Effect of Oxybenzone on Fertility and Early Embryonic Development in Sprague-Dawley Rats (Segment I)

NCTR Experiment E0218601

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[a]Note: Between the end of the in-life portion of this study and completion of the final report there were changes in the organizational structure at NCTR so that affiliations of some of the listed personnel changed. Namely, the computer support changed from ICF International to the Office of Scientific Coordination, NCTR. Patrick Clayton (Animal Care) left Priority One Services prior to the end of the in-life portion of the study and was replaced by Vicki Thompson (Animal Care) who subsequently left Priority One Services prior to completion of the final study report. Mark Moore (Animal Care, Priority One Services) and Dr. Neera Gopee (Animal Care, OSC, NCTR) also left prior to completion of the final report. Dr. Jeff Carraway (Animal Care, OSC, NCTR) and Dr. John R. Latendresse (Pathology, TPA, Inc.) retired prior to completion of the final study report.

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EXECUTIVE SUMMARY

Background

Oxybenzone (OXY; CAS No. 131-57-7) is an organic ultra-violet absorbing compound used in a variety of personal care products, including sunscreens as well as plastic products, because of its ability to block UVA and UVB radiation and prevent UV-induced photodecomposition. There has been recent interest in the biological activity of oxybenzone due to its high volume of use, its detection in the urine of a large percentage of the U.S. population and its reported estrogenic activity. This study is designed to address data gaps identified by the Center for Drug Evaluation and Research (CDER) on the potential reproductive toxicity of oxybenzone.

Methods

The study examined the effects of oxybenzone on fertility and early embryonic development. Male and female Sprague-Dawley rats (Harlan) were given dosed feed that contained 0, 3,000, 10,000 or 30,000 parts per million (ppm) of oxybenzone or 0.05 ppm of the reference estrogen ethinyl estradiol (EE2). Males were dosed at least 10 weeks and females for at least two weeks prior to mating in order to assess effects on fertility; vaginal cytology was monitored to assess effects on estrus cyclicity. For mating, one male and one female were placed together for up to 15 days or until mating occurred. A sperm positive vaginal lavage or a vaginal plug preventing lavage was used as an indication of mating and was designated as gestational day 0 (GD 0). Dosing was continuous throughout the mating period and continued until the time of implantation (GD 6 of the female's pregnancy) when the animals were returned to control chow. Females were sacrificed on GD 15 and males sacrificed after GD 6. Evaluations of the females included assessment of the effects of oxybenzone on the estrus cycle, body weights, feed consumption, organ weights and on various reproductive and pregnancy parameters. For males, investigations included body weight, feed consumption and organ weight analyses in addition to evaluation of sperm parameters and hormone level determinations. Hematology and clinical chemistry analyses were also evaluated in both males and females from each dose group.

Results

Oxybenzone treatment did not impact fertility or early embryonic development in male and female Sprague-Dawley rats. There were no significant differences related to oxybenzone exposure on the percentage of animals that mated, the time to mating, the percentage of pregnant females or on the number of implantations and resorptions. Estrus cycles in treated females were not affected nor were sperm parameters or hormone levels in males. Treatment with the reference estrogen EE2 did, however, lead to an increase in the number of females with extended diestrus cycles and overall lower gravid uterine weights. No other fertility or early embryonic developmental endpoints were affected by treatment with EE2.

Female and male rats dosed with oxybenzone had lower overall body weights. Statistically significant reductions were observed on pre-mating treatment days 5 and 9 for females in the OXY 10,000 and OXY 30,000 ppm treatment groups. Significant reductions were also observed on pre-mating day 12 and throughout gestation for females treated with 30,000 ppm of oxybenzone. Similar observations were also noted for females dosed with 0.05 ppm EE2. For males, reduced body weights were observed after the first pre-mating week of treatment with 30,000 ppm of oxybenzone. Weights of males in the high dose group continued to be significantly lower during the 10-week treatment period prior to mating and post-mating. As with females, males treated with 0.05 ppm EE2 had a similar reduction in body weight to males dosed with 30,000 ppm of oxybenzone.

Various organ weights, organ histopathology, hematology and clinical chemistry parameters were also significantly affected in the treated adult males and females. Absolute liver and kidney weights in males at the oxybenzone 10,000 and 30,000 ppm doses were significantly higher than controls; a significant increase in trend for absolute liver weights was noted for females. Significant trends were also noted for absolute testis weights (increase) and seminal vesicle and coagulating gland weights (decrease).

Gross observation of the kidneys indicated an increase in lesions in the males treated with 30,000 ppm of oxybenzone; changes were consistent with previously reported observations of exposure. Histopathological analysis indicated a statistically significant increase in the severity of mammary

gland alveolar hyperplasia in the OXY 3,000 ppm treatment group males, no significant effects were observed at the higher doses. Similar observations in mammary gland alveolar hyperplasia were noted for males in the EE2 0.05 ppm treatment group where an increase in incidence was also found. A significant increase in the severity of thyroid gland ultimobranchial cysts were observed for both the OXY 30,000 and EE2 0.05 ppm treatment group males.

Few changes were noted in the hematological parameters of treated females. However, in males significant changes were primarily observed in the OXY 30,000 ppm group and included lower hemoglobin (HGB), hematocrit (HCT), and packed cell volume (PCV) levels compared to controls. In clinical chemistry parameters, sorbitol dehydrogenase (SDH) levels were significantly lower in the OXY 3,000 and OXY 30,000 ppm treated females while creatine kinase (CK) levels were lower and glucose (GLU) levels were higher in the OXY 30,000 ppm group. For males, aspartate aminotransferase (AST) levels were significantly lower in the OXY 10,000 and 30,000 ppm treated groups. The only clinical chemistry parameter affected that was shared between male and females was that of AST, which was lower relative to controls.

Conclusions

Oxybenzone exposure for at least two weeks prior to mating for females and at least 10 weeks prior to mating for males, with continuous dosing through implantation, had no effects on fertility or early embryonic development at dose levels that induced minimal toxicity. Exposure to oxybenzone did lead to minor decreases in female (OXY 10,000 and 30,000) and male (OXY 30,000) body weights and affected various organ weights, organ histopathology, hematology and clinical chemistry parameters, primarily in the highest dose group. However, estrus cycles, sperm parameters, and various reproductive and pregnancy parameters were not influenced by oxybenzone exposure.

ABSTRACT
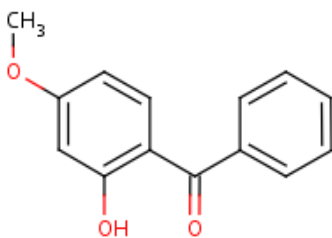
OXYBENZONE

CAS No. 131-57-7

Chemical Formula: $C_{14}H_{12}O_3$ Molecular Weight: 228.24

Synonyms: 2-hydroxy-4-methoxybenzophenone, HMB, benzophenone-3, (2-hydroxy-4-methoxyphenyl)-phenylmethanone

Oxybenzone (OXY) is a ultra-violet absorbing compound (UV-filter). Due to its ability to absorb both UVA and UVB, it is synthesized for incorporation into a wide variety of commercial personal care products including sunscreens, creams and lotions. It is also commonly found in nail polishes, perfumes, lipsticks, hair sprays and conditioners as a photostabilizer and/or a fragrance enhancer (Environmental Working Group (EWG); http://www.ewg.org/skindeep). Oxybenzone is an active sunscreen ingredient in the U.S. Food and Drug Administration's (FDA) sunscreen monograph 21 CFR 352.10 (Code of Federal Regulations (CFR), 2013b). In addition to personal care products, oxybenzone is also commonly found in plastics due to its ability to act as an ultraviolet light absorber and stabilizer (Hazardous Substance Data Bank (HSDB); http://toxnet.nlm.gov/cgi-bin/sis/htmlgen?HSDB). It has also been approved by the FDA as an indirect food additive used in food contact substances (21 CFR 177.1010) (CFR, 2013a).

Concerns have been raised regarding the biological activity of oxybenzone. A number of studies have examined the endocrine disrupting potential of the compound. These studies have indicated that oxybenzone or its primary metabolite, 2,4-dihydroxybenzophenone (DHB; 2,4OH-BP), may have estrogenic activity. Also of primary concern is the high volume of oxybenzone use and its detection in the urine of a large percentage of the U.S. population (Calafat et al., 2008). However,

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the effects of oxybenzone on fertility and early embryonic development have not been thoroughly assessed. Therefore, this study was undertaken to examine its effects on fertility and early embryonic development to implantation according to the International Conference on Harmonisation (ICH) Guideline S5(R2) 4.1.1 (ICH, 1993). An additional objective was to compare the results obtained from typical ICH fertility and early embryonic development studies to results obtained from a modified one-generation study conducted in parallel and concurrently with the National Toxicology Program (NTP).

For this study, 125 male and 125 female Sprague-Dawley rats were obtained from Harlan Industries (Indianapolis, IN). Males were approximately 5-7 weeks old at arrival and females were approximately 9-11 weeks old. After completion of quarantine, 25 animals of each sex were assigned to control (CTRL), oxybenzone (OXY 3,000, OXY 10,000, OXY 30,000) or a reference estrogen, ethinyl estradiol (EE2 0.05), treatment group by a weight-ranked randomization procedure. Data from a preliminary reproductive and developmental dose-finding feed study (Nakamura et al., 2015) in conjunction with the results from a NTP dose-finding feed study were used to select the dietary oxybenzone concentrations in the current study. To maintain consistently low background exposure to phytoestrogens, all animals were fed a soy- and alfalfa-free Purina 5K96 diet.

Males were dosed for at least 10 weeks and females for at least two weeks prior to mating. Vaginal cytology was monitored prior to the females being placed on dosed chow and throughout the pre-mating dosing period to determine whether oxybenzone affected the estrus cycle. For mating, one male and one female were housed together for up to 15 days or until mating occurred. A sperm positive lavage or a vaginal plug preventing lavage was used as an indication of mating and was designated as GD 0. Rats received dosed chow throughout the cohabitation period until GD 6; at GD 6 males and females were placed back on control chow until sacrifice. Males were sacrificed after GD 6; females were sacrificed on GD 15 and evaluated for possible effects on pregnancy and early embryonic development.

Female and male rats given oxybenzone or the reference estrogen, EE2, had lower overall body weights compared to unexposed animals. Significant reductions were observed on pre-mating

treatment day 5 for females in the OXY 10,000 and OXY 30,000 ppm treatment groups when compared to controls. Significant reductions were also observed in the same two treatment groups on pre-mating day 9 relative to controls; significant reductions on pre-mating treatment day 12 were limited to the OXY 30,000 ppm group. After mating, gestational body weights of females in the OXY 30,000 ppm treatment group were significantly reduced and remained at levels lower than controls until sacrifice on GD 15. A similar trend in reduced body weights was also observed for females given 0.05 ppm EE2. The reductions in female body weight, however, did not appear to be the result of decreased food consumption as those in the OXY 30,000 ppm group appeared to consume more chow on treatment days 6-9 prior to mating and post-mating from GDs 0-6. An increase in food consumption in the EE2 0.05 ppm treatment group was also observed post-mating. For males, reduced body weights were observed after the first week of treatment with 30,000 ppm of oxybenzone or 0.05 ppm EE2 and continued throughout the 10-week treatment period prior to mating. Reductions were also observed in the same two treatment groups at GD 0 and GD 6 of the female's pregnancy. Similar to what was observed with females, the reduction in body weight did not appear to be related to a decrease in food consumption. No decrease in food consumption was observed throughout the pre-mating treatment period or post-mating.

Although reductions were observed in body weights of both males and females, oxybenzone treatment did not impact endpoints used to measure fertility or early embryonic development parameters. There were no significant differences related to oxybenzone treatment on the percentage of animals that mated, the time to mating, the percentage of pregnant females or the number of implantations and resorptions among treated dams. Estrus cycles in treated females were not affected nor were sperm parameters or hormone levels in treated males.

While oxybenzone treatment did not influence fertility or early embryonic development, it significantly impacted various organ weights, organ histopathology, hematology and clinical chemistry parameters among the treated adult animals (both males and females). Absolute liver and kidney weights among males treated with 10,000 and 30,000 ppm of oxybenzone were significantly higher than controls; while no significant differences were observed in pairwise comparisons to controls for absolute liver weights of treated females, a significant increasing trend

for liver weights was noted. Significant trends were also noted for absolute testis weights (increase) and seminal vesicle and coagulating gland weights (decrease).

Gross observation of the kidneys indicated an increase in lesions in the males treated with 30,000 ppm of oxybenzone. Changes in the kidney have previously been documented in response to oxybenzone exposure and are characterized by protein casts, dilated renal tubules, regeneration of renal tubules and inflammation (French, 1992); similar changes were noted in this study. However, due to the low numbers of control animals assessed, a statistical analysis was not performed on the non-neoplastic findings reported for the kidneys. Histopathological analysis also indicated a significant increase in the severity of mammary gland alveolar hyperplasia in the OXY 3,000 ppm treatment group males compared to controls, no significant effects were observed at the higher doses. A significant increase in severity and incidence of mammary gland alveolar hyperplasia was also noted for males in the positive control EE2 0.05 ppm treatment group. Additionally, significant increases in the severity of thyroid gland ultimobranchial cysts were noted for the OXY 30,000 and EE2 0.05 ppm treatment group males.

Few changes were noted in the hematological parameters of treated females; the only significant difference from controls was in the lower percentage of neutrophils (NEU) in the OXY 30,000 ppm treatment group. For males, significant differences were primarily confined to the high dose group of oxybenzone and included lower HGB concentration, the percentage of total blood volume that is composed of red blood cells (HCT) and PCV. The clinical chemistry parameters significantly affected by oxybenzone treatment also varied between males and females. For females, SDH levels were significantly lower in the OXY 3,000 and OXY 30,000 ppm treatment groups relative to controls. CK levels were also lower while GLU levels were higher between control and OXY 30,000 ppm treated females. For males, AST levels were significantly lower in pairwise comparisons of controls to OXY 10,000 and 30,000 ppm treated males. The only clinical chemistry parameter affected that was shared between male and females was that of AST which was lower than levels in control animals.

Summary

Oxybenzone exposure for at least two weeks prior to mating for females and at least 10 weeks prior to mating for males, with continuous exposure through implantation (GD 6), had no effects on fertility or early embryonic development in Sprague-Dawley rats at levels that produced some toxicity among treated males and females. Findings included decreases in female (OXY 10,000 and OXY 30,000) and male (OXY 30,000) body weights and alternations in various absolute organ weights, organ histopathology, hematology and clinical chemistry parameters, primarily in the highest dose group of both sexes. There were no significant differences related to oxybenzone exposure on the percentage of animals that mated, the time to mating, the percentage of pregnant females or the number of implantations and resorptions. Oxybenzone exposure did not affect estrus cyclicity among treated females or sperm parameters and hormone levels among treated males.

