal agents of photoallergic contact dermatitis diagnosed in the national institute of dermatology of Colombia. Photodermatol Photoimmunol Photomed. 2006; 22(4):189-192. 10.1111/j.1600-0781.2006.00212.x

33. Ang P, Ng SK, Goh CL. Sunscreen allergy in Singapore. Am J Contact Dermat. 1998; 9(1):42-44.

34. Darvay A, White IR, Rycroft RJ, Jones AB, Hawk JL, McFadden JP. Photoallergic contact dermatitis is uncommon. The British Journal of Dermatology. 2001; 145(4):597-601. 10.1046/j.1365-2133.2001.04458.x

35. National Toxicology Program (NTP). UV filters. Research Triangle Park, NC: U.S. Department of Health and Human Services, Public Health Service, National Toxicology

Program; 2020. https://ntp.niehs.nih.gov/whatwestudy/topics/uvfilters/index.html#:~:text=NTP%20is%20working%20to%20assess,chemical%20in%20rats%20and%20mice.

36. Blystone CR, Kissling GE, Bishop JB, Chapin RE, Wolfe GW, Foster PM. Determination of the di-(2-ethylhexyl) phthalate NOAEL for reproductive development in the rat: Importance of the retention of extra animals to adulthood. Toxicol Sci. 2010; 116(2):640-646.

37. U.S. Environmental Protection Agency (USEPA). Guidelines for developmental toxicity risk assessment. Washington, DC: U.S. Environmental Protection Agency, Risk Assessment Forum; 1991. EPA Document No. EPA/600/FR-91/001.

38. Makris SL, Solomon HM, Clark R, Shiota K, Barbellion S, Buschmann J, Ema M, Fujiwara M, Grote K, Hazelden KP. Terminology of developmental abnormalities in common laboratory mammals (version 2). Congenit Anom. 2009; 49(3):123-246. http://dx.doi.org/10.1111/j.1741-4520.2009.00239.x

39. Cora MC, Kooistra L, Travlos G. Vaginal cytology of the laboratory rat and mouse: Review and criteria for the staging of the estrous cycle using stained vaginal smears. Toxicol Pathol. 2015; 43(6):776-793. 10.1177/0192623315570339

40. Staples RE. Detection of visceral alterations in mammalian fetuses. Teratology. 1974; 9(3):A37-A38.

41. Stuckhardt JL, Poppe SM. Fresh visceral examination of rat and rabbit fetuses used in teratogenicity testing. Teratog Carcinog Mutagen. 1984; 4(2):181-188. 10.1002/tcm.1770040203

42. Thompson RF. Chapter 4: Basic neuroanatomy. Foundations of Physiological Psychology. New York, NY: Harper and Row Publishers; 1967. p. 79-82.

43. Marr MC, Price CJ, Myers CB, Morrissey RE. Developmental stages of the CD (Sprague-Dawley) rat skeleton after maternal exposure to ethylene glycol. Teratology. 1992; 46(2):169-181. 10.1002/tera.1420460210

44. Tyl RW. Commentary on the role of maternal toxicity on developmental toxicity. Birth defects research Part B, Developmental and reproductive toxicology. 2012; 95(3):262-266. 10.1002/bdrb.21015

45. Robb GW, Amann RP, Killian GJ. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J Reprod Fertil. 1978; 54(1):103-107. 10.1530/jrf.0.0540103

46. Maronpot RR, Boorman GA. Interpretation of rodent hepatocellular proliferative alterations and hepatocellular tumors in chemical safety assessment. Toxicol Pathol. 1982; 10(2):71-78. 10.1177/019262338201000210

47. Boorman GA, Haseman JK, Waters MD, Hardisty JF, Sills RC. Quality review procedures necessary for rodent pathology databases and toxicogenomic studies: The National Toxicology Program experience. Toxicol Pathol. 2002; 30(1):88-92. 10.1080/01926230252824752

48. Kupper L, Portier C, Hogan M, Yamamoto E. The impact of litter effects on dose-response modeling in teratology. Biometrics. 1986; 42(1):85-98. 10.2307/2531245

49. Rao JN, Scott AJ. A simple method for the analysis of clustered binary data. Biometrics. 1992; 48(2):577-585.

50. Fung KY, Krewski D, Rao JN, Scott AJ. Tests for trend in developmental toxicity experiments with correlated binary data. Risk Anal. 1994; 14(4):639-648.

