APPENDIX XIII

Statistical Analysis of Pre-Mating Food Consumption – Females and Males

Statistical Report

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

Task: Statistical Analysis of Pre-Breeding Food Consumption
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 Pre-Breeding Food Consumption

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 effects of oxybenzone on pre-breeding food consumption of females and males prior to breeding.

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 mating. 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.

Food consumption was to be measured twice weekly throughout the study beginning at the time of allocation. Water consumption was not to be measured. Food consumption was not to be determined while the animals were housed together for mating.

3. Statistical Methods

Treatment group means of daily food consumption per animal were analyzed using data collected twice weekly for each cage (study 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, week, and interaction. Within-group correlations were modeled using a heterogeneous first-order autoregressive (ARH(1)) correlation structure, which allows for correlated differences in variability across time points. Test of trend, increasing treatment effect with increasing dose, was performed for the oxybenzone and control groups. Comparisons of dosed groups to control were performed with Dunnett's method for adjusted contrasts using two-sided tests.

4. Results

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

Summary statistics for mean daily food consumption per animal by treatment are given in Table 1 for females and in Table 2 for males.

The ANOVA omnibus test results for females are given in Table 3 for the null hypothesis that all of the dosed treatment and control means for food consumption are equal. The effects of treatment, day on study and treatment by day interaction were significant (p=0.047, <0.001, and =0.008, respectively).

For females, least squares mean comparisons of dosed treatments to the control group are presented in Table 4. There were significant trends overall and at days 6-9 and 10-12 (p=0.017, =0.004, and =0.003, respectively). In pairwise comparisons of dosed treatments to control, there was a significant difference for oxybenzone 30,000 ppm at days 6-9 (p=0.046), with the dosed treatment showing mean 26.4% higher compared to control. There were no other significant pairwise differences for any treatment.

The ANOVA omnibus test results for males are given in Table 5 for the null hypothesis that all of the dosed treatment and control means for food consumption are equal. The effects of week and treatment by week interaction were significant (p<0.001, and =0.005, respectively).

For males, least squares mean comparisons of dosed treatments to the control group are presented in Table 6. In pairwise comparisons of dosed treatments to control, there were significant trends at weeks 2 and 3 (p=0.002 and =0.004, respectively). There was a significant difference for oxybenzone 30,000 ppm at week 3 (p=0.045), with the dosed treatment showing mean 16.7% higher compared to control. There were no other significant pairwise differences.

5. Conclusions

In pairwise comparisons of dosed treatments to control for females, there was a significant difference for oxybenzone 30,000 ppm at days 6-9, with the dosed treatment showing

higher mean compared to control. For males, there was a significant difference for oxybenzone 30,000 ppm at week 3, with the dosed treatment showing higher mean compared to control. There were no other significant pairwise differences.

A1. Tables

Table 1. Summary Statistics for Female Daily Food Consumption by Treatment and Days on Study															
	Treatment														
	CTRL			OXY 3,000			OXY 10,000			OXY 30,000			EE2 0.05		
Day	N	Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE
1-5	13	18.2	0.8	13	18.6	1.2	13	17.0	2.5	13	20.6	4.0	13	13.2	1.8
6-9	13	20.6	0.6	13	20.3	1.1	13	24.0	1.7	13	26.0	2.4	13	16.6	1.2
10-12	13	23.0	1.0	13	26.1	1.4	13	23.4	2.4	13	29.7	2.9	13	27.4	1.9

1. N is the number of cages; data were collected for 25 animals in each treatment group at each day.

Table 2. Summary Statistics for Male Daily Food Consumption by Treatment and Week															
	Treatment														
	CTRL			OXY 3,000			OXY 10,000			OXY 30,000			EE2 0.05		
Week	N	Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE
1	13	22.0	0.3	13	20.5	0.4	13	21.0	0.8	13	23.3	1.5	13	20.0	1.0
2	13	21.0	0.7	13	18.7	0.9	13	21.6	0.6	13	24.4	1.8	13	20.9	1.0
3	13	22.2	0.9	13	22.2	0.8	13	21.8	0.7	13	25.9	1.2	13	24.1	1.5
4	13	22.7	0.7	13	23.4	1.3	13	21.6	1.1	13	23.8	1.2	13	26.1	1.6
5	13	23.3	1.1	13	24.5	2.0	13	21.3	0.8	13	21.4	0.8	13	27.0	2.2
6	13	21.1	0.6	13	22.4	1.2	13	21.7	0.5	13	22.0	0.9	13	23.8	1.4
7	13	22.8	1.2	13	23.1	1.3	13	21.3	0.7	13	21.8	0.8	13	25.4	1.5
8	13	20.2	1.0	13	22.4	1.8	13	20.1	0.6	13	20.2	0.6	13	22.8	1.0
9	13	21.2	0.6	13	23.0	1.8	13	20.9	0.6	13	21.9	1.0	13	22.8	1.2
10	13	22.4	0.8	13	23.1	1.4	13	20.5	0.5	13	21.4	0.6	13	22.7	0.8