Table S1: Summary of the Fertility/Early Embryonic Development Feed Study of Oxybenzone[a,b]

Endpoint	
Pre-Mating Body Weights - Females	
Treatment Day 1	-
Treatment Day 5	↓ (OXY 10,000; OXY 30,000; EE2 0.05; Trend)
Treatment Day 9	↓ (OXY 10,000; OXY 30,000; EE2 0.05; Trend)
Treatment Day 12	↓ (OXY 30,000; EE2 0.05; Trend)
Overall (Treatment Days 1-12)	↓ (OXY 10,000; OXY 30,000; EE2 0.05; Trend)
Pre-Mating Food Consumption - Females	
Treatment Days 1-5	-
Treatment Days 6-9	↑ (OXY 30,000; Trend)
Treatment Days 9-12	↑ (Trend)
Overall (Treatment Days 1-12)	↑ (Trend)
Pre-Mating Body Weights - Males	
Treatment Week 1	↓ (OXY 30,000; EE2 0.05;Trend)
Treatment Week 2	↓ (OXY 30,000; EE2 0.05;Trend)
Treatment Week 3	↓ (OXY 30,000; EE2 0.05;Trend)
Treatment Week 4	↓ (OXY 30,000; EE2 0.05;Trend)
Treatment Week 5	↓ (OXY 30,000; EE2 0.05;Trend)
Treatment Week 6	↓ (OXY 30,000; EE2 0.05;Trend)
Treatment Week 7	↓ (OXY 30,000; EE2 0.05;Trend)
Treatment Week 8	↓ (OXY 30,000; EE2 0.05;Trend)
Treatment Week 9	↓ (OXY 30,000; EE2 0.05;Trend)
Treatment Week 10	↓ (OXY 30,000; EE2 0.05;Trend)
Overall (Treatment Weeks 1-10)	↓ (OXY 30,000; EE2 0.05;Trend)
Pre-Mating Food Consumption - Males	
Treatment Week 1	-
Treatment Week 2	↑ (Trend)
Treatment Week 3	↑ (OXY 30,000; Trend)
Treatment Week 4	-
Treatment Week 5	-
Treatment Week 6	-
Treatment Week 7	-
Treatment Week 8	-
Treatment Week 9	-
Treatment Week 10	-
Overall (Treatment Weeks 1-10)	-
Gestational Body Weights - Females	
GD 0	↓ (OXY 30,000; EE2 0.05; Trend)
GD 6	↓ (OXY 30,000; EE2 0.05; Trend)
GD 10	↓ (OXY 30,000; EE2 0.05; Trend)
GD 15	↓ (OXY 10,000; OXY 30,000; EE2 0.05; Trend)
Overall (GD 0-GD 15)	↓ (OXY 10,000; OXY 30,000; EE2 0.05; Trend)
Gestational Food Consumption - Females	
GD Interval 1-6 (dosed chow)	↑ (OXY 30,000; EE2 0.05;Trend)
GD Interval 7-15 (control chow)	-
Overall (GD Interval 1-15)	↑ (OXY 30,000; EE2 0.05;Trend)

Table S1: Summary of the Fertility/Early Embryonic Development Feed Study of Oxybenzone Cont.[a,b]

Endpoint	
Post-Mating Body Weights - Males	
GD 0	↓ (OXY 30,000; EE2 0.05; Trend)
GD 6	↓ (OXY 30,000; EE2 0.05; Trend)
Overall (GD 0-GD 6)	↓ (OXY 30,000; EE2 0.05; Trend)
Post-Mating Food Consumption - Males	
GD Interval 1-6 (dosed chow)	-
Organ Weights - Females	
Adrenal Gland (paired)	↑ (OXY 3,000)
Liver	↑ (Trend)
Kidney (paired)	-
Ovary (paired)	↑ (OXY 10,000); ↓ (EE2 0.05)
Thyroid Gland	-
Organ Weights - Males	
Adrenal Glands (paired)	-
Epididymis (paired)	-
Liver	↑ (OXY 10,000; OXY 30,000; EE2 0.05; Trend)
Kidney (paired)	↑ (OXY 10,000; OXY 30,000; Trend)
LABC	-
Preputial Glands (paired)	-
Dorsolateral Prostate	-
Ventral Prostate	↓ (Trend)
Seminal Vesicles/Coagulating Glands	↑ (OXY 10,000)
Testis (paired)	↑ (Trend)
Thyroid Gland	-
Cowper's Glands (paired)	-
Vaginal Cytology	
Proportion in Proestrus	-
Proportion in Estrus	-
Proportion in Diestrus	-
Abnormal Cycles (%) - Proestrus	-
Abnormal Cycles (%) - Estrus	-
Abnormal Cycles (%) - Diestrus	↑ (EE2 0.05)
Abnormal Cycles (%) - Total	-
Estrus Stage Transitions	-
Estrus Cycle Length	-
Reproductive/Pregnancy Parameters	
Percent Mated	-
Time to Mating	-
Percent Pregnant	↑ (OXY 10,000)
Gravid Uterine Weights	↓ (EE2 0.05)
Number of Implants	-
Number of Resorptions	-

Table S1: Summary of the Fertility/Early Embryonic Development Feed Study of Oxybenzone Cont.[a,b]

Endpoint	
Sperm Parameters	
Morphology	-
Motility	-
Testicular Spermatid Head Counts	-
Cauda Epididymal Sperm Counts	-
Hematology Parameters - Females	
WBC	-
NEU (%)	↓ (OXY 30,000; Trend)
LYM (%)	-
MON (%)	-
EOS (%)	-
BAS (%)	-
NEU	-
LYM	-
MON	-
EOS	-
BAS	-
RBC	↑ (EE2 0.05)
HGB	↑ (EE2 0.05)
HCT	-
MCV	-
MCH	-
MCHC	-
PLT	-
PCV	-
Retic	-
Hematology Parameters - Males	
WBC	-
NEU (%)	-
LYM (%)	-
MON (%)	-
EOS (%)	-
BAS (%)	-
NEU	-
LYM	-
MON	-
EOS	-
BAS	-
RBC	↓ (Trend)
HGB	↓ (OXY 30,000; Trend)
HCT	↓ (OXY 30,000; Trend)
MCV	↓ (OXY 3,000)
MCH	-
MCHC	-
PLT	-
PCV	↓ (OXY 30,000; Trend)

Table S1: Summary of the Fertility/Early Embryonic Development Feed Study of Oxybenzone Cont.[a,b]

Endpoint	
Hematology Parameters Cont. - Males	
Retic	-
Clinical Chemistry Parameters - Females	
SDH	↓ (OXY 3,000; OXY 30,000; Trend)
TBA	-
ALB	-
ALT	-
ALP	-
AST	↓ (OXY 30,000; Trend)
TRIG	-
CHOL	↑ (EE2 0.05)
TP	-
CK	↓ (OXY 30,000; Trend)
CREAT	↑ (Trend)
BUN	-
GLU	↑ (OXY 30,000; Trend)
Clinical Chemistry Parameters - Males	
SDH	-
TBA	↑ (EE2 0.05; Trend)
ALB	-
ALT	↑ (EE2 0.05)
ALP	-
AST	↓ (OXY 10,000; OXY 30,000; Trend)
TRIG	-
CHOL	↑ (Trend)
TP	↑ (Trend)
CK	-
CREAT	-
BUN	-
GLU	-
Testosterone	-
FSH	-
Histopathology - Females	
Mammary Gland	
Alveolus, Hyperplasia	-
Histopathology - Males	
Mammary Gland	
Alveolus, Hyperplasia	↑ (OXY 3,000; EE2 0.05)
Pituitary Gland	
Pars Distalis, Cyst	-
Thyroid Gland	
Ultimobranchial, Cyst	↑ (OXY 30,000; EE2 0.05)
Prostate, Ventral Lobe	
Infiltration Cellular, Lymphocyte	-

[a] GD = gestational day; LABC = levator ani bulbocavernosus muscle complex; WBC = white blood cell; NEU = neutrophils; LYM = lymphocytes; MON = monocytes; EOS = eosinophils; BAS = basophils; RBC = red blood cells; HGB = hemoglobin concentration; HCT = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; PLT = platelets; PCV = packed cell volume; Retic = reticulocytes; SDH = sorbitol dehydrogenase; TBA = total bile acids; ALB = albumin; ALT = alanine aminotransferase; ALP = alkaline phosphatase; AST = aspartate aminotransferase; TRIG = triglycerides; CHOL = cholesterol; TP = total protein; CK = creatine kinase; CREAT = creatinine; BUN = blood urea nitrogen; GLU = glucose; FSH = follicle stimulating hormone.

[b] ↑ or ↓, significant increase or decrease relative to controls at the exposure indicated in parentheses, or, where indicated, significant overall exposure concentration trend (analysis of trend excluded the EE2 0.05 ppm treatment group); "-", no exposed group significantly different from the control group in pairwise comparisons. Details of the specific statistical analysis used for each endpoint are described in the body of the report.

INTRODUCTION AND STUDY RATIONALE
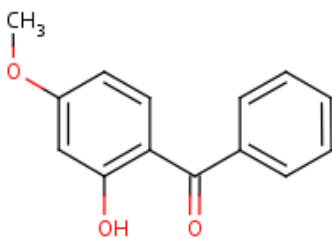
OXYBENZONE

CAS No. 131-57-7

Chemical Formula: $C_{14}H_{12}O_3$ Molecular Weight: 228.24

Synonyms: 2-hydroxy-4-methoxybenzophenone, HMB, benzophenone-3, (2-hydroxy-4-methoxyphenyl)-phenylmethanone

Physical Properties, Production, and Uses

Oxybenzone [OXY; IUPAC name: (2-hydroxy-4-methoxyphenyl)-phenylmethanone] is a monomethoxylated derivative of 2-hydroxybenzophenone (Cosmetic Ingredient Review, 1983). It occurs naturally in flower pigments (Stecher, 1958) and is a white to light yellow crystalline powder that is insoluble in water, has a melting point of $66°C$, low volatility and is stable at room temperature (O'Neil et al., 2012).

Oxybenzone is synthesized for commercial use by the Friedel-Crafts reaction of benzoyl chloride with 3-hydroxyanisole. The resulting product is then re-crystallized from water/methanol and dried (Cosmetic Ingredient Review, 1983). Oxybenzone is included on the U.S. Environmental Protection Agency's (EPA) High Production Volume (HPV) list (http://www.epa.gov/hpv/index.htm); the HPV list is based on the 1990 Inventory Update Rule (IUR) (40 CFR part 710 subpart B; 51FR21438). Chemicals listed as high production volume were either produced or imported into the U.S. in >1 million pounds in 1990 and/or 1994. As of April 4, 2011 thirteen companies were listed as manufacturers of oxybenzone according to the HSDB (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).

Commercially, oxybenzone is widely used in sunscreens and sunscreen containing products because of its ability to act as a UV filter, blocking both UVA and UVB rays (Klein, 1992). In addition to sunscreens, it is found in a wide range of personal care products including nail polishes, lipsticks, perfumes, hair sprays and shampoos where it is included as a photostabilizer and/or fragrance enhancer (EWG; http://www.ewg.org/skindeep). The incorporation of oxybenzone in personal care products has risen over the years. In 1976 oxybenzone was found in 47 products compared to 62 products in 1979 (Cosmetics Ingredient Review, 1983) and 451 products in 2002 (Cosmetics Ingredient Review, 2005). The largest increase in product incorporation during this time period was in lipsticks, moisturizers and suntan gels, creams and liquids (Cosmetics Ingredient Review, 2005). A more recent search using the EWG's Skin Deep Cosmetics Database found oxybenzone listed as a component in 1,607 products (as of May 28, 2015). Sunscreens and sunscreen containing items continue to be the most common oxybenzone containing personal care products followed by nail polish, lipsticks/lip balms and fragrances (EWG; http://www.ewg.org/skindeep).

Concentrations of oxybenzone in products vary and have been reported to range from <0.1-1% in 1976 to 0.005-7% in 2002 (Cosmetics Ingredient Review, 2005). Oxybenzone is a sunscreen active ingredient in the U.S. FDA's sunscreen monograph 21 CFR 352.10 (CFR, 2013b). In addition to personal care products, oxybenzone is also commonly found in plastics because of its ability to absorb ultraviolet light and act as a stabilizer (HSDB; http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB). It has also been approved by the FDA as an indirect food additive used in food contact substances (21 CFR 177.1010) (CFR, 2013a).

Exposures

Exposure to oxybenzone may occur through occupational sources or through contact with consumer products. The National Occupational Exposure Survey conducted by the National Institute for Occupational Safety and Health (NIOSH) in 1981-1983 estimated that 27,518 workers were potentially exposed to oxybenzone on the job-site with 8,664 being female (http://www.cdc.gov/noes//noes2/occssyna.html). More recent analyses, however, have estimated the prevalence of oxybenzone exposure to be much higher due to its widespread incorporation in personal care products (Calafat et al., 2008; Centers for Disease Control and Prevention, 2015).

The levels of oxybenzone or its metabolites were measured in urine samples collected from 2,517 individuals from 2003-2004 as part of the National Health and Nutrition Examination Survey (NHANES) (Calafat et al., 2008). Upon analysis over 96.8% of the samples had measurable levels of oxybenzone with a geometric mean value of 22.9 µg/L (22.2 µg/g creatinine) and a 95th percentile concentration of 1,040 µg/L (1,070 µg/g creatinine). The values for non-Hispanic whites were significantly higher than those for other racial groups. Additionally, females had significantly higher levels than did males, and non-Hispanic white women were 6.8 times and Mexican American women were 4 times more likely to have levels above the 95th percentile than were non-Hispanic black women (Calafat et al., 2008).

Measurable levels of oxybenzone have also been reported in 96% of urine samples collected and analyzed from 90 6- to 9-year old girls with levels ranging from <0.2 – 26,700 µg/L (geometric mean of 30.8 µg/g creatinine) (Wolff et al., 2007). In addition, oxybenzone has been detected in the urine of 40 out of 42 premature infants with a geometric mean of 3.4 µg/L (range of LOD – 176 µg/L) (Calafat et al., 2009). Additional studies have also detected measurable levels in amniotic fluid. A survey of 69 pregnant woman found 61% (range of LOD – 15.7 µg/L) had detectable levels in their amniotic fluid (Philippat et al., 2013).

Updated NHANES levels for the most recent survey years (2011-2012) indicate little change in the urine concentration of oxybenzone for the U.S. population (Centers for Disease Control and Prevention, 2015). In survey years 2011-2012, samples were collected from 2,489 individuals. The mean geometric mean value for oxybenzone was reported to be 23.2 µg/L (26.4 µg/g creatinine) with a 95th percentile concentration of 1,200 µg/L (1,680 µg/g creatinine). Individuals in the 12-19 year old age group had higher geometric mean levels (27.8 µg/L; n=388) compared with those 6-11 years (18.7 µg/L; n=396) or 20 years and older (23.1 µg/L; n=1705). Females still had significantly higher levels (geometric mean 31.0 µg/L) than males (geometric mean 17.2 µg/L) and non-Hispanic whites had the highest levels when analyzed by race/ethnicity.

Recent studies have also compared the levels of oxybenzone and its metabolites in the urine of children and adults from the U.S. and China (Wang and Kannan, 2013). While oxybenzone was

detected in over 99% of all samples analyzed (n=164 total participants; 38 U.S. children versus 70 Chinese children; 30 U.S. adults versus 26 Chinese adults), the concentrations in the urine of Chinese participants were significantly lower than those from U.S. participants. Geometric means from the U.S. samples were 9.97 and 15.7 ng/mL for children and adults, respectively, while the geometric means for the corresponding Chinese samples were 0.62 and 0.98 ng/mL, respectively. Lower levels of oxybenzone have previously been reported in the Chinese population (Chen et al., 2012; Zhang et al., 2013) and are thought to be in part due to the relatively low usage of sunscreen and/or sunscreen containing personal care products by the Asian population. In addition, oxybenzone is not the predominant compound used in sunscreens and sunscreen containing personal care products in China; instead methoxy ethylhexyl acrylate and butyl methoxy dibenzoylmethane are more commonly used (Gao et al., 2011).