51. Bailer AJ, Portier CJ. Effects of treatment-induced mortality and tumor-induced mortality on tests for carcinogenicity in small samples. Biometrics. 1988; 44(2):417-431.

52. Piegorsch W, Bailer AJ. Statistics for environmental biology and toxicology. London, England: CRC Press; 1997. p.

53. Portier CJ, Bailer AJ. Testing for increased carcinogenicity using a survival-adjusted quantal response test. Fundam Appl Toxicol. 1989; 12(4):731-737. 10.1016/0272-0590(89)90004-3

54. Bieler GS, Williams RL. Ratio estimates, the delta method, and quantal response tests for increased carcinogenicity. Biometrics. 1993; 49(3):793-801.

55. Nam JM. A simple approximation for calculating sample sizes for detecting linear trend in proportions. Biometrics. 1987; 43(3):701-705.

56. Dixon W, Massey F. Introduction to Statistical Analysis. New York, NY: McGraw Hill Book Company Inc; 1957. http://dx.doi.org/10.2307/2332898

57. Tukey J. Easy summaries – numerical and graphical. Exploratory Data Analysis. Reading, MA: Addison-Wesley; 1977. p. 43-44.

58. Dunnett CW. A multiple comparison procedure for comparing several treatments with a control. J Am Stat Assoc. 1955; 50(272):1096-1121. http://dx.doi.org/10.1080/01621459.1955.10501294

59. Williams D. A test for differences between treatment means when several dose levels are compared with a zero dose control. Biometrics. 1971; 27(1):103-117. http://dx.doi.org/10.2307/2528930

60. Williams D. The comparison of several dose levels with a zero dose control. Biometrics. 1972; 28(2):519-531. http://dx.doi.org/10.2307/2556164

61. Hsu JC. The factor analytic approach to simultaneous inference in the general linear model. J Comput Graph Stat. 1992; 1(2):151-168. 10.1080/10618600.1992.10477011

62. Shirley E. A non-parametric equivalent of Williams' test for contrasting increasing dose levels of a treatment. Biometrics. 1977; 33(2):386-389. http://dx.doi.org/10.2307/2529789

63. Williams DA. A note on Shirley's nonparametric test for comparing several dose levels with a zero-doe control. Biometrics. 1986; 42(1):183-186. http://dx.doi.org/10.2307/2531254

64. Dunn OJ. Multiple comparisons using rank sums. Technometrics. 1964; 6(3):241-252. http://dx.doi.org/10.1080/00401706.1964.10490181

65. Jonckheere A. A distribution-free k-sample test against ordered alternatives. Biometrika. 1954; 41:133-145. http://dx.doi.org/10.1093/biomet/41.1-2.133

66. Davison AC, Hinkley DV. Bootstrap Methods and Their Application. Cambridge, UK: Cambridge University Press; 1997.

67. Datta S, Satten GA. Rank-sum tests for clustered data. J Am Stat Assoc. 2005; 100(471):908-915.

68. Hommel G. A stagewise rejective multiple test procedure based on a modified Bonferroni test. Biometrika. 1988; 75(2):383-386.

69. Hothorn LA. Statistical evaluation of toxicological bioassays – a review. Toxicology Research. 2014; 3(6):418-432. 10.1039/c4tx00047a

70. Kalbfleisch JD, Lawless JF. The analysis of panel data under a Markov assumption. J Am Stat Assoc. 1985; 80(392):863-871. 10.1080/01621459.1985.10478195

71. Code of Federal Regulations (CFR). 21(Part 58).

72. National Toxicology Program (NTP). DART-06: Growth and clinical finding tables (I), pathology tables (PA), developmental and reproductive tables (R) from NTP modified one generation dose range finding study and modified one generation main study studies. Research Triangle Park, NC: U.S. Department of Health and Human Services, National Institutes of Health, National Toxicology Program; 2019. https://doi.org/10.22427/NTP-DATA-DART-06

73. Engelbregt MJT, Houdijk MECAM, Popp-Snijders C, Delemarre-van de Waal HA. The effects of intra-uterine growth retardation and postnatal undernutrition on onset of puberty in male and female rats. Pediatr Res. 2000; 48(6):803-807. 10.1203/00006450-200012000-00017

