APPENDIX XX

Statistical Analysis of Reproductive Parameters

Statistical Report

Project #: E02186.01
Project Title: Effect of oxybenzone on fertility and early embryonic development in Sprague-Dawley rats (Segment I)
PI: Amy Inselman

Task: Statistical Analysis of Reproductive Parameters
Statistician: Beth Juliar, Division of Bioinformatics and Biostatistics
Reviewer: Paul Felton, Division of Bioinformatics and Biostatistics

Signatures:

[redacted]

Statistician Date

[redacted]

Reviewer Date

[redacted]

Team Leader – Statistical Support Group Date

Statistical Analysis of Reproductive Parameters

1. Objectives

1.1 Project Objectives

The objective of the study is to examine the reproductive toxicity of oxybenzone in male and female rats and is designed to focus specifically on fertility and early embryonic development to implantation [ICH Guideline S5(R2) 4.1.1]. An additional objective is to compare the results of a typical Segment I, II, III study design with results from a modified one-generation study proposed by the NTP.

1.2 Analysis Objectives

The goal of this analysis is to test the effect of oxybenzone on time to mating, a reproductive parameter.

2. Experimental Design

A total of 262 rats were to be requested for this study. Of this number 125 male rats were to be requested along with 125 female rats. Males were to be approximately 5-7 weeks old when delivered to the NCTR, and females were to be approximately 9-11 weeks of age when delivered. All males were to be delivered in one shipment, and all females were to be delivered in a separate shipment. After a two week quarantine period the animals were to be weighed and allocated to the study.

The test article in this study is 2-hydroxy-4-methoxybenzophenone (synonyms: HMB, benzophenone-3, oxybenzone). The animals were to be divided into five treatment groups with 25 male and 25 female rats assigned to each group. The treatment groups were to be four oxybenzone dose levels 0 ppm (control), 3000 ppm, 10,000 ppm, and 30,000 ppm and one estrogen ethinyl estradiol (EE_2) 0.05 ppm treatment.

Males were to be dosed for 10 weeks and females for approximately 2 weeks prior to randomization to breeding pairs. Dosing was to continue until gestational day (GD) 6 for all animals. From GD 6 to GD 15, dams were to receive control chow. All dams were to be sacrificed on GD 15; males were to be sacrificed soon after breeding (approximately GD 6).

All animals were to be housed in pairs in cages prior to breeding. For breeding, males and females were to be housed one male: one female for up to 15 days or until animals have mated. Males and females were to be housed individually upon indication of mating (GD 0) until the time of sacrifice.

3. Statistical Methods

Counts and percentages of mated and pregnant females in each treatment group were calculated. 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. The test for trend was performed for the oxybenzone and control treatments (excluding the EE_2 treatment). Tests were conducted as two-sided at the 0.05 significance level

4. Results

Tables are presented in Appendix A1 and figures are presented in Appendix A2.

For 125 paired females, counts and percentages of the observed data for mated and pregnant females in each treatment are given in Table 1. There were 12 pregnant females that did not have dates of mating based on sperm positive vaginal lavage or presence of plug (UIN=5A000002579, 5A000002580, 5A000002600, 5A000002607, 5A000002630, 5A000002636, 5A000002639, 5A000002648, 5A000002652, 5A000002653, 5A000002661, and 5A000002678). In addition, one female in the control group (UIN=5A000002671) was not monitored for evidence of mating until the 3rd day of pairing, but was plug positive by day 3.

Table 2 presents observations of study pathologists regarding littering and fetal development at sacrifice for dams without mating dates. In consultation with the pathologists and the Principle Investigator, pathology observations for 11 dams with missing dates of mating was used to estimate GD 0 and time to mating. For dams that littered, litter day was assumed to have been at GD 21. For dams with comments regarding GD of fetal development, time to mating was calculated using the study pathologists' observation. Time to mating was not estimated for 2 dams, the dam that was unmonitored for mating in the control group (UIN=5A000002671) and the dam with no mating date or pathology observation in the oxybenzone 30,000 ppm group (UIN=5A000002678).

Using pathology observations to calculate of time to mating for females with unobserved mating dates, data from 123 paired females were analyzed. The 2 females without mating dates or pathology observations were excluded from the analysis. There were 99 pregnant females of 123 paired females (80.5%), 10 mated females that were not pregnant (8.1%) and 14 females that were not mated and not pregnant (11.4%).