Assessment of urine specimens from a sub-sample of participants in the Study of Use of Products and Exposure Related Behavior (SUPERB), a study aimed at investigating human behaviors and their impact on exposure to environmental pollutants, also revealed higher concentrations of oxybenzone in adults (median concentration 128 µg/L) compared to their children (median concentration 55 µg/L) (Philippat et al., 2015). Oxybenzone concentrations reported by Philippat et al. (2015), however, were approximately nine times higher than those found in the NHANES survey by the Centers for Disease Control and Prevention (2015) for the same study period (2007-2009) (median concentration 13.7 µg/L for 2007-2008; 16.5 µg/L for 2008-2009). The demographic composition of the SUPERB study was limited to California mothers with young children who tended to be highly educated and white of non-Hispanic origin. Previous studies have also linked increased oxybenzone exposure and sunscreen product usage to specific demographic groups and socio-economic status within the U.S. (Calafat et al., 2008; Wu et al., 2010; Kunisue et al., 2012; Tyrrell et al., 2013).

In the environment, oxybenzone has been detected in wastewater samples (31-168 ng/L; 11-286 ng/L) (Rodil et al., 2008; Pedrouzo et al., 2009), in wastewater treatment plants (<LOD-16 ng/L) (Rodil et al., 2008; Balmer et al., 2005) as well as in the surface waters of lakes (<2-125 ng/L) (Poiger et al., 2004) and in the coastal waters of beaches (46.6 ng/L) (Rodríguez et al. 2015). Concentrations were found to be seasonal and tended to be higher in the warmer months, perhaps

as a result of increased swimming and/or sunscreen usage (Poiger et al., 2004; Rodil et al., 2008). Oxybenzone has also been found in sediments (1.6 ng/g) collected in Southern California (Schlenk et al., 2005), but was below the limit of quantification (LOQ, 8.2 ng/g) in livers from the hornyhead turbot, a demersal fish (Kwon et al., 2009).

Absorption, Metabolism and Distribution

Oxybenzone has been shown to be metabolized to 2,4-dihydroxybenzophenone (DHB; 2,4 OH-BP or BP-1), 2,2'-dihydroxy-4-methoxybenzophenone (DHMB; 2,2'-OH-4-MeO BP or BP-8) and 2,3,4-trihydroxybenzophenone (THB) in animal studies. DHB and DHMB are the major detectable metabolites while THB is present at much lower levels (Jeon et al., 2008). Oxybenzone and its metabolites can be conjugated at the hydroxyl groups as glucuronides or as sulfates (El Dareer et al., 1986; Jeon et al., 2008).

Oxybenzone was found to be quickly absorbed from the gastrointestinal tract after a single dose (100 mg/kg body weight) when administered orally to male Sprague-Dawley rats with the maximum plasma concentration reached within 3 hours of administration and declining rapidly thereafter (Jeon et al., 2008). This is in contrast to the predominant metabolite, DHB, which decreases more slowly with time (Jeon et al., 2008). The main route of elimination was via the urine (over 75% urinary excretion occurred in the first 6-12 hours), followed by the feces (90% of fecal excretion in the first 24 hours) (Jeon et al., 2008). Previous studies which examined tissue distribution determined that the liver accumulated the highest amount of compound followed by the kidney (Okereke et al., 1993; Kadry et al., 1995). These studies are in general agreement with a previously sponsored NTP study that examined oral, intravenous (IV) and topical administration of the compound. In this study they also found the compound to be rapidly absorbed, metabolized and excreted (El Dareer et al., 1986).

Additional studies have evaluated absorption and metabolite levels of oxybenzone in humans. Three separate studies investigated the ability of sunscreen products, which contained oxybenzone as an active ingredient, to penetrate the skin and be absorbed into the bloodstream. Hayden and colleagues (1997) found that between 1-2% of the applied amount of oxybenzone (12.4 mg/cm^2) was absorbed into the internal circulation within 10 hours of application in nine healthy volunteers.

Janjua et al. (2008) measured plasma and urine levels of oxybenzone in 32 study volunteers who were exposed to daily whole-body topical application of 2.0 mg/cm^2 over a four day period. Levels of oxybenzone were detected within one to two hours of the first application in the plasma. Jiang et al. (1999), using excised human epidermis, reported that roughly 10% of a dermally applied dose (2.0-2.5 mg/cm^2) penetrated and passed through the skin. Five other common sunscreen agents were tested alongside oxybenzone (octyl methoxycinnamate, titanium dioxide, octylsalicylate, octocrylene and butyl methoxydibenzoylmethane); none of which were able to readily penetrate the skin in significant amounts (Jiang et al., 1999).

Liquid-chromatography (LC)-tandem mass spectrometry (MS) has been used to analyze oxybenzone metabolites, derivatives and concentrations in human urine samples. A study by Kunisue et al. (2012) analyzed samples from 625 women in California and Utah for the presence and concentration of oxybenzone, its metabolites (DHB and DHMB), and the benzophenone derivatives 2,2',4,4'-tetrahydroxybenzophenone (2,2',4,4'-OH BP; BP-2) and 4-hydroxybenzophenone (4-OH-BP). Levels of DHMB and 2,2',4,4'-OH BP were the lowest and detected in only 2.6% and 5.6% of samples, respectively. Levels of oxybenzone were the highest (median concentration 6.1 ng/mL) and found in 99.0% of samples analyzed; levels of the major metabolite, DHB, were detected in 93.3% of samples (median concentration of 6.1 ng/mL). 4OH-BP, on the other hand, was detected in about 84% of samples (median concentration of 0.36 ng/mL). A study by Wang and Kannan (2013) reported similar results. They identified DHB as the most prevalent metabolite found in the urine of 100% of adult samples (n=30) with a geometric mean concentration of 4.37 ng/mL. Levels of 4OH-BP were detected in 93% of samples (compared to 84% by Kunisue et al., 2012) at a concentration of 0.330 ng/mL (geometric mean). Levels of 2,2',4,4'-OH BP and DHMB were not quantified in the study by Kunisue et al. (2012); however, the prevalence of the metabolites were much higher in the study by Wang and Kannan (2013) (60% of samples versus 5.6% and 53% versus 2.6%, respectively).

Endocrine Disruptor Potential

A number of studies have examined the endocrine disruption potential of oxybenzone. These studies have included both *in vitro* (Schlumpf et al., 2001; Nakagawa and Suzuki., 2002; Wada et

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al., 2004; Suzuki et al., 2005; Kunz et al., 2006) and *in vivo* (French, 1992; Datson et al., 1993; Schlumpf et al., 2001; Schlecht et al., 2004; Suzuki et al., 2005; Kunz et al., 2006; Coronado et al., 2008) analyses. Overall, the studies demonstrate that oxybenzone and/or the primary metabolite, DHB, have weak estrogenic activity. These compounds are approximately 10^5 to 10^6 less potent than estrogens such as estradiol and have similar estrogenic potency as bisphenol A.

Dose Selection for the Embryo/Fetal Developmental Feed Study of Oxybenzone
Results from a developmental dose range-finding feed study of oxybenzone are presented in Nakamura et al. (2015) which utilized exposure concentrations of up to 50,000 ppm. These results, in conjunction with the results of an NTP dose-finding feed study (unpublished), were used to select dietary exposure concentrations of 0, 3,000, 10,000 and 30,000 ppm for the current study.

Dietary exposures of 0, 1,000, 3,000, 10,000, 25,000 and 50,000 ppm were evaluated in the developmental dose range-finding study (Nakamura et al., 2015). Time-mated female Sprague-Dawley rats (Harlan) were administered oxybenzone via Purina 5K96 chow beginning on GD 6. Dosing continued until the time of sacrifice on GD 10, GD 15, GD 20 or postnatal day (PND) 23. A decrease in maternal body weight was observed as early as GD 10 for the highest oxybenzone dose group and continued through GD 20. Although oxybenzone at the two highest treatment concentrations produced maternal toxicity evidenced by weight loss, no differences were observed in the number of implantations, live fetuses or fetal weights at GD20. At PND 23, there were no body weight differences between the treated groups of maternal animals, however, administration of 10,000 ppm and greater was associated with higher relative liver weights and increases in clinical chemistry parameters suggestive of liver toxicity. Oxybenzone did affect postnatal pup weight at 50,000 ppm where a decrease in postnatal pup weight was observed as early as PND 4 for females and PND 7 for males; significant differences for both sexes were observed at PND 14. Overall, the results from the developmental dose range-finding feed study of oxybenzone indicated a lowest adverse effect level (LOAEL) to be 10,000 ppm for maternal toxicity and 25,000 ppm for pups. Additionally, the LOAEL was deemed to be 25,000 ppm for reproductive and developmental effects.

Study Objective

This study was designed to determine the potential effects of oxybenzone on fertility and early embryonic development to implantation in Sprague-Dawley rats. There has been recent interest in the biological activity of oxybenzone due to its high volume of use and its detection in the urine of a large percentage of the U.S. population.

The study was designed and conducted according to the recommendations outlined in ICH Guideline S5(R2) 4.1.1 (ICH, 1993); a modified one-generation study was also conducted on oxybenzone by the NTP to compare results from the two study designs.

MATERIALS AND METHODS

Materials and methods and experimental design are outlined in Table 1. Additional information is provided below and in the referenced Appendices.

Procurement and Characterization of Oxybenzone

Oxybenzone (CAS No. 131-57-7) was obtained in a single lot (1F100604) from Ivy Fine Chemicals (Cherry Hill, NJ). Identity and purity analyses were conducted by the Chemistry Support Group located in the Division of Biochemical Toxicology at the National Center for Toxicological Research (NCTR; Jefferson, AR). Reference standards were purchased from Sigma-Aldrich (St. Louis, MO; Lot S42088).

Bulk chemical was stored in the original shipping container(s) protected from light at room temperature. Purity and identity was assessed prior to study initiation using the methods described in brief below; detailed descriptions on analyses performed can be found in Appendix IV. Proton nuclear magnetic resonance (NMR) spectroscopy of the test article indicated that the test article was consistent with the structure of oxybenzone and matched the reference standard. Other than the resonances of the residual non-deuterated solvent and water, no other significant traces of contaminants were detected, indicating the test article was of high purity. The purity of the test article was also assessed by reverse phase high-performance liquid chromatography (HPLC) with photodiode array (PDA) and MS detection. HPLC-MS analysis revealed a single UV peak that was identical to that of the reference standard. HPLC-PDA analysis also revealed one major peak with an identical UV spectrum to that of the oxybenzone standard; purity was determined to be greater than 99%.

Procurement and Characterization of Ethinyl Estradiol

The reference estrogen, ethinyl estradiol (EE2; CAS No. 57-63-6), was purchased from Sigma-Aldrich (Lot 071M14392V). Identity and purity analyses were conducted by the Chemistry Support Group located in the Division of Biochemical Toxicology, NCTR (Jefferson, AR). Reference standards for EE2 were obtained from Sigma-Aldrich (Lot 028K141188) and Steraloids (Newport, RI; Batch G745).

EE2 was stored in the original shipping container(s) at room temperature. As with oxybenzone, purity and identity of EE2 was confirmed prior to study initiation using the methods described in brief below; detailed descriptions can be found in Appendix IV. Proton NMR spectroscopy indicated the spectrum of the test article was consistent with the structure for EE2, matching that of the reference standards. No significant traces of contaminants were detected indicating high purity of the test article. HPLC-MS analysis revealed a single UV peak in the EE2 test article and reference standards; the mass spectra of each peak were identical indicating EE2 was the major component of the test article with little to no impurities present. HPLC-PDA analysis also revealed a single UV peak for the test article that was equivalent to that of the reference standards. Purity of EE2 was determined to be greater than 99%.

Background Isoflavone Content of Purina 5K96 Chow

In an attempt to minimize background exposure to phytoestrogens, animals were maintained on a soy- and alfalfa-free rodent chow, designated 5K96 [verified casein diet 10 IF, irradiated], obtained through Purina Mills, Inc. (St. Louis, MO). Isoflavone levels, defined as the total amounts of genistein and daidzein, in each lot of feed were determined by the Chemistry Support Group, Division of Biochemical Toxicology, NCTR (Jefferson, AR) prior to utilization. The composition of the diet and results of isoflavone/contaminant analyses are presented in Appendices V and IV, respectively, for feed used throughout the study. Based on historical data on lots of 5K96 previously used at NCTR the tolerance level was set at 2 ppm for total isoflavone levels. Analyses indicated that the total levels of daidzein and genistein did not exceed the specified limit of 2 ppm and averaged 0.07 ± 0.07 ppm for daidzein and 0.15 ± 0.14 ppm for genistein (mean total for daidzein and genistein: 0.23 ± 0.20 ppm).

Preparation and Analysis of Oxybenzone Dose Formulations

The Diet Preparation Group, Priority One Services at NCTR prepared all dose formulations for the current study. Dose formulations were prepared as needed by mixing oxybenzone with 5K96 chow. Mixing was performed in a Patterson-Kelly blender using an intensifier bar. Formulations were stored in stainless steel cans at $4^{\circ}C \pm 2^{\circ}$. Details on the dose formulation procedure can be found in Appendix V.

Homogeneity and stability studies using lot 1F100604 of oxybenzone were previously conducted as part of the dose range-finding study (Nakamura et al., 2015). Homogeneity (1,000 and 50,000 ppm dose levels) was verified and stability of the 1,000 ppm dose formulation was confirmed through seven weeks[1] at refrigerated temperature (see Appendix IV for detailed description).

Dose certifications and periodic homogeneity analyses were conducted by the Chemistry Support Group, Division of Biochemical Toxicology, NCTR (Jefferson, AR) for each batch of dosed chow that was mixed. Samples were prepared for analysis by HPLC-PDA as outlined in Appendix IV. Oxybenzone was prepared at three dose concentrations (3,000; 10,000; 30,000 ppm). One sample from each concentration was supplied as aliquots labeled top, middle and bottom for analysis. In all instances, except for one (SCR# 2186 98 00017), the dose formulations of oxybenzone were within 10% of the target concentrations with a CV of 10% or less (Appendix IV). The dose formulation that failed to meet the defined specifications (only 59% of the target dose of 10,000 ppm) was re-mixed and certified prior to use. A sample of the 0 ppm diet was also supplied at the time of analysis for each batch of dosed chow that was mixed.

Preparation and Analysis of Ethinyl Estradiol Dose Formulations
The stability and homogeneity of EE2 was determined previously in E0213801 conducted at the NCTR with results presented in NTP Technical Reports 547 (NTP, 2010a) and 548 (NTP, 2010b). Homogeneity was verified and stability of a 5 parts per billion (ppb) dose in 5K96 feed was confirmed for at least 24 weeks when stored in stainless steel cans at 2-8°C or for up to 16 days under simulated animal room conditions.

Dose formulations were prepared as needed by mixing a concentrated solution of EE2, dissolved in 95% ethanol, with the 5K96 feed in a Patterson-Kelley blender using an intensifier bar. The ethanol was removed from the preparation by heating under vacuum in the blender. Samples were then submitted to the Chemistry Support Group, Division of Biochemical Toxicology, NCTR (Jefferson, AR) for confirmation of the target concentration (0.05 ppm) and for homogeneity evaluation. Quantitative analysis of the samples was conducted using Ultra

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[1]Stability at the time the protocol was approved was six weeks. Refer to the Analytical Chemistry Report (Appendix IV) for data supporting stability of seven weeks.

Performance Liquid Chromatography (UPLC) tandem MS analysis as described in Appendix IV. The target concentration of 0.05 ppm was achieved for each of the study samples, and the distribution of EE2 in the feed was determined to be homogenous.