74. Bronson FH. Puberty in female rats: Relative effect of exercise and food restriction. Am J Physiol. 1987; 252(1 Pt 2):R140-144. 10.1152/ajpregu.1987.252.1.R140

75. Chapin RE, Gulati DK, Barnes LH, Teague JL. The effects of feed restriction on reproductive function in Sprague-Dawley rats. Fundam Appl Toxicol. 1993; 20(1):23-29. https://doi.org/10.1006/faat.1993.1003

76. Ritter EJ, Scott Jr WJ, Randall JL, Ritter JM. Teratogenicity of di(2-ethylhexyl) phthalate, 2-ethylhexanol, 2-ethylhexanoic acid, and valproic acid, and potentiation by caffeine. Teratology. 1987; 35(1):41-46. 10.1002/tera.1420350107

77. Pennanen S, Tuovinen K, Huuskonen H, Komulainen H. The developmental toxicity of 2-ethylhexanoic acid in Wistar rats. Fundam Appl Toxicol. 1992; 19(4):505-511. 10.1016/0272-0590(92)90088-y

78. Tyl RW, Fisher LC, Kubena MF, Vrbanic MA, Gingell R, Guest D, Hodgson JR, Murphy SR, Tyler TR, Astill BD. The developmental toxicity of 2-ethylhexanol applied dermally to pregnant Fischer 344 rats. Fundam Appl Toxicol. 1992; 19(2):176-185. 10.1016/0272-0590(92)90149-c

79. LabDiet. Advanced Protocol® Verified Casein Diet 10 IF. 2017. https://www.labdiet.com/cs/groups/lolweb/@labdiet/documents/web_content/mdrf/mdi4/~edisp/ducm04_028427.pdf

Appendix A. Chemical Characterization and Dose Formulation Studies

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A.1. Procurement and Characterization

2-Ethylhexyl p-methoxycinnamate (EHMC) was obtained from Acros Organics (Fair Lawn, NJ) in a single lot (A0293319). Identity, purity, and stability analyses were conducted by the analytical chemistry lab at MRIGlobal (Kansas City, MO). Reports on analyses performed in support of the EHMC study are on file at the National Institute of Environmental Health Sciences.

EHMC is a clear, colorless liquid. The identity of lot A0293319 was evaluated using Fourier Transform infrared (FT-IR) spectroscopy, ^{1}H nuclear magnetic resonance (NMR) spectroscopy, ^{13}C NMR spectroscopy, and gas chromatography (GC) with mass spectrometry (MS) (Table A-1).

The FT-IR, ^{1}H NMR, and ^{13}C NMR spectra (Figure A-1,Figure A-2,Figure A-3) were consistent with the structure of EHMC and reference spectra for the *trans*-isomer in the National Institute of Advanced Industrial Science and Technology Spectral Database (No. 19199). The GC/MS spectra corresponded with the National Institute of Standards and Technology Mass Spectral Library reference for EHMC.

Elemental analysis was conducted at Prevalere Life Science, Inc. (Whitesboro, NY) and found to be consistent with the composition of EHMC. The relative amount of carbon (74.50%), hydrogen (9.16%), and nitrogen (0.06%) in lot A0293319 were within 2% of anticipated ratios. Karl Fisher titration indicated a water content of <0.1%. Triplicate analysis of the boiling point of lot A0293319 indicated a boiling point of 250.5°C–275.1°C at 30 inHg. The relative density of 1.012 at 21.5°C agreed with anticipated specific gravity of 1.007–1.012 at 25°C indicated by the supplier. The log P_{ow} value determined was 5.27.

The purity of lot A0293319 was determined using GC with flame ionization detection (FID) conducted with two different column types. The GC/FID analysis conducted with a DB-5 column (Table A-1, System B) indicated a purity of 99.17%. Similarly, the GC/FID analysis using a Rtx-200 column (Table A-1, System C) determined a purity of 98.99%. Both methods identified three impurities having an area ≥0.05%. The purity of lot A0293319 was determined to be >98%.