The mean and median time to mating for pregnant females (N=99) using pathology observations for females with missing mating dates are presented in Table 3.

The median time to mating for paired females (N=123), using the product-limit analysis method, is presented in Table 4. For each treatment, upper and lower 95% confidence limits for median time to mating, counts of mated and unmated females, and the percent censored are given. Females were considered censored at 14 days if there was no evidence of mating.

Results of the proportional hazards model analysis of time to mating are presented in Table 5 for paired females. The test of trend was not statistically significant and there were no significant differences for the dosed groups compared to the control group.

5. Conclusions

In the analysis of time to mating for paired females, there were no statistically significant differences for the dosed groups compared to the control group.

A1. Tables

Table 1. Counts and Percents for Mated (Observed) and Pregnant Females[1] from 125 Breeding Pairs

Treatment	*Mated*	*Pregnant*	*Count*	*Percent*
CTRL	N	N	5	20.0
		Y	2	8.0
	Y	N	4	16.0
		Y	14	56.0
OXY 3,000	N	N	3	12.0
		Y	0	0.0
	Y	N	3	12.0
		Y	19	76.0
OXY 10,000	N	N	0	0.0
		Y	5	20.0
	Y	N	1	4.0
		Y	19	76.0
OXY 30,000	N	N	3	12.0
		Y	4	16.0
	Y	N	1	4.0
		Y	17	68.0
EE2 0.05	N	N	3	12.0
		Y	1	4.0
	Y	N	1	4.0
		Y	20	80.0

1. Twelve pregnant animals had unobserved mating dates; one additional pregnant animal was not monitored during the first 2 days of pairing but had mated by day 3 ("N" is no and "Y" is yes).

Table 2. Listing of Pregnant Females with Unobserved Mating[1]

Treatment	*UIN*	*Observation*
CTRL	5A000002607	pups noted as day 20
	5A000002639	pups noted as day GD19
OXY 10,000	5A000002579	animal littered
	5A000002630	animal littered
	5A000002636	pups noted as GD21
	5A000002648	pups noted as day 19
	5A000002653	pups noted as day 19
OXY 30,000	5A000002580	animal littered
	5A000002600	sacrificed early; missed VSSE; pups noted as day 16
	5A000002652	pups noted as day 18
	5A000002678	
EE2 0.05	5A000002661	pups noted as day 18

1. For analysis, observations by study pathologists were used to estimate mating date; data from one female was considered missing due to absence of observation.

Table 3. Time to Mating for Pregnant Females[1]					
Treatment	***N[2]***	***Mean[3]***	***SE***	***Median***	***95% CL***
CTRL	15	4.1	0.6	4.0	(2.0-5.0)
OXY 3,000	19	4.5	0.6	4.0	(2.0-6.0)
OXY 10,000	24	5.5	0.7	5.0	(3.0-7.0)
OXY 30,000	20	4.1	0.5	4.5	(2.0-5.0)
EE2 0.05	21	6.1	0.8	5.0	(3.0-8.0)

1. The product-limit method was used for estimation of time to mating.
2. Data were considered missing for one female with no mating data or pathology observation and for the female that was not monitored until the 3rd day of pairing.

Table 4. Time to Mating for Paired Females[1]						
Treatment	***Median***	***95% CL***	***N[2]***	***Mated***	***Not Mated***	***% Censored[3]***
CTRL	4.5	(3.0-8.0)	24	19	5	20.8
OXY 3,000	4.0	(2.0-6.0)	25	22	3	12.0
OXY 10,000	5.0	(4.0-7.0)	25	25	0	0.0
OXY 30,000	5.0	(3.0-6.0)	24	21	3	12.5
EE2 0.05	6.0	(4.0-9.0)	25	22	3	12.0

1. The product-limit method was used for estimation of time to mating.
2. Missing mating dates of pregnant females were estimated using pathology observations; data were excluded for the female with no observation and for the female that was not monitored until the 3rd day of pairing.
3. Females were considered censored at 14 days if there was no evidence of mating.