Source and Specification of Animals

The study design was modeled on the recommendations presented in ICH Guideline S5(R2) 4.1.1 for assessing effects on fertility and early embryonic development to implantation (ICH, 1993). A total of 250 Sprague-Dawley rats (125 male; 125 female) were obtained from Harlan Industries (Indianapolis, IN). Twenty-five animals of each sex were assigned per treatment group and fed diets containing 0, 3,000, 10,000 or 30,000 ppm oxybenzone or 0.05 ppm of the reference estrogen EE2.

Vaginal cytology was monitored for 14 days prior to the females being placed on dosed chow and for 14 days beginning at the start of dosing to determine whether oxybenzone affected the estrus cycle. Males were dosed for at least 10 weeks and females for at least two weeks prior to mating; mating was staggered over a three week period. Randomization tables, stratified by treatment, were generated to determine mating pairs. Animals were housed together for up to 15 days or until they mated. A sperm positive lavage or a vaginal plug preventing lavage was used as an indication of mating. The date of sperm positive lavage or vaginal plug detection was designated as GD 0. Animals received dosed chow throughout the mating period until GD 6 of the female's pregnancy; at GD 6 animals were placed back on control chow until sacrifice. Males were sacrificed after GD 6; females were sacrificed on GD 15 and evaluated as described below.

Animal Maintenance and Health

Animals were allocated to treatment groups by a weight-ranked randomization procedure upon completion of quarantine such that the mean weight of each treatment group was approximately the same (Appendix VIII). All animals were identified by tail tattoo. Forty-eight hours prior to mating, the males were separated and singly housed until the introduction of the female into the breeding cage. For breeding, one male was housed with one female. Once mating was indicated, the males and females were separated and housed individually for the remainder of the study. Animals were also housed individually if after 15 days of cohabitation there was no indication of

mating (sperm positive lavage or vaginal plug). Solid-bottom polysulfone cages with hardwood chip bedding were used throughout the study.

Animals were maintained on a soy- and alfalfa-free Purina 5K96 chow for the duration of the study. All animals received control chow until the designated start of dosing (see Table 1 for male and female start dates). Males received dosed chow for at least 10 weeks prior to mating; females for at least two weeks prior to mating. Dosing continued through the mating period until GD 6 of the female's pregnancy for those pairs determined to have mated. For pairs where mating had not been indicated, dosing continued until the breeding pair was dissolved after 15 days of cohabitation. Animals were returned to control chow on GD 6 of the female's pregnancy or after 15 days of cohabitation until sacrifice. Purina 5K96 chow and Millipore®-filtered tap water were available *ad libitum*. The 5K96 diet underwent routine concentration analysis as well as analysis for isoflavone concentrations as described above. Feed consumption was measured at least twice weekly beginning at the time of allocation and continuing until mating. Feed consumption measurements were not recorded during the time the animals were placed together for mating. Feed consumption measurements resumed at GD 0 (day of sperm positive vaginal lavage or presence of a vaginal plug) and continued through GD 6 of the female's pregnancy for both the males and females with measurements occurring on GD 0 and GD 6. Feed consumption for females continued until GD 15. Information on feed consumption (Tables 4, 5, 10 and 12) and contaminants (Appendix IV) can be found in the referenced tables and appendix. Water consumption was not measured; water bottles were changed weekly or as often as necessary to maintain a constant supply. Animal cages were changed at least once weekly throughout the study.

The health of the animals was monitored according to the protocols of the study laboratory's Microbiology Surveillance and Diagnostic Program (Appendix VI). Twelve animals (six male; six female) were ordered; eight animals were used as sentinels (four males; four females). Two animals of each gender were sacrificed after four weeks at the study laboratory; the remaining four animals were sacrificed at study completion. Two animals per shipment (four animals total) were ordered for microbiology surveillance. All males were ordered and received in one shipment and all females in another.

In-Life Examinations and Pathology

Data collected during the in-life phase of the study and at necropsy are detailed in Table 1. The Multigeneration Support System (MGSS), which was developed by the former R.O.W. Sciences at the NCTR, was used to collect in-life animal data. Twice daily morbidity and mortality checks were performed; clinical observations were recorded twice weekly. Body weights of males and females were recorded twice per week from the day of allocation until the determination of pregnancy which was designated GD 0. The body weights of the males were also recorded on GD 0, GD 6 (end of dosing) and at sacrifice; in addition, body weights of females were recorded on GD 0, GD 6 (end of dosing), GD 10 and at GD 15 (sacrifice).

Vaginal lavage was performed daily for 14 days prior to the start of dosing on all females to determine the length of the estrus cycle. It was also performed daily through the two week, pre-mating dosing period to determine if exposure to oxybenzone had any effect on estrus cyclicity. In addition to determining estrus cyclicity, vaginal lavage was used to establish the date of mating/pregnancy (GD 0). The morning following establishment of a breeding pair, vaginal lavage was performed daily up to 14 days or until evidence of mating on each female. A sperm positive lavage was considered evidence of mating; however, a vaginal plug preventing lavage was also considered evidence of mating.

Males and females were euthanized by over-exposure to carbon dioxide. Males were sacrificed after GD 6; females on GD 15. Blood was removed by cardiac puncture from all animals at sacrifice. Ten males and 10 pregnant females from each treatment group (50 total/sex) were selected for analysis of clinical chemistry and hematology parameters (see Notes to Study File – Randomization for Clinical Chemistry, Appendix IX). Males were selected based on randomization lists generated by the NCTR statisticians; for females the first 10 pregnant animals at sacrifice in each treatment group were used for analysis. In addition to hematological and clinical chemistry parameters, hormone levels (testosterone, follicle stimulating hormone and luteinizing hormone) were analyzed in the males selected for hematological and clinical chemistry analyses. All remaining blood samples were retained for analysis at the discretion of the Study Director or were transferred to NCTR Protocol E0750301 (non-GLP) "Effect of Fetal or Postnatal Exposure to Oxybenzone on Reproduction in Male Rats."

All males and females underwent a gross examination and a complete necropsy of the thoracic and abdominal cavities at terminal sacrifice. Any gross lesions identified were collected, weighed and processed for histopathology. To serve as a reference for any gross lesions identified, all tissues from one male and one female per dose group were collected and processed to slides. The testes, epididymides, prostate gland (dorsolateral and ventral), seminal vesicles with coagulating glands, preputial glands, Cowper's glands, levator ani bulbocavernosus muscle complex (LABC), ovaries, uterus (with right ovary, vagina and cervix attached), adrenal glands, liver, kidneys, and the thyroid gland were removed at sacrifice and weighed. Weights of the dorsolateral prostate, ventral prostate and the thyroid gland were obtained following fixation. The mammary glands, the pituitary gland, and any retained nipples were also collected; however, weights were not obtained.

For assessment of pregnancy and the number of implantations, the uterus (with the right ovary, vagina and cervix attached) were removed from the females and weighed. The uterus was opened to allow evaluation of the number and status (early, late, live or dead) of the implantation sites on GD 15. After examination of the uterus, the right ovary was removed and the individual weight was recorded. For animals that did not appear pregnant, the uterus was stained with ammonium sulfide to detect any implantation sites. Prior to fixation of the ovaries, corpora lutea counts were performed.

Histopathology was performed on the tissues of the males and females in the oxybenzone high dose (OXY 30,000 ppm) group, the control (CTRL) group, and the ethinyl estradiol (EE2 0.05 ppm) reference estrogen group. Other dose groups were evaluated only if triggered. For the males, histopathology analysis was conducted on the right testis, right epididymis, dorsolateral prostate, ventral prostate, seminal vesicles with coagulating glands, retained nipples (if observed), mammary glands, the adrenal, pituitary and thyroid glands. Histopathology on the preputial glands, Cowper's glands, and LABC was at the discretion of the Study Pathologist and/or Study Director. The left testis and epididymis from the 50 males selected for hematological and clinical chemistry analysis were not fixed, instead they were used for assessment of testicular spermatid head counts, epididymal sperm counts, sperm morphology and sperm motility evaluations. If gross lesions were observed at necropsy on the left testis or epididymis, then the right organs were used for sperm

evaluations and the left used for histopathology observations. Tissues (e.g. testis and epididymis) from male animals not needed for histopathology analysis (i.e. no gross lesions identified) or sperm analysis were transferred to NCTR Protocol E0750301 "Effect of Fetal or Postnatal Exposure to Oxybenzone on Reproduction in Male Rats" for gene/protein expression. This was a non-GLP protocol, and the data are not included in this report.

Histopathological analysis on the females included the mammary gland. The vagina, cervix and a representative sample of the uterus along with the adrenal, pituitary and thyroid glands were processed to block and held. The liver and kidneys from both males and females were also processed to block and held with histopathology conducted only if requested by the Study Pathologist and/or Study Director. Tissues were fixed in either 10% neutral buffered formalin (NBF) or Modified Davidson's according to the Specifications for the Conduct of Studies to Evaluate Animals for the National Toxicology Program (May 2011), except for the ovaries, which instead were fixed in 10% NBF. Histopathology samples collected during the course of the study are stored in the NCTR Archives.

Records Storage

The signed and dated copy of the final report and supporting study information will be placed into the NCTR archives for permanent storage. Raw data, including any handwritten data, collected during the course of the study will also be placed into the NCTR archives.

STATISTICAL METHODS

The following paragraphs outline the statistical methods used for analysis of the study data. Detailed statistical reports for all endpoints evaluated are included as Appendices XII – XXIV. For *post hoc* analysis, comparisons of treatment groups to control were performed with Dunnett's method for adjusted contrasts (Dunnett, 1955), unless noted. Tests of trend, increasing treatment effect with increasing dose, were performed for the oxybenzone and control groups; the EE2 0.05 ppm treatment group was excluded from the analysis of trend. Tests were conducted as two-sided at the 0.05 significance level.

Pre-Mating Body Weights – Females and Males (Appendix XII)

Treatment group means of body weight were determined from the twice weekly observations. For females, data was analyzed by day (days 1, 5, 9 and 12); for males the last data collected for each animal by week (weeks 1-10) was used for analysis. Pairwise comparisons of means were performed using contrasts within a two-way repeated measures mixed model analysis of covariance (ANOCOVA) for females and males, separately. Model terms were treatment group, days or weeks on study, interaction and covariate baseline (defined as pre-dosing weight). Within-group correlations were modeled using a heterogeneous first-order autoregressive (ARH(1)) correlation structure, which allowed for correlated differences in variability across time points.

Pre-Mating Food Consumption – Females and Males (Appendix XIII)

Treatment group means of daily food consumption per animal were analyzed using data collected twice weekly for each cage (days 1-5, 6-9 and 10-12 for females and weeks 1 through 10 for males). Pairwise comparisons of means were performed using contrasts within a two-way repeated measures mixed model analysis of variance (ANOVA) for females and males separately, with terms for treatment group, days or week, and the interaction between treatment and days or treatment and week. Within-group correlations were modeled using a ARH(1) correlation structure.

Gestational Body Weights – Females (Appendix XIV)

Pairwise comparisons of means were performed using contrasts within a two-way repeated measures mixed model ANOVA with terms for treatment group, GD and interaction of treatment group and GD. Analysis was performed on body weight data collected at GD 0, GD 6, GD 10 and GD 15 (weight at sacrifice). Within-group correlations were modeled using a ARH(1) correlation structure. Excluded from the analyses were 24 females that were not pregnant and 13 pregnant dams with undetermined or unknown mating dates (GD 0). GD 10 and GD 15 weights were excluded for four dams that were dosed past the specified stop date (GD 6) (see Appendix XIV for a complete list of exclusions). One dam in the OXY 10,000 ppm treatment group and one in the EE2 0.05 ppm treatment group were sacrificed early (GD 14 and GD 13, respectively) due to availability of Toxicological Pathology Associates (TPA), Inc. personnel. Results for these two dams, however, were included in the GD 15 analyses as exclusion did not result in any differences in conclusions.

Gestational Food Consumption – Females (Appendix XV)

Treatment group means of food consumption during gestation were analyzed using the daily food consumption of each dam during the following intervals: GD 0-6 (dosed chow) and GD 7-15 (control chow). Pairwise comparisons of means were performed using contrasts within a two-way repeated measures mixed model ANOVA with terms for treatment group, GD interval, and the interaction between treatment and GD interval. Within-group correlations were modeled using a ARH(1) correlation structure. Food consumption data from females that were not pregnant or had an unknown or undetermined mating dating were not included in the analysis. Also excluded, food consumption data collected from GD 7-15 for four dams that were dosed past GD 6 (see Appendix XV for a complete list of exclusions).

Post-Mating Body Weights – Males (Appendix XVI)

Analyses were performed using data from males with a known mating date (GD 0) and where mating resulted in pregnancy (see Appendix XVI for the list of exclusions). Pairwise comparisons of body weight means were performed using contrasts within a two-way repeated measures mixed model ANOVA with terms for treatment group, dam's GD, and the interaction between treatment group and GD. Analysis was performed on body weight data collected at GD 0 and GD 6 of the dam's pregnancy. Within-group correlations were modeled using a ARH(1) correlation structure.

Post-Mating Food Consumption – Males (Appendix XVII)

Pairwise comparisons of daily food consumption means were performed using contrasts within an ANOVA to test for treatment effect. Analysis was performed on male food consumption data collected from the day of mating (GD 0) through the end of dosing (GD 6). Analyses were performed using data from males with a known mating date (GD 0) and where mating resulted in pregnancy. Due to missing food consumption data, two males with pregnant female partners and a known mating date were excluded (see Appendix XVII for a complete list of exclusions).

Organ Weights – Females and Males (Appendix XVIII)

Analyses of organ weights were conducted on the combined weights of paired organs, if applicable. Treatment group means of organ weights were analyzed for males and for pregnant females with a known GD 6 (end of dosing date). Pairwise comparisons of means were performed using contrasts within a one-way ANOCOVA to test for treatment effect with the covariate receiving weight for pregnant females and males, separately. Organ weights for animals in breeding pairs with an unknown mating date (GD 0) were excluded from analysis as were organ weights for four dams that were dosed past the GD 6 stop date (see Appendix XVIII for a complete list of exclusions).

Vaginal Cytology (Appendix XIX)

To monitor the effect of oxybenzone on estrus cyclicity, vaginal swabs were collected daily for 14 days at the start of dosing. Daily swabs were reported as estrus, proestrus or diestrus. Analyses were conducted on the proportion of days in estrus stages, the proportions of animals with abnormal cycles, daily cycling transitions (between normal, extended estrus and extended diestrus) and cycle length.

Proportion of days spent in estrus, diestrus and proestrus for each animal were analyzed using a multivariate analysis of variance (MANOVA) using the arcsine-square root data transformation with treatment as the predictor. Dunnett's method of adjustment was performed for multiple comparisons.

For abnormal cycling the Cochran-Armitage method for binomial proportions was used to evaluate the pairwise differences in proportions. The two-sided p-value for the Fisher's exact test is reported for comparisons of dosed groups to control, and the Cochran-Armitage trend test was performed. The endpoints evaluated included abnormal cycling, extended estrus, extended diestrus and excessive proestrus. Extended estrus was defined as more than two consecutive days of estrus; extended diestrus was defined as more than four consecutive days of diestrus, and excessive proestrus was defined as two or more consecutive days of proestrus in a given cycle.