Accelerated stability studies were conducted on samples stored protected from light at ambient (approximately 22°C), refrigerated (approximately 5°C), elevated (approximately 60°C), and frozen (approximately −20°C) temperatures using GC/FID (Table A-1). Stability was confirmed for at least 2 weeks under these conditions. Upon receipt by the analytical laboratory, the 150 kg drum of lot A0293319 was homogenized by blending all portions of the drum with an air-driven stirrer. The chemical was then transferred to 1-gallon narrow-mouthed amber glass bottles sealed with Teflon-lined lids. Periodic reanalysis of the bulk chemical performed during and after the studies showed no degradation.

A.2. Preparation and Analysis of Dose Formulations

Dose formulations of EHMC in LabDiet 5K96 Verified Casein Diet 10 IF feed were prepared following the protocols outlined in Table A-2. Dose formulations of 1,000, 3,000, and 6,000 ppm were used for the modified one-generation study. Formulations were stored at approximately 5°C and were considered stable for 35 days.

Dose formulations and homogeneity were evaluated using GC/FID (Table A-1, System D). The method of preparation was validated for concentration ranges of 400–25,000 ppm, as well as high-dose formulations of 40,000 and 80,000 ppm used as stock feed. Homogeneity was confirmed in 22 kg preparations of dose formulations at 1,000, 2,250, and 20,000 ppm.

Prior to study start, the stability and homogeneity of the dose formulations were determined using GC/FID. Stability of the 1,000 ppm formulation was confirmed for 35 days at refrigerated temperatures (5°C). A 7-day simulated dose study of the 1,000 ppm formulations was conducted to determine stability in animal room conditions. Isolated formulations and formulations mixed with 5% w/w rodent urine and feces reflective of anticipated conditions were stable for <4 days at a concentration of 1,000 ppm.

Analyses of preadministration and postadministration dose formulations were conducted throughout the study by the study laboratory, RTI International (Research Triangle Park, NC). Postadministration samples were collected from the animal room at the end of the first exposure period. All samples were within 10% of the target concentration (Table A-3). One batch of the 6,000 ppm dose formulation prepared on December 3, 2012 was 9.2% below the target concentration and was subsequently replaced by a freshly prepared batch (9.0% below target).

Table A-1. Chromatography Systems Used in the Modified One-Generation Study of 2-Ethylhexyl p-Methoxycinnamate

Chromatography	Detection System	Column	Mobile Phase
System A			
Gas chromatography	Mass spectrometer	HP-5MS (30 m × 0.25 mm ID, 0.25 µm film thickness)	Helium, 1.5 mL/min flow rate
System B			
Gas chromatography	Flame ionization detector	J&W Scientific DB-5 (30 m × 0.53 mm ID, 1.5 µm film thickness)	Helium, 10 mL/min flow rate
System C			
Gas chromatography	Flame ionization detector	Restek, Rtx-200 (30 m × 0.25 mm ID, 0.25 µm film thickness)	Helium, 2.5 mL/min flow rate
System D			
Gas chromatography	Flame ionization detector	Agilent DB-5 (30 m × 0.53 mm ID, 1.5 µm film thickness)	Helium, 10 mL/min flow rate

ID = internal diameter.

Table A-2. Preparation and Storage of Dose Formulations in the Modified One-Generation Study of 2-Ethylhexyl p-Methoxycinnamate

Preparation
A premix of 2-ethylhexyl p-methoxycinnamate (EHMC) (Lot A0293319) and LabDiet 5K96 Verified Casein Diet 10 IF feed was diluted with additional feed to reach the target concentration. To make the premix, an appropriate amount of LabDiet 5K96 Verified Casein Diet 10 IF feed was weighed into a plastic bag. A small portion was transferred from the bag into a stainless-steel container and a well was shaped in the middle of the feed (feed well). An appropriate amount of EHMC was weighed into a stainless-steel beaker and poured into the feed well. The contents were mixed thoroughly with a spatula. The remaining feed was used to wash residual EHMC from the weighing container and sides of the stainless-steel mixing container. The contents were mixed thoroughly using the spatula between additions until all feed was incorporated into the premix. To prepare the formulations from the premix, feed was weighed into a plastic bag. Feed was transferred to an 8-quart twin shell blender and evenly distributed into each. An appropriate amount of premix was added to the blender and also evenly distributed between ports. The remaining feed was used to rinse the premix container into the blender. The blender ports were sealed, and the formulation was blended for approximately 15 minutes using an intensifier bar for the first 5 minutes.
Chemical Lot Number
A0293319 (Acros Organics)
Maximum Storage Time
35 days
Storage Conditions
Polyethylene bags stored at 5°C (refrigerated)
Study Laboratory
RTI International (Research Triangle Park, NC)