Table 5. Cox[1] Proportional Hazards Analysis of Time to Mating for Paired Females			
Treatment (ppm)	***Hazard Ratio[2]***	***P-value***	***Adjusted P-value[3]***
CTRL	-	0.651	-
OXY 3,000	1.307	0.393	1.000
OXY 10,000	1.410	0.262	1.000
OXY 30,000	1.274	0.445	1.000
EE2 0.05	0.938	0.838	1.000

1. A Cox proportional hazards model was used for the trend test (shown for control) and comparisons to control.
2. Hazard ratios are relative to the control.
3. Holm's adjusted p-value.
4. For analysis, missing mating dates of pregnant females were estimated using pathology observations; data were excluded for the female with no observation and for the female that was not monitored until the 3rd day of pairing.

A2. Figures

Figure 1. Kaplan-Meier Curve of Time to Mating for Pregnant Females

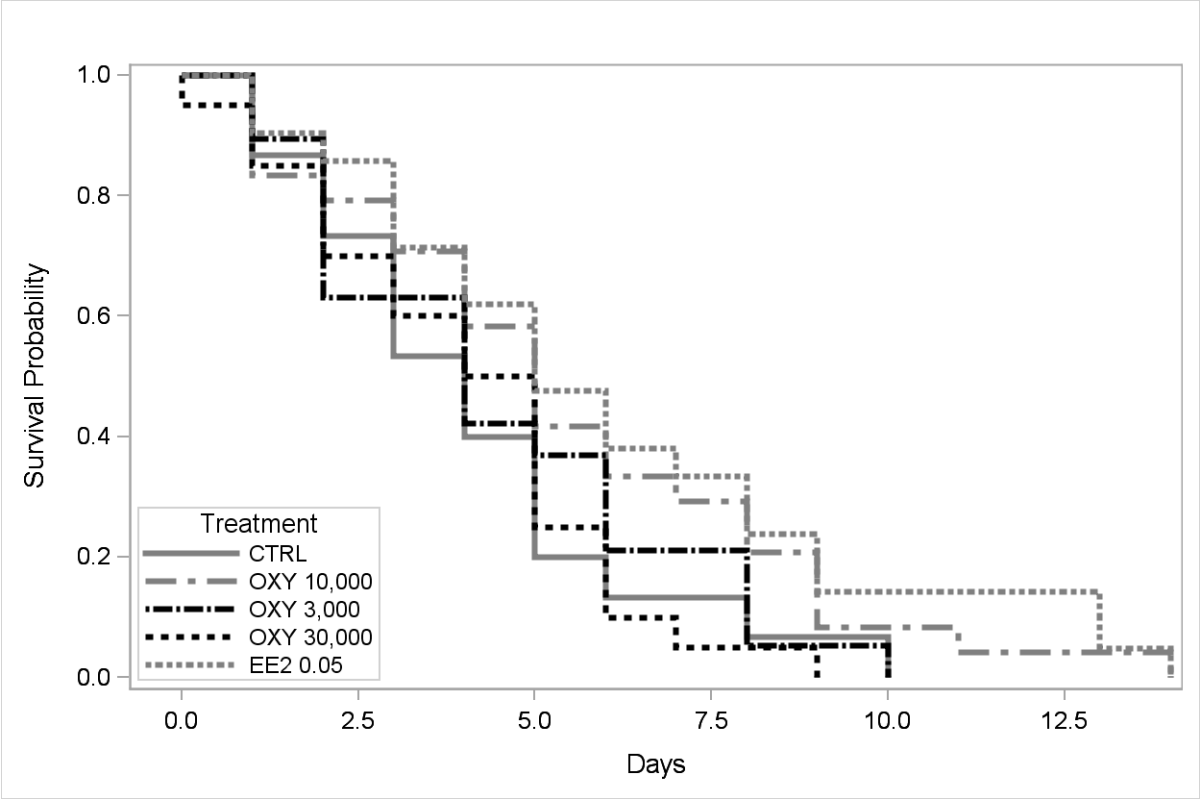
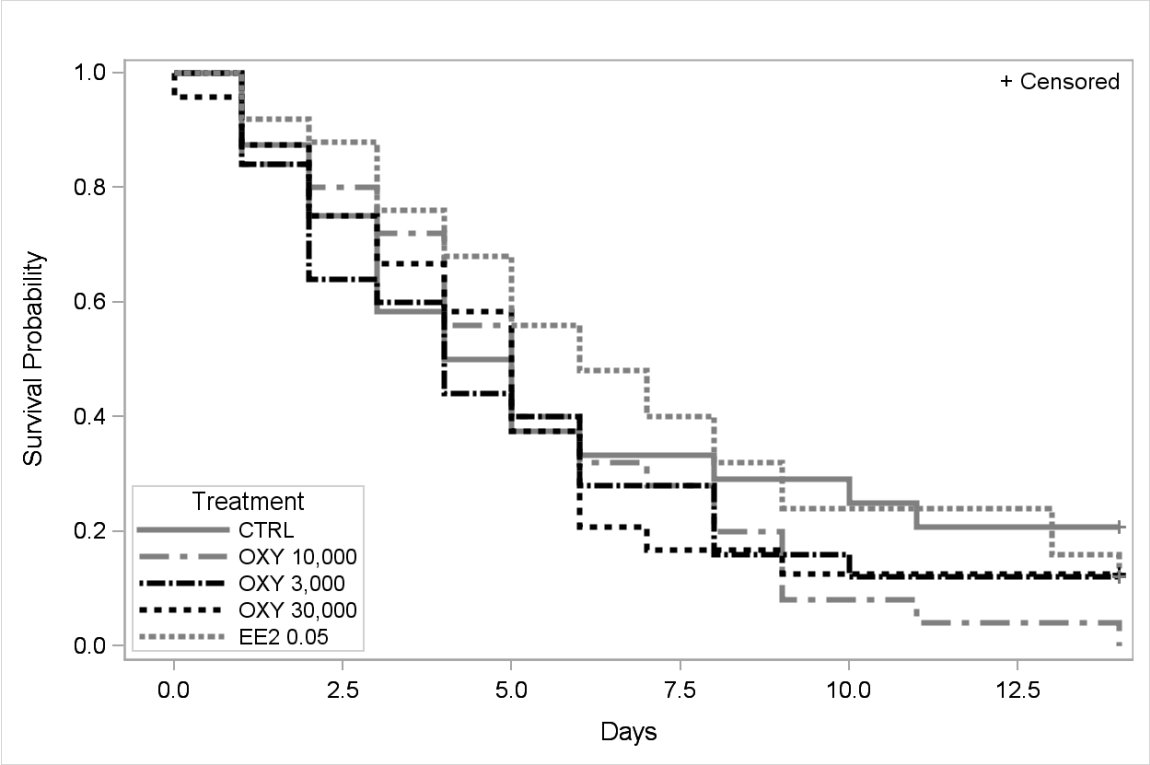