Transition matrices of treatment groups were compared to the control group for analyses of normal, extended estrus and extended diestrus based on the Markov chain model of Girard and Sager (1987). From the first day of estrus, transition to a second day of estrus was defined as normal. Subsequent transitions to estrus were defined as extended estrus. After four consecutive days of diestrus, subsequent transitions to diestrus were defined as extended diestrus. Although abnormal cycling transitions were not defined for proestrus, the analysis only considered the transition to the first proestrus as "normal". Subsequent transitions to proestrus (second or third proestrus, for example) were treated as missing. Other transitions were defined as normal. Analysis was performed using Markov chains with the chi square statistic to test for differences between dosed groups and control. No adjustment for multiple comparisons was performed.

For analysis of estrus cycle length, cycle days were defined from the first day of estrus in a sequence of stages until the first day of estrus in the following sequence. Pairwise comparisons of means were performed using contrasts within a one-way repeated measures mixed model to test for treatment effect accounting for animal effect and a compound symmetric correlation structure. Dunnett's method was performed for multiple comparisons adjustment.

Reproductive Parameters (Appendix XX)

Counts and percentages of mated and pregnant females in each treatment group were calculated. TPA, Inc. Study Pathologist's observations regarding littering and fetal development at sacrifice were used to estimate mating dates (GD 0) and time to mating for 11 pregnant females that had missing dates based on negative vaginal lavages. One pregnant animal littered; for this animal litter day was assumed to be GD 21 for estimation of mating date and time to mating determination.

Two dams were excluded from the time to mating analysis; one dam in the control group was not monitored for mating until the third day of pairing and one dam in the OXY 30,000 ppm group had a missing mating date and no pathology observations noted.

Survival analysis was performed for time to mating with unmated females treated as censored at the end of the maximum of 14 days for breeding. Among pregnant females (uncensored), the product-limit method was used for the Kaplan-Meier survival curve and to estimate the mean and median time to mating. For mated and unmated females, the product-limit method was used for the Kaplan-Meier survival curve to estimate the median time to mating and to present counts of censored and uncensored animals.

A Cox proportional hazards regression analysis was performed to test for dose trend and to compare hazard ratios of dosed groups to the control group. In Cox regression, the survival time of each member of a population is assumed to follow its own hazard function, and the hazard functions of any two groups are assumed to be proportional at any particular time. Multiple comparisons were adjusted using Holm's (step-down Bonferroni) adjustment for pairwise comparisons to the control group.

Pregnancy Parameters (Appendix XXI)

Counts and percentages of mated and pregnant females in each treatment group were calculated. Pregnant females were assumed to have mated even in the absence of an observed vaginal plug or a positive vaginal lavage. Summary statistics were performed for gravid uterine weights. Pregnant females were excluded from the analysis of gravid uterine weights if they had unknown mating dates (GD 0) and/or were dosed past the protocol specified stop date (GD 6). Mean counts per litter for each treatment group were calculated for corpora lutea, number of implants, number of resorptions and live fetuses. Mean litter percentages were computed for pre-implantation loss and post-implantation loss. For analyses, pre-implantation loss was defined as the percentage of corpora lutea that did not result in implantation. Post-implantation loss was defined as the percentage of implantations that were resorbed. The number of live fetuses, resorptions and post-implantation loss percentages are not included in the analysis for pregnant females with an underdetermined mating date.

Proportions of mated and pregnant females were analyzed using Fisher's Exact test for comparisons of treated groups to control and using the Cochran-Armitage test for trend. Analysis of gravid uterine weight was performed using contrasts within a one-way ANOVA. Counts of implantation sites were analyzed using Poisson regression with terms for treatment and covariate number of corpora lutea. Counts of resorptions were analyzed using Poisson regression with terms for treatment and covariate number of implantation sites. Comparisons of pregnancy proportions of treated groups to control were performed using Holm's method of adjustment for multiple comparisons. For all other analyses, the Dunnett's method for adjusted contrasts was used for comparison of treated groups to controls.

Sperm Parameters (Appendix XXII)

Analysis of sperm morphology data was performed using a generalized linear model with a Poisson distribution and a log link function. Each treatment was compared to the control group, and adjustment for multiple comparisons was performed using Hochberg's method. Percent sperm motility, testis spermatid head counts and cauda epididymial sperm counts were analyzed using an ANOVA model with Kenward-Roger estimated degrees of freedom (Kenward and Roger, 1997). Males with undetermined mating dates (GD 0) that remained in the breeding cage past the specified removal date and continued to be dosed past the protocol specified stop date (GD 6) were excluded from analysis.

Hematology and Clinical Chemistry Parameters – Females and Males (Appendix XXIII)

An ANOVA was performed separately for males and females for each endpoint to determine the effect of treatment on hematology and clinical chemistry parameters using a nonparametric method with midranks (using the average of left and right ranks for ties) and an unstructured covariance (Brunner et al., 2002). Treatment percent of controls was determined using the nonparametric analysis of ranked data. Hormone analysis for selected males was also performed as described above.

Two females were excluded from hematological/clinical chemistry analysis. One female had an undetermined mating date (GD 0) and another continued to be dosed past the protocol specified

stop date (GD 6). Six males were excluded from analysis that had undetermined mating dates (GD 0); these same males were excluded from analysis of sperm parameters.

Histopathology – Females and Males (Appendix XXIV)

Histopathology data were obtained from NARSS AD HOC PROD on NCTR Citrix using the PTHEXTRT program in Natural written by Brett Thorn (NTP Support Group, Division of Bioinformatics and Biostatistics (DBB), NCTR) to extract data and save to ASC files. Exported data files were read into SAS via the import macros written by the NTP Support Group, DBB. The imported data were checked against the TPA, Inc. histology report and found to match. All female and male animals were included in the analysis of pathology; inclusion was not dependent on dosing duration, mating or pregnancy. Each lesion with an incidence of at least two was included in the analysis.

For statistical analyses of histopathological findings the exact Cochran-Armitage trend test was used to test for trends in nonneoplastic incidences with dose, and the Fisher's Exact test was used to compare incidences between treated groups and the control group (CAFÉ). Tests for trend and comparisons of treated groups to control were performed as one-sided tests. For the analysis of lesion severity scores, the Jonckheere-Terpstra (JT) test was used to test for trends (Terpstra, 1952; Jonckheere, 1954). Shirley's method, modified by Williams (JT-SW), was used to compare lesion severity for treated groups to the control group (Shirley, 1977; Williams, 1986).

RESULTS

In-Life Observations and Clinical Signs

No in-life observations or clinical signs were noted that were considered related to chemical exposure.

Pre-mating Body Weights – Females and Males

Exposure to dietary oxybenzone resulted in statistically significant reductions in body weights of both female and male Sprague-Dawley rats. For females, dietary exposure began after initial vaginal cytology evaluations were completed to assess estrus cyclicity; analysis was by day of treatment. On pre-mating treatment day 5, females in the OXY 10,000 ppm and OXY 30,000 ppm treatment groups showed a 2.8% (p = 0.001) and 4.5% (p = <0.001) reduction in body weight, respectively, when compared to controls (Figure 1 and Table 2). A statistically significant reduction (2.4%; p = 0.011) at pre-mating treatment day 9 was also observed for females in the OXY 10,000 ppm group. Significant reductions in body weight were also observed in pairwise comparisons of the OXY 30,000 ppm treatment group and controls at pre-mating day 9 and day 12 of treatment. Females in the OXY 30,000 ppm treatment group weighed 5.3% less (p = <0.001) at pre-mating day 9 and 5.9% less (p = <0.001) at pre-mating day 12. The overall mean female body weights (defined as the mean body weight for pre-mating days 1, 5, 9 and 12) for the two highest OXY treatment groups were also statistically different from controls (OXY 10,000 ppm, 1.8% reduction, p = 0.015; OXY 30,000 ppm, 3.9% reduction, p = <0.001) (Table 2). No significant differences in female body weights were observed in the OXY 3,000 ppm treatment group throughout the dosing period (Figure 1 and Table 2).

A reduction in female body weight was also observed in the positive control EE2 0.05 ppm group. During the pre-mating dosing period a 6.6% reduction was observed at day 5, a 7.9% reduction at day 9 and a 7.8% reduction at day 12 (p = <0.001 for each time point) when compared to the control group (Figure 1 and Table 2).

Males were exposed to dietary oxybenzone for at least 10 weeks prior to mating. Males treated with 30,000 ppm of oxybenzone showed an 8.1% (p = <0.001) reduction in body weight relative to controls at the end of the first week of treatment. A statistically significant reduction in body weight was also observed at the end of each subsequent week of treatment (range 4.1% at week 3 to 7.7% at week 9; p = ≤0.001 for each time point) (Figure 2 and Table 3). No significant reductions in male body weight were observed in either the OXY 3,000 ppm or OXY 10,000 ppm dose groups throughout the pre-mating exposure period. Similar to what was observed in the females, treatment with 0.05 ppm EE2 also resulted in a statistically significant body weight reduction in males. In the reference estrogen treatment group, weight loss ranged from a low of 3.4% (p= 0.001) at week 1 to a high of 11.3% (p = <0.001) at week 9. Additionally, the overall mean body weights (defined as the mean body weight at the end of weeks 1 through 10) were significantly reduced for males in the OXY 30,000 ppm (6.3%; p = <0.001) and EE2 0.05 ppm (8.7%; p = <0.001) groups (Table 3).

Pre-mating Food Consumption – Females and Males

Food consumption was monitored by twice weekly measurements that began at the time of allocation for both females and males. Food consumption data for females receiving dosed chow were analyzed at the following intervals: pre-mating days 1-5, days 6-9 and days 10-12 (Table 4). An overall analysis of food consumption was also performed and covered pre-mating treatment days 1-12. Pairwise comparisons indicated a statistically significant difference between the OXY 30,000 ppm treatment group and controls at pre-mating days 6-9 (p = 0.046) with the OXY 30,000 ppm group consuming 26.4% more feed than controls. There were no other significant pairwise differences in food consumption for the females prior to mating. However, significant trends were noted at pre-mating days 6-9 (p = 0.004), pre-mating days 10-12 (p = 0.003) and overall (p = 0.017).

Food consumption data for males receiving dosed chow were analyzed in weekly intervals (Table 5). In pairwise comparisons to controls the OXY 30,000 ppm treatment group was significantly different (p = 0.045) at week 3 with the treated group consuming 16.7% more feed than controls. Significant trends were also noted at weeks 2 and 3 (p = 0.002 and p = 0.004, respectively).

No significant differences in pre-mating food consumption were noted for the positive control EE2 reference estrogen group for either the females or males. Estimated ingested doses during the pre-mating period for oxybenzone and EE2 are reported in Table 6 for females and Table 7 for males.

Gestational Body Weights – Females

Dietary exposure to oxybenzone impacted the body weights of pregnant females during gestation. Treatment with 30,000 ppm of oxybenzone resulted in significantly reduced body weights at the time of mating (GD 0); weights of females in this treatment group were 7.4% lower ($p = 0.002$) than those in the control group and continued to be lower throughout gestation (Figure 3 and Table 8). Body weights of pregnant females in the OXY 30,000 ppm dose group at GD 6, when dosing was stopped, were 7.6% lower, at GD 10 they were 4.4% lower and at the time of sacrifice (GD 15) they were 5.4% lower ($p = <0.001$ for all time points). Overall, the mean body weights of pregnant females in the OXY 30,000 ppm treatment group were 6.2% lower ($p = <0.001$) during gestation when compared to the control group. A statistically significant reduction in gestational body weight was also observed for females in the OXY 10,000 ppm group on GD 15; body weights of females in this group were 2.9% lower than controls ($p = 0.029$). A statistically significant overall reduction in gestational body weight was also noted for females in the OXY 10,000 ppm group with animals weighing 2.5% less ($p = 0.032$). There were no significant effects observed on gestational weights of pregnant females in the OXY 3,000 ppm treatment group.

Reductions in gestational body weight were also observed in the EE2 0.05 ppm treatment group. Statistically significant reductions in gestational weight were observed at GD 0 (7.7%), GD 6 (9.1%), GD 10 (4.3%) and GD 15 (6.8%) ($p = \leq0.001$ for all time points). The overall mean gestational weight, when compared to controls, was also significantly reduced (6.9%) ($p = <0.001$; Figure 3 and Table 8).

Non-pregnant animals, confirmed by ammonium sulfide staining of the uterus, were excluded from analysis of gestational body weights. Thirteen dams with unknown mating dates (GD 0) were also excluded; four dams were excluded from GD 10 and GD 15 analyses because dosing continued past the protocol specified stop date (see Appendix XIV for a complete list).

Post-Mating Body Weights – Males

The body weights of males at the time of mating (GD 0 of the female's pregnancy) and at the end of dosing (GD 6 of the female's pregnancy) were significantly lower for males in the OXY 30,000 ppm treatment group and in the EE2 0.05 ppm reference estrogen group. At the time of mating (GD 0) males in the OXY 30,000 ppm treatment group weighed 9.6% less than controls (p = <0.001); at GD 6 males weighed 9.2% less (p = < 0.001; Table 9). Similar reductions were observed upon treatment with 0.05 ppm EE2. At the time of mating (GD 0), males weighed 10.9% less (p = <0.001) than corresponding controls and 10.7% less (p = <0.001) at the end of dosing (GD 6; Table 9). Overall post-mating body weights (mean weights at GD 0 and GD 6) for both treatment groups were also significantly reduced (9.4% OXY 30,000 ppm; 10.8% EE2 0.05 ppm; p = <0.001 for each time point). No statistically significant reductions in post-mating body weights were observed for either the OXY 3,000 ppm or the OXY 10,000 ppm treated males.

Post-mating body weights for seven males of the 88 included in the analysis were missing, although each animal had a body weight corresponding to either GD 0 or GD 6 of the female's pregnancy. Thirteen males were excluded from analysis because the mating date for the breeding pair was undetermined, 14 males were excluded because the animals failed to mate, and 10 were excluded because mating did not result in pregnancy (see Appendix XVI for a complete list).

Gestational Food Consumption – Females

Gestational food consumption for pregnant females was analyzed in two intervals. The first interval included GDs 0-6 when the females were receiving dosed chow; the second covered GDs 7-15 when the animals were consuming control chow. During the first interval (GDs 0-6) pregnant females in both the OXY 30,000 ppm and in the EE2 0.05 ppm treatment groups consumed significantly more chow than controls in pairwise comparisons (Table 10). Females in the OXY 30,000 ppm group on average consumed 73.1% more feed than controls (p = <0.001) while females in the EE2 0.05 ppm group consumed 98.0% more (p = <0.001). There were no differences in food consumption for any of the treatment groups when the animals were receiving control chow (GDs 7-15) and may indicate palatability issues with the highest oxybenzone and reference estrogen dosed feed, leading to increased spillage. There was also a significant increase

when the overall food consumption, defined as the mean food consumption covering GDs 0-15, was analyzed. Again, pregnant females in the OXY 30,000 ppm and EE2 0.05 ppm treatment groups consumed 43.2% (p = 0.001) and 52.5% (p = <0.001), respectively, more chow overall than controls (Table 10). Animals excluded from analysis are reported in Appendix XV and include females who were not pregnant or had unknown mating dates (GD 0). Dosing for four females extended past the protocol specified stop date (GD 6); these females were excluded from the GD 7-15 interval of analysis.

Estimated ingested dose amounts of oxybenzone and EE2 by females during gestation are reported in Table 11.