Table A-3. Results of Analyses of Dose Formulations Administered to Rats in the Dose Range-finding Study of 2-Ethylhexyl p-Methoxycinnamate

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)[a]	Difference from Target (%)
February 1, 2012	February 6–8, 2012	0	BLOQ	NA
		2,250	2,170	−3.6
		5,000	4,910	−1.8
		10,000	9,900	−1.0
		20,000	20,500	2.5
March 15, 2012	March 12–20, 2012	0	BLOQ	NA
		2,250	2,190	−2.7
		5,000	4,880	−2.4
		10,000	9,690	−3.1
		20,000	19,200	−4.0
Animal Room Samples				
February 1, 2012	March 12, 2012	0	BLOQ	NA

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)[a]	Difference from Target (%)
		2,250	2,020	−10.2
		5,000	4,540	−9.2
		10,000	9,080	−9.2
		20,000	18,000	−10.0
March 15, 2012	April 6, 2012	0	BLOQ	NA
		2,250	1,980	−12.0
		5,000	4,420	−11.6
		10,000	9,300	−7.0

BLOQ = below the limit of quantification; NA = not applicable.
[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-Ethylhexyl p-Methoxycinnamate

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)[a]	Difference from Target (%)
September 17, 2012	September 7–20, 2012	0	BLOQ	NA
		1,000	953	−4.7
		3,000	2,840	−5.3
		6,000	5,710	−4.8
December 3, 2012	December 5–7, 2012	0	BLOQ	NA
		1,000	981	−1.9
		1,000	979	−2.1
		3,000	2,870	−4.3
		3,000	2,900	−3.3
		6,000	5,820	−3.0
		6,000	5,450	−9.2[b]
December 11, 2012	December 11, 2012	6,000	5,460	−9.0
January 14, 2013	January 16–17, 2013	0	BLOQ	NA
		1,000	964	−3.6
		3,000	3,010	+0.3
		6,000	6,150	+2.5
February 18, 2013	February 21–22, 2013	0	BLOQ	NA
		1,000	957	−4.3
		3,000	2,810	−6.3
		6,000	5,700	−5.0

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)[a]	Difference from Target (%)
Animal Room Samples				
September 17, 2012	October 23, 2012	0	BLOQ	NA
		1,000	907	−9.3
		3,000	2,740	−8.7
		6,000	5,440	−9.3

BLOQ = below the limit of quantification; NA = not applicable.
[a]Average of triplicate analysis.
[b]The formulation was not used in the study and was replaced by the formulation prepared on December 11, 2012.