Figure 2. Kaplan-Meier Curve of Time to Mating for Paired Females



A3. Data

Reproductive parameter data were provided in an Excel spreadsheet from the Principle Investigator and data were extracted from the Genesis database using SAS Proc SQL, utilizing the Vortex ODBC driver.

Statistical Analysis of Reproductive Parameter Data– QC

1. Data Verification

The extraction of the data into SAS was verified by the reviewer, Paul Felton, by review of the SAS code used to extract and verify the data.

2. Computer Program Verification

SAS programs were used to extract the data, explore the distributional properties of the data, and perform the statistical analysis.

The SAS programs were verified by detailed review of the program code, the program log, and the program output.

3. Statistical Report Review

3.1 Statistical Report Text

The statistical report was reviewed for logic, internal completeness, technical appropriateness, technical accuracy, and grammar. Technical appropriateness was reviewed based on statistical expertise.

Comments and questions were provided from the reviewer to the statistician. The statistician made appropriate changes and returned the report to the reviewer for final verification.

The text of the final statistical report was considered by the reviewer to be logical, internally complete, and technically appropriate and accurate. The statistical results stated in the text accurately presented those presented in the tables.

3.2 Table Verification

Analysis results were output from SAS to an .rtf file using PROC REPORT, which were then copied into the statistical report.

Statistical report tables were verified by checking the procedure used to create the tables and, additionally, by checking numbers sufficiently to conclude that the tables are correct.

3.3 Graph Verification

Graphs were verified by review of the SAS code used to generate them, and by calculation of summary statistics , and by checking numbers sufficiently to conclude that the graphs are correct. Graphs appear to be appropriate and correct.

4. Conclusions

The final statistical report has been fully reviewed and is considered by the reviewer to be logical, internally complete, and technically appropriate and accurate.