Post-Mating Food Consumption – Males

For males post-mating food consumption measurements resumed the day of mating (GD 0 of the female's pregnancy) and continued through the end of dosing (GD 6 of the female's pregnancy). Analysis of this time period indicated no significant differences in food consumption in pairwise comparisons to controls for any of the oxybenzone treatment groups or the reference estrogen EE2 group (Table 12). Estimated ingested doses of oxybenzone and EE2 by males during the post-mating period can be found in Table 11.

Excluded from the analysis were males with unknown mating dates, males that failed to mate or where mating failed to result in pregnancy (see Appendix XVII for a complete list). Food consumption values for two males were missing, and they were also excluded from analysis.

Organ Weights – Females and Males

The liver, kidneys, thyroid and adrenal glands were removed from pregnant females and males at sacrifice and weighed. Ovaries were collected and weighed from the females while the testes, epididymides, dorsolateral prostate, ventral prostate, seminal vesicles with coagulating glands, preputial glands, bulbourethral glands (Cowper's glands), and the LABC were collected and weighed from the males. All fat associated with the collected organs was trimmed before weighing. Individual weights of the left and right kidneys, ovaries, testes, epididymides and seminal vesicles

were recorded and are reported in Appendix XVIII; however, for analysis the paired weights of the organs were used (Tables 13 and 14 and Appendix XVIII). Relative organ weight (mg) to body weight (g) ratios were calculated for relevant tissues (i.e. changes in organ weights proportional to changes in body weight) (Bailey et al., 2004), and the summary statistics are presented in Table 15 for pregnant females and Table 16 for males.

ANOCOVA analysis of pregnant female organ weights indicated a significant treatment effect on the adrenal glands (p = 0.003), liver (p = 0.003) and paired ovarian (p = <0.001) weights after adjustment for body weight at sacrifice (Table 13 and Appendix XVIII). In pairwise comparisons an 8.6% increase (p = 0.042) in the adrenal gland weights of pregnant females was significant in the OXY 3,000 ppm treatment group when compared to controls. A significant increase of 12.1% (p = 0.016) was also noted for paired ovarian weights of pregnant females in the OXY 10,000 ppm treatment group. However, while both findings are statistically significant, they appear sporadic and unrelated to dose. Increases of only 2.4% (p = 0.855) and 1.4% (p = 0.984) were observed in adrenal gland weights in the OXY 10,000 and OXY 30,000 ppm treatment groups, respectively. An increase of 9.4% (p = 0.072) in paired ovarian weights was observed in the OXY 3,000 ppm treatment group, while a decrease of 1.9% (p = 0.977) was observed in response to the highest dose of oxybenzone. A significant trend (p = 0.002) was noted for liver weights (increasing weight with increasing dose), but in pairwise comparisons to controls there were no significant differences (Table 13 and Appendix XVIII).

Analysis of organ weights from pregnant females in the EE2 0.05 ppm treatment group revealed no statistically significant differences in adrenal gland, liver, kidney or thyroid gland weights. Ovarian weights were decreased by 11.8% (p = 0.050), just meeting the statistical significance threshold in pairwise comparisons with controls.

For males, ANOCOVA analyses of organ weights indicated significant treatment effects of oxybenzone on the liver (p <0.001), paired kidneys (p <0.001) and paired testes (p = 0.008) after adjustment for body weight. There were significant trends and differences for the liver and paired kidneys in the OXY 10,000 and OXY 30,000 ppm treatment groups in pairwise comparisons to controls (Table 14 and Appendix XVIII). Males in the OXY 10,000 ppm treatment group had an 8.1% increase in mean liver weights (p = 0.006) and a 9.9% increase in mean kidney weights (p =

0.001) relative to controls. For the OXY 30,000 ppm treatment group the mean liver weight increased by 18.4% relative to controls (p = <0.001), and mean kidney weights increased by 14.8% (p = <0.001). The mean liver weights of the positive control EE2 0.05 ppm treatment group were also significantly different from the control group in pairwise comparisons with a 7.5% increase (p = 0.022) observed in the EE2 0.05 ppm treatment group. Although there was also a statistically significant increase of 12.4% (p = 0.049) in the paired seminal vesicle weights between the OXY 10,000 ppm treatment group and controls in pairwise comparisons, the difference did not appear related to dose and thus was considered a sporadic finding. Significant trends were also noted for the ventral prostate and the paired testes weights (p = 0.031 and p = 0.029, respectively), but in pairwise comparisons to control there were no significant differences (Table 14 and Appendix XVIII).

Exclusions of animals and specific organs from organ weight analyses are listed in Appendix XVIII. Exclusions of organs from analysis include those that were weighed after being placed in fixative. Animal exclusions include mating pairs with unknown mating dates (GD 0), non-pregnant females and females that were dosed past the protocol specified stop date (GD 6).

Vaginal Cytology Evaluations

Vaginal cytology evaluations were conducted on female animals for 14 days prior to the start of dosing and for 14 days beginning at the time of dosing. The analyses described below include data collected from the initiation of dosing. Analyses conducted included the proportion of days in estrus stages (proestrus, estrus or diestrus), the proportion of females with abnormal cycles, differences in daily cycling transitions and estrus cycle length.

There were no significant trends or differences in pairwise comparisons between control and oxybenzone treatment groups when the proportion of days spent in proestrus, estrus and diestrus were analyzed. Control females spent 6.0% of time in proestrus, 25.4% in estrus and 65.7% in diestrus while those in the OXY 30,000 ppm treatment group spent 4.3% in proestrus (p=0.799), 22.7% in estrus (p=0.875) and 70.2% in diestrus (p=0.873; Table 17 and Appendix XIX). There were also no significant differences observed between control females and those in the EE2 0.05

ppm treatment group in the proportion of days spent in each phase of the estrus cycle (Table 17 and Appendix XIX).

For the analysis of estrus cycle abnormalities, abnormal cycling was defined for each animal. Endpoints evaluated were excessive proestrus, extended estrus, extended diestrus and any pattern of abnormal cycling. Excessive proestrus was defined as two or more consecutive days of proestrus in a cycle. Extended estrus was defined as more than two consecutive days of estrus, while extended diestrus was defined as more than four consecutive days of diestrus. Total counts and percentages are included in Appendix XIX; percentages are shown in Table 18. Overall there were no significant trends or differences in the number of normal versus abnormal cycles evaluated either by specific stage (proestrus, estrus or diestrus) or overall between the control and oxybenzone treatment groups. There was, however, a significant difference (p=0.042) between the control and EE2 0.05 ppm positive control group in the number of extended diestrus cycles observed. Only 24% of control females had an extended diestrus cycle while 56% of EE2 0.05 ppm treated females had an extended diestrus cycle. There were, however, no other differences in the patterns or percentages of abnormal cycles, overall or at specific estrus stages between the two groups.

Analysis of estrus cycle stage transitions was based on the Markov chain model of Girard and Sager (1987) with abnormalities determined by differences in the observed and expected transitions. The first day of estrus transition to a second day was defined as normal. Subsequent transitions to estrus were defined as extended estrus. Extended diestrus was defined as more than four consecutive days of diestrus. Abnormal cycling conditions were not defined for proestrus, the analysis only considered the transition to the first proestrus as normal; subsequent transitions to proestrus were treated as missing. Observed estrus cycle stage transitions and proportions are reported in Table 19 and Appendix XIX; expected transition counts and percentages are reported in Appendix XIX. Analysis indicated no significant differences for any of the treatment groups, including the positive control EE2 0.05 ppm group, when compared to the cycle stage transitions of the control animals.

Comparison of estrus cycle length between the control and oxybenzone treatment groups also revealed no significant trend or differences in pairwise comparisons. Estrus cycle length averaged 4.83 ± 0.23 days in controls animals and 5.22 ± 0.25 days in OXY 30,000 ppm treated animals (p=0.624; Table 20 and Appendix XIX). Estrus cycle length was defined from the first day of estrus in a sequence of stages until the first day of estrus in the following sequence. Cycles were considered censored if the last stage was either diestrus or proestrus; analysis was only performed using non-censored cycles.

Reproductive and Pregnancy Parameters

The results of exposure to oxybenzone on reproductive and pregnancy related parameters are reported in Tables 21-23 and Appendices XX and XXI. For determination of the number mated, females were included in the analysis, if pregnant, regardless of negative vaginal sperm smear evaluations (VSSEs). For time to mating analysis, TPA, Inc. observations by Study Pathologists regarding GD at sacrifice were used to calculate a mating date; time to mating, however, could not be determined for two dams and they were excluded from analysis (Appendix XX).

Treatment with dietary oxybenzone for at least two weeks prior to mating had no effect on the overall number of females that mated per treatment group. Mating percentages ranged from a low of 80.0% for control animals to a high of 100.0% for the OXY 10,000 ppm treatment group, while the positive control EE2 group had a mating percentage of 88.0% (Table 21 and Appendix XX). There were also no significant differences noted in the time to mating determinations between the treatment groups. The median time to mating for control animals was 4.5 days (95% CI: 3.0-8.0) compared to a median of 5.0 days (95% CI: 3.0-6.0) for the OXY 30,000 ppm dose group. Those in the positive control EE2 0.05 ppm treatment group had a slightly longer median time to mating at 6.0 days (95% CI: 4.0-9.0), but this was not significantly different from controls.

Analysis of pregnancy rates which included all females that were pregnant regardless of VSSE results, ranged from 64.0% to 96.0% (Table 22 and Appendix XXI). Pregnancy rates for the control animals were the lowest at 64% while those for the OXY 10,000 ppm group were the highest at 96.0%. Females in the OXY 30,000 ppm dose group had a pregnancy rate of 84.0%. In pairwise comparisons, the percentage of pregnant females in the OXY 10,000 ppm treatment group

was statistically significant (p = 0.042) when compared to the controls; however, while statistically significant the result is not considered to be a direct result of oxybenzone treatment as the pregnancy rate for control females is lower than historical control values for reproductive toxicity studies previously estimated at approximately 85% (Parker, 2006) influencing the statistical analysis.

Analysis of gravid uterine weights was conducted only on those animals with a known or determined mating date (GD 0). Pregnant females were also excluded from the analysis if dosing continued past the GD 6 protocol specified stop date. Analysis of gravid uterine weights, which included the weights of the right ovary, were not statistically significant when analyzed for treatment effect using contrasts within a one-way ANOVA. Additionally, no statistically significant trend or statistically significant differences in pairwise comparisons were observed between control and oxybenzone treated females. However, the gravid uterine weights of females in the EE2 0.05 ppm treatment group were significantly lower than controls in pairwise comparisons with those in the positive control group weighing 17.8% less (p=0.030; Table 22 and Appendix XXI).

Counts of implantation sites, adjusted for the number of corpora lutea, and the number of resorption sites, adjusted for the number of implantations, were not statistically different between oxybenzone treatment and control groups. There was a significant covariate effect (p=0.008) for the number of corpora lutea (Appendix XXI) in the analysis of implants. For females that mated, but did not appear pregnant, the uterus was stained with ammonium sulfide to detect whether any implantation sites were present. Of the resorptions detected, all were classified as early resorptions. No late resorptions or dead fetuses were observed on study. Excluded from the analysis of resorptions were females with unknown/undetermined mating dates or those dosed past the protocol specified end date; analysis of implantations only included those animals that did not litter.

Summary statistics for the number of corpora lutea, the number of live fetuses, the percentage of pre-implantation loss and the percentage of post-implantation loss are reported in Table 23 and Appendix XXI.

Sperm Parameters

Results of the sperm analyses are presented in Tables 24-27 and Appendix XXII. Of 50 randomly selected males, six were excluded from analyses due to undetermined mating dates and were continued to be fed dosed chow past the GD 6 stop date specified in the protocol (see Appendix XXII for a complete list of exclusions).

There were no statistically significant trends or differences for any treatment group when compared to controls for the following sperm parameters: percent motility, cauda epididymal sperm counts and testis spermatid head counts (Tables 24, 25 and 26, respectively and Appendix XXII). One animal in the control group and one in the OXY 10,000 ppm treatment group were noted as having very low motility (5% and 8%, respectively), and because of the very low motility a nonparametric analysis was also conducted. However, there was no difference in the conclusions when the second analysis was performed (data not shown).

In the analysis of sperm morphology, no head abnormalities were reported in any of the treatment groups. For sperm tail abnormalities, equal to the total number of observed abnormalities, the test of trend was not significant and there were no statistically significant differences between control and oxybenzone or control and EE2 treatment groups (Table 28 and Appendix XXII).

Hematology and Clinical Chemistry Parameters – Females and Males

A total of 10 females and 10 males from each treatment group were randomly selected for hematological and clinical chemistry analysis. The selected males were also used in the analysis of sperm parameters (discussed above) and hormone levels. Two females in the control group were excluded from the final data analysis; one female had an undetermined mating date (GD 0) and the other remained on dosed chow past the protocol specified end date (GD 6). Six males (one in the control, two in the OXY 10,000 ppm and three in the OXY 30,000 ppm group) were also excluded from the final data analysis. Each of the males had undetermined mating dates which resulted in dosing continuing past the protocol specified end date (see Appendix XXIII for a complete list of exclusions for females and males).

Each animal selected for hematology and clinical chemistry analysis was sacrificed by 10:30 AM on GD 15 of pregnancy for females and after GD 6 for males (sacrifice of the males was dependent on TPA, Inc. availability). An ANOVA was performed using a nonparametric method with midranks and an unstructured covariance (Brunner et al., 2002) to determine whether oxybenzone or EE2 treatment had any effect on the hematological parameters listed in Table 28 for females and Table 29 for males. ANOVA results indicated a significant treatment effect on the following hematological measurements in females: the portion of NEU, the portion and absolute count of eosinophils (EOS), the absolute count of lymphocytes (LYM) and red blood cells (RBC), and on the concentration of HGB. For males, ANOVA analysis indicated a significant treatment effect on HGB concentration, which was also observed for females, and on the proportion (by volume) of blood that consists of RBCs defined as the HCT, the mean corpuscular volume (MCV) and PCV.

In pairwise comparisons of the female oxybenzone treatment groups to controls, however, only a significant difference in the portion of NEU (p=0.003) was observed between the OXY 30,000 ppm and control groups with the mean treatment rank proportion of NEU, from nonparametric analysis of ranked data, being 55.9% lower in the oxybenzone group relative to mean controls (Appendix XXIII). Analysis of trend was also significant for the proportion of NEU (p=0.007). There were no other significant differences for hematological parameters in trends or in pairwise comparisons between females in the oxybenzone treatment groups and controls. There were, however, statistically significant differences in pairwise comparisons between the control and EE2 treatment groups in the counts of RBC (p=0.027) and HGB concentration (p=0.007). In each instance levels for the EE2 0.05 ppm reference estrogen group were higher than those of the control group. For RBC, treatment mean ranks were 70.7% higher than in controls; treatment mean ranks for HGB concentrations were 106.6% higher in the EE2 treatment group compared to control levels (Appendix XXIII).

For males, a significant trend was noted for RBC numbers, HGB concentration, and HCT and PCV measurements (p=0.027, p=0.005, p= 0.001 and p =0.001, respectively). While the trend was significant for RBC numbers, there were no significant differences in pairwise comparisons between control and oxybenzone treatment groups. However, significant differences in pairwise comparisons to controls for HGB, HCT, MCV and PCV levels were observed. HGB, HCT and

PCV levels were significantly lower in the OXY 30,000 ppm treatment group when compared to controls. HGB treatment mean rank levels were lower by 58.7% (p=0.013), HCT treatment mean rank levels by 62.9% (p=0.005) and PCV treatment mean rank levels by 61.9% (p=0.004) in the OXY 30,000 ppm treatment group. There was also a significant difference observed in MCV measurements between the control and OXY 3,000 ppm treatment group with a 55.7% (p=0.0003) reduction reported in the treatment mean ranks for the OXY 3,000 ppm treatment group (Appendix XXIII).