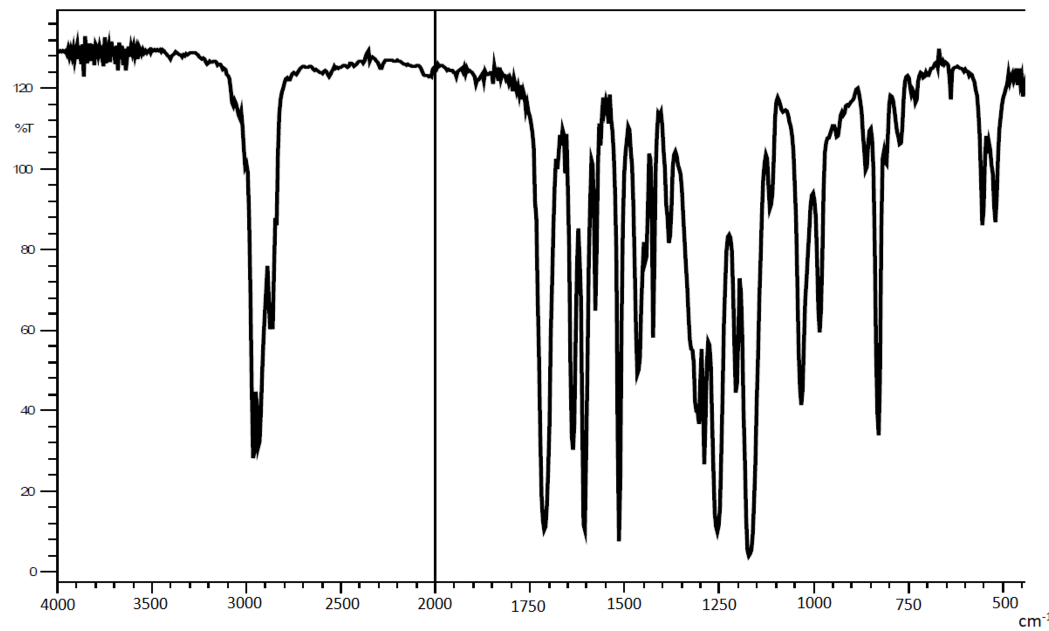

Figure A-1. Fourier Transform Infrared Absorption Spectrum of 2-Ethylhexyl p-Methoxycinnamate (Lot A0293319)

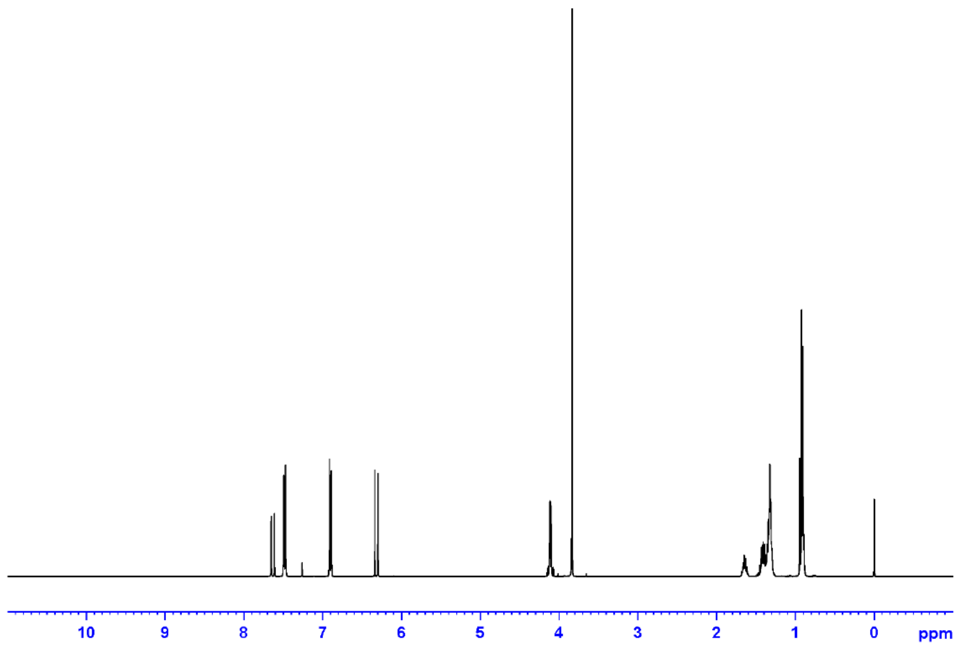

Figure A-2. Fourier Transform ^{1}H Nuclear Magnetic Resonance Spectrum of Reference Sample of 2-Ethylhexyl p-Methoxycinnamate (Lot A0293319)