There were no significant differences noted in pairwise comparisons of the EE2 0.05 ppm treatment group to controls for any of the hematological parameters measured.

In the analysis of clinical chemistry parameters, ANOVA indicated significant treatment effects on the measurements of SDH, cholesterol (CHOL) and GLU in females (Table 30 and Appendix XXIII) and on total bile acids (TBA), alanine aminotransferase (ALT), AST, CHOL and total protein (TP) levels in males (Table 31 and Appendix XXIII).

Pairwise comparisons of clinical chemistry parameters in the females revealed significant differences in SDH levels in the OXY 3,000 and OXY 30,000 ppm treatment groups relative to controls (p=0.003 and p=0.002, respectively). In each instance the treatment mean ranks for SDH were lower relative to controls by over 54%. In addition to SDH, levels of AST, CK and GLU were significantly different between pregnant females in the OXY 30,000 ppm treatment group and control group (p=0.032, p=0.038 and p=0.002, respectively). OXY 30,000 ppm treatment group mean ranks for AST and CK were each 44.7% lower relative to controls while levels of GLU were 98.2% higher. Analysis of trends also indicated significant dose trends for SDH (p=0.010), AST (p=0.031), CK (p=0.022), creatinine (CREAT; p=0.007) and GLU (p=0.005) (Table 30 and Appendix XXIII).

Pairwise comparisons of clinical chemistry parameters also indicated a significant difference between females in the control and reference estrogen group. EE2 0.05 ppm dosed females had elevated CHOL treatment mean rank levels that were 135.8% higher than controls (p=<0.001; Appendix XXIII). An increase in CHOL was also observed in response to oxybenzone treatment

with treatment mean rank levels 61.8% higher in the OXY 30,000 ppm treatment group relative to controls. However, the increase in the OXY 30,000 ppm group was not statistically significant in pairwise comparisons (p=0.239), and there was no significant trend observed (p=0.072).

In the analysis of clinical chemistry parameters in males, a significant trend was indicated in TBA, AST, CHOL and TP measurements (p=0.001, p=0.016, p=0.033 and p=0.001, respectively; Table 31 and Appendix XXIII). However, in pairwise comparisons there were no significant differences for CHOL and TP levels. Similar to what was observed in females, treatment with oxybenzone tended to increase CHOL levels in a dose dependent manner. TP levels in males treated with oxybenzone were lower relative to controls at the OXY 3,000 ppm treatment level and were higher at the OXY 30,000 ppm levels. TP levels at the OXY 10,000 ppm dose were equal to that of controls (Appendix XXIII).

Significant differences in pairwise comparisons of AST levels were observed in males treated with 10,000 and 30,000 ppm of oxybenzone. AST mean rank levels were 47.0% (p=0.018) and 54.7% (p=0.018) lower, respectively in the OXY 10,000 and OXY 30,000 ppm treatment groups relative to controls. A 26.5% reduction in treatment mean ranks was observed in the OXY 3,000 ppm group, but was not statistically significant (Appendix XXIII). A significant trend was also noted for TBA levels; however, there were no significant differences in pairwise comparisons of control and oxybenzone treatment groups. TBA levels were reduced in the two lowest oxybenzone treatment groups relative to control levels and were increased relative to control levels at the highest oxybenzone dose. There was, however, a significant difference in pairwise comparisons between males in the control and the EE2 0.05 ppm treatment group. Mean ranks of TBA were 77.4% (p=0.007) higher upon EE2 treatment. An increase of 72.7% in treatment mean ranks was also observed for ALT levels in the EE2 treatment group (p=<0.001); this increase relative to control levels was also significant (p=<0.001; Appendix XXIII).

For hormone level analysis, measurements were made for testosterone, follicle stimulating hormone and luteinizing hormone. However, measurements for luteinizing hormone were above the limit of quantification of 0.1 ng/ml in only four animals; therefore, levels of luteinizing hormone were not included in the final data analysis. Analysis of testosterone and follicle

stimulating hormone levels indicated no significant dose trends or differences between the control, oxybenzone or EE2 treatment groups (Table 32 and Appendix XXIII).

Histopathology – Females and Males

In the analyses of pathology data, all female and male animals were included independent of dosing durations or pregnancy status. Histopathology on the males included the right testis, right epididymis, dorsolateral prostate, ventral prostate, seminal vesicles with coagulating glands, mammary glands, adrenal, pituitary and thyroid glands. For females, analysis included the mammary glands. Histopathological analysis was also performed on any gross lesions detected throughout the study.

The identified tissues/organs of females and males in the control group, the OXY 30,000 ppm dose group and the EE2 0.05 ppm positive control group were assessed. To serve as a reference for any gross lesions identified all tissues from one male and one female from each dose group were collected and processed to slides. Mammary gland whole mounts from both sexes were not histopathologically evaluated due to the thickness of the specimens. The opinion of the TPA, Inc. Study Pathologists was that assessment would result in a highly variable data set; therefore, the specimens were placed into individual heat-sealed clear plastic pouches containing methyl salicylate for long-term storage and archival.

One female in the control group was identified as having a mammary gland adenocarcinoma; no other neoplasms were identified in any of the treated animals on study. Nonneoplastic lesions with an incidence of at least two in a targeted dose group were analyzed for their incidence and severity profile (lesions graded as minimal/mild/moderate/marked). Analysis of the mammary glands in females revealed two instances of alveolus hyperplasia in the OXY 30,000 ppm treatment group. However, the incidence and the severity profiles of the lesions were not statistically different from controls (Table 33, Appendix XI and XXIV). Analysis of the mammary glands in males also revealed an increase in alveolus hyperplasia with males in the OXY 3,000 and OXY 10,000 ppm treatment groups having a higher incidence than controls. Incidence levels in the OXY 30,000 ppm treatment group, however, were not increased over the incidence levels observed in controls. Although the lower two oxybenzone dose groups had increased incidence levels they were not

statistically different from controls in pairwise comparisons suggesting the findings are sporadic with little to no biological significance (Table 34, Appendix XXIV). There was, however, a statistically significant difference in the severity of the lesions identified in the OXY 3,000 ppm dose group compared to control males (p=0.045; Table 34 and Appendix XI and XXIV).

An increase in mammary gland alveolus hyperplasia was also observed in males treated with 0.05 ppm of EE2. Ten males (40.0%) in the EE2 0.05 ppm dose group were identified as having alveolus hyperplasia compared to only two control males (8.0%). Statistical analysis indicated this difference was significant in pairwise comparison to controls (p=0.009); the analysis of trend was also significant (p=0.010; Table 36 and Appendix XXIV). The severity profile of the lesions were also statistically significant (p=0.005) with half being graded as minimal and the other half as mild.

Aside from the mammary gland alveolus hyperplasia, there was one incidence of a cyst located on the distal portion (pars distalis) of the anterior pituitary gland in an OXY 30,000 ppm male. However, two incidences were also noted in control animals (Table 35 and Appendix XI and XXIV). A non-significant increase in the incidence of ultimobranchial cysts of the thyroid gland were also noted in high dose oxybenzone males (Table 35 and Appendix XI and XXIV). Treated males had a 20.0% increase in incidence compared to controls (7/25 versus 2/25, respectively), and while the increase in the incidence was not significant, there was a statistically significant difference in the severity profiles of lesions identified in the OXY 30,000 ppm dose group (p=0.030). An increased incidence of ultimobranchial cysts were also noted in the EE2 0.05 ppm dose group. Similar to what was observed with the OXY 30,000 ppm dose group males, the total number of incidences was not significant, but the severity of the lesions were significant in pairwise comparison to controls (p=0.032; Table 36 and Appendix XI and XXIV).

TPA, Inc. Study Pathologists also noted in the Pathology Report (Appendix XI) kidney changes in the OXY 30,000 ppm treatment group males. Kidney changes were characterized by the presence of protein casts, dilated renal tubules, regeneration of renal tubules and inflammation. However, due to the low numbers of tissues assessed histopathologically (n=1 for controls, OXY 3,000 ppm and OXY 10,000 ppm and n=7 for OXY 30,000 ppm) the results were not analyzed.

However, these types of changes in the kidney have been well documented in previous studies with oxybenzone (French, 1992).

DISCUSSION

Oxybenzone is an organic ultra-violet absorbing compound that is widely used in commercial personal care products because of its ability to block both UVA and UVB radiation (Klein, 1992). It is commonly incorporated into sunscreens and sunscreen containing products, but is also found in nail polishes, lipsticks and perfumes (EWG; http://www.ewg.org/skindeep). Oxybenzone is a sunscreen active ingredient in the U.S. FDA's sunscreen monograph 21 CFR 352.10 (CFR, 2013b). It is also a common component of plastics and has been approved by the FDA as an indirect food additive (21 CFR 177.1010) (CFR, 2013a). There has been heightened interest in the biological activity of oxybenzone due to its high volume of use, detection in the urine of a large percentage of the U.S. population (Calafat et al., 2008; Centers for Disease Control and Prevention, 2015) and numerous *in vitro* (Schlumpf et al., 2001; Nakagawa and Suzuki., 2002; Wada et al., 2004; Suzuki et al., 2005; Kunz et al., 2006) and *in vivo* (French, 1992; Datson et al., 1993; Schlumpf et al., 2001; Schlecht et al., 2004; Suzuki et al., 2005; Kunz et al., 2006; Coronado et al., 2008) studies indicating endocrine disruptor activity. The present study was designed to examine the effects of oxybenzone exposure on fertility and early embryonic development to implantation (GD 6) and was conducted using the recommendations in ICH guideline S5(R2) 4.1.1 (ICH, 1993).

A dose finding study was previously conducted to gather preliminary data on the effects of oxybenzone exposure to pregnant females and their offspring (Nakamura et al., 2015). The results of that study were used to determine the dosing levels in the current study and approximate a relevant exposure level to humans. In the preliminary study, pregnant dams and their offspring were dosed with up to 50,000 ppm of oxybenzone from implantation (GD 6) until weaning on PND 23. The LOAEL for reproductive function and developmental effects was determined to be 25,000 ppm of oxybenzone; minimal effects on liver enzyme levels and/or liver/body weight ratios, however, were observed at 10,000 ppm for PND 23 dams and female offspring, respectively (Nakamura et al., 2015). Serum levels of oxybenzone and its three metabolites (DHB, THB and DHMB) were measured at the time of sacrifice in the dams. Oxybenzone and DHB levels were found to increase in a dose-dependent manner while levels of THB and DHMB were below the limit of detection (LOD for THB 0.05 to 0.1 µg/ml; LOD for DHMB 0.005 µg/ml). The method of Reagan-Shaw et al. (2008), which utilizes a body surface area normalization method, was then used to calculate an approximate human equivalent dose (HED) for comparison of human and

animal levels. Based on comparisons of the plasma levels in the rat to dermal doses used by either Janjua et al. (2008) (2 mg/cm^2) or Wang et al. (2011) (1 mg/cm^2) it appears that the OXY 10,000 ppm dose used in the dose finding study was within five fold or was twofold higher, respectively, of what might be considered a typical human dose. All higher doses (>10,000 ppm) could be considered in excess of typical human exposures (Nakamura et al., 2015).

Prior to the current study there was limited and conflicting information regarding the effects of oxybenzone on reproduction and fertility in animal models (NTP, 1990, 1991; French, 1992; Datson et al., 1993; Nakamura et al., 2015). The results of the present study indicate that exposure to oxybenzone for at least 10 weeks for males and for at least two weeks for females did not adversely affect fertility or reproduction in Sprague-Dawley (Harlan) rats at doses that could be considered equivalent or in excess of typical human exposures. Estrus cycle evaluations which included the proportion of days spent in each estrus stage, the proportion of females with abnormal cycles and differences in cycling transitions revealed no significant differences in pairwise comparisons to controls. The estrus cycle length in the OXY 30,000 ppm females was slightly longer (5.22 $\pm$ 0.25) than that of controls (4.83 $\pm$ 0.23) or EE2 0.05 ppm positive control (5.04 $\pm$ 0.24) animals (Table 20); however, the result was not statistically significant in pairwise comparisons. There were also no significant differences in the analysis of sperm parameters in males. Analyses included testicular spermatid head counts and cauda epididymal sperm counts along with assessment of morphology and motility. There were also no differences in follicle stimulating hormone levels in males; testosterone levels increased in a dose-dependent manner (1.9 $\pm$ 0.4 ng/ml in controls versus 4.5 $\pm$ 2.2 ng/ml in OXY 30,000 ppm group; Table 32 and Appendix XXII). However, this increase was not significant in pairwise comparisons and further analysis of measurements from individual animals indicates that the observed dose-dependent increase can be attributed to only one or two animals within each treatment group that had significantly higher levels.

Evaluation of reproductive organs/tissues also revealed few effects upon exposure to oxybenzone. Pairwise comparisons of adrenal gland weights and ovarian weights in females revealed significant differences in the weights of the adrenal glands with an 8.6% increase (p = 0.042) observed between OXY 3,000 ppm females and controls while ovarian weights were significantly increased

(12.1%; p = 0.016) between OXY 10,000 ppm females and controls. However, both findings were considered sporadic and unrelated to dose. For males, a significant difference in the paired weight of the seminal vesicles was observed between controls and the OXY 10,000 ppm dose group with those treated with oxybenzone having a 12.4% increase in seminal vesicles weights (p = 0.049). However, similar to the findings in female reproductive organ weights, this observation was not considered related to dose as weights were equivalent to or slightly elevated in the OXY 3,000 and OXY 30,000 ppm treatment groups, respectively, when compared to controls. Significant trends were noted for the ventral prostate (p = 0.031) and paired testes (p = 0.029) with weights of the testes increasing with the highest dose (3.3%) while those of the ventral prostate were decreased (13.8%); no significant difference was observed in pairwise comparisons though (Table 16). In males, histopathological analysis of the mammary glands indicated an increased incidence of alveolus hyperplasia in the two lowest oxybenzone dose groups (OXY 3,000 and OXY 10,000 ppm). The increased incidence, however, was not statistically different from controls (Table 34); incidence in the highest OXY 30,000 ppm dose group was at a level equivalent to controls suggesting the increase observed at the two lowest doses were not directly related to treatment. There was also a 20% increase in the incidence of ultimobranchial thyroid gland cysts in the OXY 30,000 ppm dose group when compared to controls (Table 35). Again, the increase in incidence was not significant, but there was a difference in the severity profile between control and treated animals. A similar observation of thyroid gland ultimobranchial cysts was also noted in the EE2 0.05 ppm positive control group (Table 36). The incidence of ultimobranchial cysts in the Sprague-Dawley strain of rats has previously been documented and ranged from a high of approximately 37% in eight week old male animals to 11% in animals at 108 weeks of age (Rao-Rupanagudi et al., 1992); therefore, the increase observed in the present study is not considered treatment related. While the severity profile of the thyroid cysts was statistically significant, all observations were still considered minimal to mild with no reports of moderate or marked occurrences.