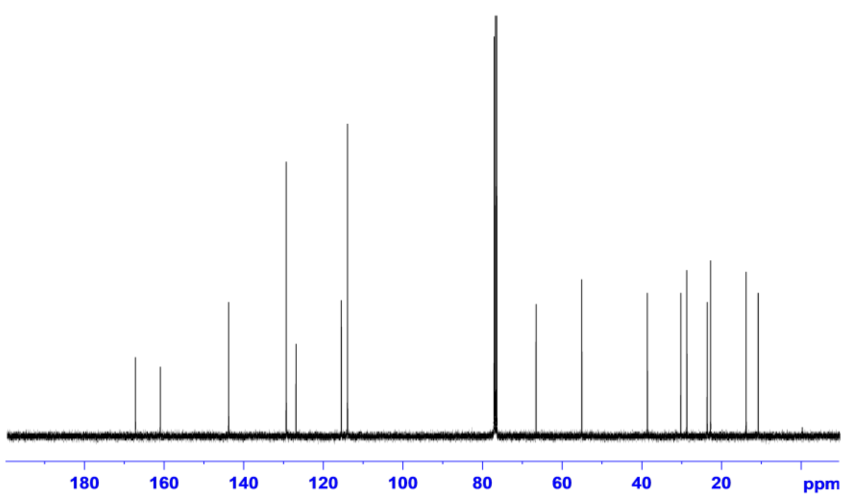

Figure A-3. Fourier Transform ^{13}C Nuclear Magnetic Resonance Spectrum of 2-Ethylhexyl p-Methoxycinnamate (Lot A0293319)

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

Tables

Additional information on ingredients, vitamins, and minerals in the 5K96 rat diet can be found online.[79]

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

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by Weight)	21.3 ± 0.6797	20.6–22.3	5
Crude Fat (% by Weight)	4.38 ± 0.0837	4.3–4.5	5
Crude Fiber (% by Weight)	3.174 ± 0.1932	2.9–3.43	5
Ash (% by Weight)	6.11 ± 0.2519	5.71–6.41	5
Vitamins			
Vitamin A (IU/kg)	18,920 ± 2,509	14,600–20,800	5
Thiamine (ppm)[a]	17.24 ± 1.718	15–19	5
Minerals			
Calcium (%)	1.228 ± 0.0497	1.16–1.29	5
Phosphorus (%)	0.930 ± 0.0227	0.901–0.955	5

[a]As hydrochloride.

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

Contaminant	Mean ± Standard Deviation	Range	Number of Samples
Arsenic (ppm)	0.3484 ± 0.0327	0.316–0.391	5
Cadmium (ppm)	0.0384 ± 0.0052	0.0328–0.0435	5
Lead (ppm)	0.2264 ± 0.0152	0.215–0.251	5
Mercury (ppm)	0.0104 ± 0.0006	0.01–0.0113	5
Selenium (ppm)	0.355 ± 0.0226	0.338–0.392	5
Aflatoxins (ppb)[a]	<2.0	–	5
Nitrate Nitrogen (ppm)[b]	13.14 ± 2.7428	10.4–17.1	5
Nitrite Nitrogen (ppm)[a,b]	<1.0	–	5
BHA (ppm)[a,c]	<1.0	–	5
BHT (ppm)[a,c]	<1.0	–	5
Aerobic Plate Count (CFU/g)[d]	<10	–	5
Coliform (MPN/g)	<3.0	–	5
Escherichia coli (MPN/g)[a]	<10.0	–	5
Salmonella (MPN/g)	<3.0	–	5
Total Nitrosamines (ppb)[e]	5.6 ± 2.5	2.0–8.4	5
N-N-dimethylamine (ppb)[e]	4.1 ± 1.8	2.0–6.3	5
N-N-pyrrolidine (ppb)[e]	1.5 ± 0.9	0.0–2.4	5
Pesticides (ppm)			
α-BHC[a]	–	–	5

Contaminant	Mean ± Standard Deviation	Range	Number of Samples
β-BHC[a]	–	–	5
γ-BHC[a]	–	–	5
δ-BHC[a]	–	–	5
Heptachlor[a]	–	–	5
Aldrin[a]	–	–	5
Heptachlor Epoxide[a]	–	–	5
DDE[a]	–	–	5
DDD[a]	–	–	5
DDT[a]	–	–	5
HCB[a]	–	–	5
Mirex[a]	–	–	5
Methoxychlor[a]	–	–	5
Dieldrin[a]	–	–	5
Endrin[a]	–	–	5
Telodrin[a]	–	–	5
Chlordane[a]	–	–	5
Toxaphene[a]	–	–	5
Estimated PCBs[a]	–	–	5
Ronnel[a]	–	–	5
Ethion[a]	–	–	5
Trithion[a]	–	–	5
Diazinon[a]	–	–	5
Methyl Chlorpyrifos	0.056 ± 0.0601	0–0.136	5
Methyl Parathion[a]	–	–	5
Ethyl Parathion[a]	–	–	5
Malathion	0.016 ± 0.0089	0–0.02	5
Endosulfan I[a]	–	–	5
Endosulfan II[a]	–	–	5
Endosulfane Sulfate[a]	–	–	5

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

C.1. Methods

Rodents used in the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that could 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 are from the same production source and weanling groups as the animals used for the studies of test compounds.

For these dose range-finding and modified one-generation studies, blood samples were collected from each sentinel animal and allowed to clot, and the serum was separated. Additionally, fecal samples were collected and tested for *Helicobacter* species. All samples were processed appropriately with serology and *Helicobacter* testing was performed by IDEXX BioResearch (formerly Rodent Animal Diagnostic Laboratory [RADIL], University of Missouri), Columbia, MO, for determination of the presence of pathogens. Evaluation for endo- and ectoparasites was performed in-house by the testing laboratory.

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).

C.2. Results

All test results were negative.

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

Collection Time Points	Dose Range-finding Study		Modified One-Generation Study					
	Quarantine	**Study Termination**	**Quarantine**	**1 Month After Arrival**	**16 Weeks After Arrival**	**12 Weeks After Birth[a]**	**22 Weeks After Birth[a]**	**Study Termination**
Number Examined (Males/Females)[b]	0/5	0/5	0/5	0/5	0/5	5/0	5/0	0/5
Method/Test								
Multiplex Fluorescent Immunoassay (MFI)								
Kilham rat virus (KRV)	–	–	–	–	–	–	–	–
Mycoplasma pulmonis	–	–	–	–	–	–	–	–
Parvo NS-1	–	–	–	–	–	–	–	–
Pneumonia virus of mice (PVM)	–	–	–	–	–	–	–	–
Rat coronavirus/sialodacryoadenitis virus (RCV/SDA)	–	–	–	–	–	–	–	–
Rat minute virus (RMV)	–	–	–	–	–	–	–	–
Rat parvo virus (RPV)	–	–	–	–	–	–	–	–
Rat theilovirus (RTV)	–	–	–	–	–	–	–	–
Sendai	–	–	–	–	–	–	–	–
Theiler's murine encephalomyelitis virus (TMEV)	–	–	–	–	–	–	–	–
Toolan's H-1	–	–	–	–	–	–	–	–
Immunofluorescence Assay (IFA)								
Mycoplasma pulmonis	NT	NT	NT	–	NT	NT	NT	NT
Pneumocystis carinii	–	NT	–	NT	NT	NT	NT	NT
Pneumonia virus of mice (PVM)	NT	NT	NT	NT	NT	NT	NT	–
Polymerase Chain Reaction (PCR)								
Helicobacter species	NT	NT	NT	NT	–	–	–	–

– = negative; + = positive; NT = not tested.
[a]Male rats born at RTI.
[b]Age-matched nonpregnant females.

Appendix D. Peer-review Report

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

Appendix E. Supplemental Data

The following supplemental files are available at: https://doi.org/10.22427/NTP-DATA-DART-06.

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 Weight Summary

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 – Summary of Litter Data

R19 – Pup Mean Body Weight Summary

R19C – Pup Growth Curves

R19G – Pup Mean Body Weight Gain

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 Weight Summary

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 with Litter Incidence

PA11 – Statistical Analysis of Survival Data

PA14 – Individual Animal Pathology Data

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

PA40 – Survival Curve

PA41 – Clinical Chemistry Summary

PA43 – Hematology Summary

PA46R – Summary of Gross Pathology with Litter Incidence

R01 – Multigeneration Cross Reference

R02 – Reproductive Performance Summary

R03 – Summary of Litter Data

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 Mean Body Weight Summary

R19C – Pup Growth Curve

R19G – Pup Mean Body Weight Gain

R20 – Pup Necropsy Summary

Vaginal Cytology Markov Model

E.2.2. Individual Animal Data

F1 Fertility Cohort Vaginal Cytology Plots

F1 Prechronic Cohort Vaginal Cytology Plots

F1 Prenatal Cohort Vaginal Cytology Plots

Individual Animal Andrology Data

Individual Animal Body Weight Data

Individual Animal Clinical Chemistry Data

Individual Animal Clinical Observations Data

Individual Animal Consumption Data

Individual Animal Developmental Markers Data

Individual Animal Gross Pathology Data

Individual Animal Hematology 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