There were no observed differences in the reproductive or pregnancy parameters assessed in the current study. Parameters assessed included the number of animals that mated, time to mating, gravid uterine weights, number of implants and the number of resorptions. Poisson regression analysis of implantation sites with terms for treatment and covariate number of corpora lutea did

indicate a significant covariate effect (p = 0.008). Summary statistics of the mean corpora lutea numbers indicate a decrease in the number of corpora lutea in the OXY 30,000 ppm treatment group (14.1 $\pm$ 0.4) versus controls (16.7 $\pm$ 0.4) (Table 23 and Appendix XXI). There was a statistically significant increase in the percentage of animals that were pregnant in the OXY 10,000 ppm treatment group when compared to the controls (Table 22). However, the increase in the number of females that were pregnant in this group was not considered treatment related as the pregnancy rates for control females was lower than previously estimated historical control values for reproductive toxicity studies (Parker, 2006) and may have influenced statistical significance.

Overall, the results from the present study are in general agreement with that reported previously for oral toxicity studies of oxybenzone where limited numbers of reproductive parameters were evaluated. Previous studies in F344/N rats and B6C3F$_1$ mice reported increased estrus cycle lengths and lower epididymal sperm counts only when animals were exposed to 50,000 ppm of oxybenzone in feed (French, 1992). Decreased testicular and epididymal weights were also reported in rats while an increased incidence of abnormal sperm were reported in mice. No effects on the reproductive system, however, were noted at lower levels of exposure (<50,000 ppm). Effects on reproduction have also previously been noted in a 13-week dermal application study of oxybenzone conducted in B6C3F$_1$ mice (French, 1992). However, a similar study using the same strain of mice reported no effect when oxybenzone was applied up to a two-fold higher concentration (Datson et al., 1993). There was also no reproductive toxicity effects observed in a 13-week dermal study in rats that received up to 200 mg/kg body weight/day of oxybenzone (French, 1992).

A continuous breeding study was also previously performed in CD1 mice to assess potential effects of oxybenzone on reproduction and fertility as well as on developmental endpoints (NTP, 1991). Groups of 20 mice per sex were fed oxybenzone up to 50,000 ppm for one week prior to mating and continuing for another 21 weeks. Mice were allowed to produce a total of five litters. No effect on sperm density or morphology was observed in treated males; estrus cycle lengths in females were also not affected. There was, however, a decrease in dam weights and effects on total litter numbers and the number of live pups per litter reported with increasing dose. However, these effects were only observed at exaggerated doses which exceed normal human exposures.

Emerging evidence, however, has linked oxybenzone, its metabolite DHB and the benzophenone derivative 2,2',4,4'-OH BP to reproductive/fertility problems in humans. A 2012 report by Kunisue et al. linked exposure to DHB to an increased risk of endometriosis, a gynecological condition defined as an estrogen dependent disease (Giudice and Kao, 2004). A significant trend was noted for DHB levels and the odds of an endometriosis diagnosis in a cohort of 473 women who underwent laparoscopy/laparotomy; a similar trend was noted in the population cohort that underwent pelvic magnetic resonance imaging (n=127), but the results were not significant in this cohort. The authors note, however, the lack of significance in the population cohort may have been due to the limited number of women diagnosed with endometriosis (n=14; Kunisue et al., 2012). In a separate analysis, using the same data set, which compared women in the highest quartile in regards to DHB concentration to those in lower quartiles, revealed an approximate 65% increase in the odds of an endometriosis diagnosis (Kunisue et al., 2012). Interestingly, previous studies have shown the metabolite DHB to have increased estrogenic activity over that of the parent compound oxybenzone (Morohoshi et al., 2005; Suzuki et al., 2005). A similar study by Pollack and colleagues (2015) investigated a possible association of oxybenzone and/or DHB to a diagnosis of uterine fibroids, which are classified as estrogen sensitive tumors of the myometrium (Hodges et al., 2000). While women with a surgical diagnosis of uterine fibroids in general had higher urinary levels of oxybenzone and DHB, the association did not confer elevated odds ratios (Pollack et al., 2015).

A recent study also investigated the association between five different UV filters, including oxybenzone, and possible effects on fecundity in both men and women (Buck Louis et al., 2014). In this study the authors assessed urinary concentration levels of oxybenzone, DHB, 2,2',4,4'-OH BP, DHMB and 4-OH BP; participants completed daily journals which included data on lifestyle factors, sexual intercourse, menstruation and pregnancy testing. Couples remained on study until they achieved pregnancy or until they had tried to conceive for 12 months. Results indicate that some UV-filters may be associated with an increased time to pregnancy with males being more sensitive than females to exposures. Specifically, males exposed to 2,2',4,4'-OH BP and 4-OH BP showed a 31% and 26% reduction in fecundity, respectively (Buck Louis et al., 2014). In an analysis using joint-exposure models, 2,2'4,4'-OH BP levels in males remained associated with

longer times to pregnancy. 2,2',4,4,-OH BP has also previously been shown to have estrogenic activity in addition to oxybenzone and its primary metabolites (Suzuki et al., 2005). Sex specific differences have also previously been noted for oxybenzone exposure in mice and rats (Wegerski et al., 2011); however, no sex specific differences were noted in the present study on fertility or reproductive parameters.

Overall, very few effects were observed in response to oxybenzone treatment on fertility or reproductive parameters in Sprague-Dawley rats at levels that could be considered equivalent to human exposures ($\leq$10,000 ppm). The most significant toxicological findings in this study can be attributed to the overt toxicity of oxybenzone and include effects on body, liver and kidney weights. Similar observations, using various study designs and treatment regimes, have previously been documented (Lewerenz et al., 1972; Christian, 1983; French, 1992; Nakamura et al., 2015). In the present study, oxybenzone exposure resulted in minimal decreases in female body weights at 10,000 and 30,000 ppm on pre-mating treatment day 5 (2.8% and 4.5%, respectively); similar levels of reductions were also observed for the same two treatment groups at day 9. On pre-mating treatment day 12 only the reduction in body weight in the OXY 30,000 ppm treatment group was considered statistically significant (5.9%) (Figure 1 and Table 2). Minimal reductions in gestational body weights of females were also observed. Females in the OXY 30,000 ppm treatment group were primarily affected with the largest decrease of 7.6% observed on GD 6; however, a decrease at GD 15 (2.9%) and an overall decrease in gestational body weights (GD 0-15) (2.5%) were noted for females in the OXY 10,000 ppm treatment group as well. While reductions were minimal and are expected to have no overall biological effect, they were statistically significant (Figure 3 and Table 8). For males, pre-mating and post-mating body weight reductions were restricted to males given the highest dose of oxybenzone and averaged 6.3% for the pre-mating period and 9.4% from GDs 0-6 (Figure 2 and Tables 3 and 9, respectively).

A reduction in body weights in both the female and male rats was not attributed to reduced food consumption which had been previously reported by French in a 1992 study. Animals on the present study consumed equivalent amounts of dosed chow or consumed more chow at specific times. An increase in food consumption was noted for females on pre-mating treatment days 6-9 (Table 4) and GDs 0-6 and overall (GDs 0-15) in the OXY 30,000 ppm treatment group (Table 10); increases were also noted for males in the OXY 30,000 ppm group during pre-mating

treatment week 3 (Table 5). Significant increases in trend were also noted for both females and males. However, it should be noted that while food consumption did increase at select time points, we cannot rule out possible palatability issues with the highest dose level that may have led to increased spillage. As an example, female daily food consumption levels were increased by 43.2% over controls from GDs 0-6; however, when the animals were returned to control chow there was no significant difference in food consumption levels (Table 10).

As mentioned previously, the liver and kidneys have been shown to be target organs for oxybenzone toxicity. Consistent findings from both the dosed-feed and dermal application studies of oxybenzone sponsored by the NTP were increases in liver and kidney weights (French, 1992). In the current study, a significant increase in the trend of liver weights was noted for females given oxybenzone; no significant difference, however, was observed in paired kidney weights (Table 13). For males, significant differences in pairwise comparisons were noted in the liver and paired kidney weights for males in the OXY 10,000 and OXY 30,000 ppm dose groups when compared to controls (Table 14). The differences observed in the liver and paired kidney weights between males and females in the current study may in part be due to length of treatment. Males were dosed eight weeks longer than females with oxybenzone and, therefore, this might be a contributing factor to the more severe organ toxicity observed in males. Sex-specific differences, however, cannot be excluded as a potential contributing factor as well. Previously reported findings of renal toxicity show an increase in severity in males compared to females (French, 1992). Strain differences or combinations of strain and sex differences may, however, account for the observed variances as well. Very limited histopathological analysis was performed on the liver and kidneys in the present study. TPA, Inc. Study Pathologists noted an increase in the number of kidney lesions in the male high dose group at sacrifice (Appendix XI). Microscopic analysis of the kidneys of seven males in the high dose group were characterized by protein casts, dilated renal tubules, regeneration of renal tubules and inflammation. However, due to the very limited number of animals assessed (n=1 control group; n=7 for OXY 30,000 ppm) statistical analysis of kidney histopathology was not performed.

A number of hematological and clinical chemistry parameters were also impacted by treatment with oxybenzone in the current study. For females, the only statistically significant difference in the hematological parameters measured was in the percentage of NEU. Females in the OXY

30,000 ppm dose group overall had a lower percentage of NEU than controls. A significant decrease in trend with increasing oxybenzone concentration was also noted (Table 26). For males, significant differences in pairwise comparisons were observed for HGB concentration levels, the HCT and PCV. Levels of all three were lower in the OXY 30,000 ppm males relative to levels in control animals. The trends of all three parameters, in addition to that of RBC, were also considered significant. In a previous study sponsored by the NTP the hematologic change that was considered relevant was a mild to moderate increase in platelet counts (French, 1992). The increase in platelets was observed at various time points in male rats and in dosed females at 12 weeks. While platelet counts in the current study did tend to increase with increasing doses of oxybenzone, there were no significant differences or trends noted (Tables 28 and 29). Interestingly, the statistically significant changes noted in the hematological profile of the male rats in the OXY 30,000 ppm dose group in this study are similar to the hematological parameters affected when pregnant female rats were given 30,000 ppm of oxybenzone from GD 6 to GD 15 (NCTR E02187.01 Technical Report, GLP, unpublished). However, in that study there was a significant increase in RBC and in PCV along with platelet counts while HGB and HCT values decreased. It is unclear as to why similar hematological parameters or reductions were not observed on the current study in the pregnant females when the animals were dosed for longer periods of time, but may in part be related to the relatively small sample sizes used in the analysis (n=10 or less). Another possibility is that the changes observed in the hematological parameters are linked to changes observed in the kidney as suggested previously (French, 1992). In the present study, no significant differences in the trend or pairwise comparisons were noted in the paired kidney weights of female animals while significant differences were noted for males in the OXY 10,000 and OXY 30,000 ppm treatment groups. Histopathology on the kidneys was limited in both sexes to organs identified as having gross lesions. No gross lesions were noted in the treated females while limited observations (n=7 for OXY 30,000 ppm) showed kidney damage in treated males.

Analysis of clinical chemistry parameters in oxybenzone exposed females also indicated significant differences in a number of parameters. Again, the OXY 30,000 ppm treatment group was the dose group primarily impacted. The levels of SDH, AST and CK were lower while GLU levels were higher. A significant reduction in SDH levels was also observed in females given 3,000 ppm of oxybenzone (Table 30). The only significant differences noted in pairwise

comparisons in clinical chemistry parameters of male animals was that of AST levels. Levels were lower in males given 10,000 and 30,000 ppm of oxybenzone (Table 31). Significant trends were noted for TBA, CHOL and TP levels. Previously, mild increases in ALT levels were reported after a three day exposure to oxybenzone in male and female F344/N rats (French, 1992). A slight, but non-significant, increase in ALT levels was noted for females in the OXY 30,000 ppm group in the current study, while increasing oxybenzone concentrations resulted in lower ALT levels in males (Tables 30 and 31). French (1992) also reported significantly higher SDH levels in females after 12 weeks of treatment; however, in the current study significant reductions in pairwise comparisons were noted for females in the OXY 3,000 and OXY 30,000 ppm treatment groups. Interestingly, evaluations of clinical chemistry parameters in the current study are not indicative of hepatotoxicity or renal toxicity as was indicated by changes in organ weights and in limited histopathological evaluations. It is possible that clinical chemistry markers for liver and kidney damage were elevated at initial exposure to oxybenzone and at the time of sacrifice had returned to levels at or below control levels, indicating that perhaps some of the observed organ toxicity is reversible and may explain the differences observed in the hematology and clinical chemistry parameters analyzed. It was noted in the previous study by French (1992) that hepatocyte vacuolization and enlarged livers where more severe in two week mouse and rat studies than in 13-week studies.

In the current study exposure to oxybenzone at levels considered equivalent to typical human exposures ($\leq$10,000 ppm) had no effect on fertility and early embryonic development. Overall, effects were primarily limited to animals exposed to 30,000 ppm of oxybenzone which is considered in excess of typical human exposure levels based on the serum levels of oxybenzone determined in a developmental dose finding study (Nakamura et al., 2015).

Summary

Oxybenzone exposure for at least two weeks prior to mating for females and at least 10 weeks prior to mating for males had no effects on fertility or early embryonic development in Sprague-Dawley rats at exposures considered equivalent to typical human exposure levels ($\leq$10,000 ppm). There were no significant differences related to oxybenzone exposure on the percentage of animals that mated, the time to mating, the percentage of pregnant females or on the number of

implantations and resorptions. Oxybenzone exposure did not affect estrus cycles in females or sperm parameters and hormone levels in males. Exposure to oxybenzone did, however, lead to minimal decreases in female (OXY 10,000 and 30,000) and male (OXY 30,000) body weights and affected various organ weights, organ histopathology, hematology and clinical chemistry parameters primarily in the highest dose group.

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NATIONAL CENTER FOR TOXICOLOGICAL RESEARCH
QUALITY ASSURANCE STATEMENT

Title of study: E02186
Effect of oxybenzone on fertility and early embryonic development in Sprague-Dawley rats –
Segment I

Study Director: Amy L. Inselman, Ph.D.

This study was audited and inspected by the National Center for Toxicological Research (NCTR)
Quality Assurance Unit (QAU) located in Jefferson, Arkansas. The NCTR Study Director and
designated Management were informed of the results of each inspection.

Phase	Date(s) of Inspection	Date Reported to the Study Director and Test Facility Management
Protocol	3/09-21/2012	3/21/2012
Amendment #1	1/14/2013	1/14/2013
Diet Preparation Inspection	1/23/2014	1/23/201
Dose Analysis Inspection	1/23/2013	1/23/2013
Animal Identification	3/08/2013	3/14/2013
Vaginal Cytology Data	12/16/13-1/17/14	1/17/2014
Sperm Analysis Data	1/17-1/21/2014	1/21/2014
Clinical Pathology Data	1/24-2/03/2014	2/03/2014
Draft Pathology Report	6/25-6/30/2014	6/30/2014
Amendment #2	8/26/2014	8/26/2014
Pathology Report	10/15-10/24/2014	10/24/2014
Draft Diet Preparation Final Report	11/17/14-1/14/15	1/14/2015
Analytical Chemistry Support Summary Report	3/18-5/01/2015	5/01/2015
Animal Room Temperature and Relative Humidity Reports	8/19/2015	8/19/2015
Clinical Pathology Methods	9/04/2015	9/04/2015
Microbiology Surveillance/Diagnostic Summary Report	9/18-9/25/2015	9/25/2015
Microbiology Surveillance/Diagnostic Summary Report	10/06-10/09/2015	10/09/2015
Statistical Reports	10/09-11/04/2015	11/04/2015
Technical Report	4/01-4/22/2016	4/22/2016
Final Technical Report Change Verification	6/22/2016	6/23/2016

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6/23/2016

**National Center for Toxicological Research
Quality Assurance Unit**

Date