1. N is the number of cages; data were collected for 25 animals in each treatment group at all weeks.

Table 3. ANOVA Results for Female Food Consumption				
Effect	***NumDF***	***DenDF***	***Fvalue***	***P value***
Treatment	4	60	2.561	0.047
Day	2	120	22.509	<.001
Treatment*Day	8	120	2.739	0.008

Table 4. Comparisons of Least Squares Mean Food Consumption for Females Across Treatments[1]																			
Treatment																			
CTRL				***OXY 3,000***				***OXY 10,000***				***OXY 30,000***				***EE2 0.05***			
Food Days	***Mean***	***SE***	***P value***	***Mean***	***SE***	***Pct***	***P value***	***Mean***	***SE***	***Pct***	***P value***	***Mean***	***SE***	***Pct***	***P value***	***Mean***	***SE***	***Pct***	***P value***
All	20.6	1.5	0.017	21.7	1.5	105.3	0.957	21.5	1.5	104.4	0.978	25.4	1.5	123.7	0.074	19.1	1.5	92.7	0.882
1-5	18.2	2.3	0.424	18.6	2.3	102.5	1.000	17.0	2.3	93.6	0.990	20.6	2.3	113.3	0.878	13.2	2.3	72.6	0.371
6-9	20.6	1.5	0.004	20.3	1.5	98.8	1.000	24.0	1.5	116.7	0.320	26.0	1.5	126.4	0.046	16.6	1.5	80.8	0.211
9-12	23.0	2.0	0.030	26.1	2.0	113.5	0.661	23.4	2.0	102.0	1.000	29.7	2.0	129.4	0.071	27.4	2.0	119.3	0.354

1. All p-values and % are relative to the control group, except p-value for trend (excluding the EE2 treatment) shown below control.

Table 5. ANOVA Results for Male Food Consumption[1]				
Effect	***NumDF***	***DenDF***	***Fvalue***	***P value***
Treatment	4	60	1.497	0.214
Week	9	540	6.018	<.001
Treatment*Week	36	540	1.750	0.005

Table 6. Comparisons of Least Squares Mean Food Consumption for Males Across Treatments[1]																		
Treatment																		
CTRL				***OXY 3,000***				***OXY 10,000***				***OXY 30,000***				***EE2 0.05***			
Week	***Mean***	***SE***	***P value***	***Mean***	***SE***	***Pct***	***P value***	***Mean***	***SE***	***Pct***	***P value***	***Mean***	***SE***	***Pct***	***P value***	***Mean***	***SE***	***Pct***	***P value***
All	21.9	0.7	0.516	22.3	0.7	102.0	0.978	21.2	0.7	96.8	0.898	22.6	0.7	103.3	0.887	23.6	0.7	107.7	0.296
1	22.0	0.9	0.079	20.5	0.9	92.9	0.568	21.0	0.9	95.2	0.837	23.3	0.9	105.9	0.714	20.0	0.9	90.9	0.344
2	21.0	1.1	0.002	18.7	1.1	89.2	0.398	21.6	1.1	103.0	0.983	24.4	1.1	116.0	0.100	20.9	1.1	99.7	1.000
3	22.2	1.1	0.004	22.2	1.1	100.0	1.000	21.8	1.1	98.3	0.997	25.9	1.1	116.7	0.045	24.1	1.1	108.7	0.508
4	22.7	1.2	0.588	23.4	1.2	102.7	0.989	21.6	1.2	94.8	0.905	23.8	1.2	104.5	0.940	26.1	1.2	114.8	0.167
5	23.3	1.5	0.210	24.5	1.5	105.3	0.940	21.3	1.5	91.7	0.770	21.4	1.5	92.0	0.792	27.0	1.5	116.1	0.230
6	21.1	1.0	0.790	22.4	1.0	106.3	0.725	21.7	1.0	102.8	0.978	22.0	1.0	104.1	0.919	23.8	1.0	112.9	0.145
7	22.8	1.2	0.465	23.1	1.2	101.5	0.999	21.3	1.2	93.3	0.756	21.8	1.2	95.8	0.941	25.4	1.2	111.6	0.305
8	20.2	1.1	0.501	22.4	1.1	110.9	0.433	20.1	1.1	99.7	1.000	20.2	1.1	99.9	1.000	22.8	1.1	112.9	0.285
9	21.2	1.1	0.980	23.0	1.1	108.8	0.601	20.9	1.1	98.8	1.000	21.9	1.1	103.6	0.970	22.8	1.1	107.8	0.694
10	22.4	0.9	0.292	23.1	0.9	103.2	0.944	20.5	0.9	91.7	0.390	21.4	0.9	95.8	0.877	22.7	0.9	101.3	0.998

1. All p-values and % are relative to the control group, except p-value for trend (excluding the EE2 treatment) shown below control.

A2. Figures

Figure 1. Food Consumption by Day on Study for Females

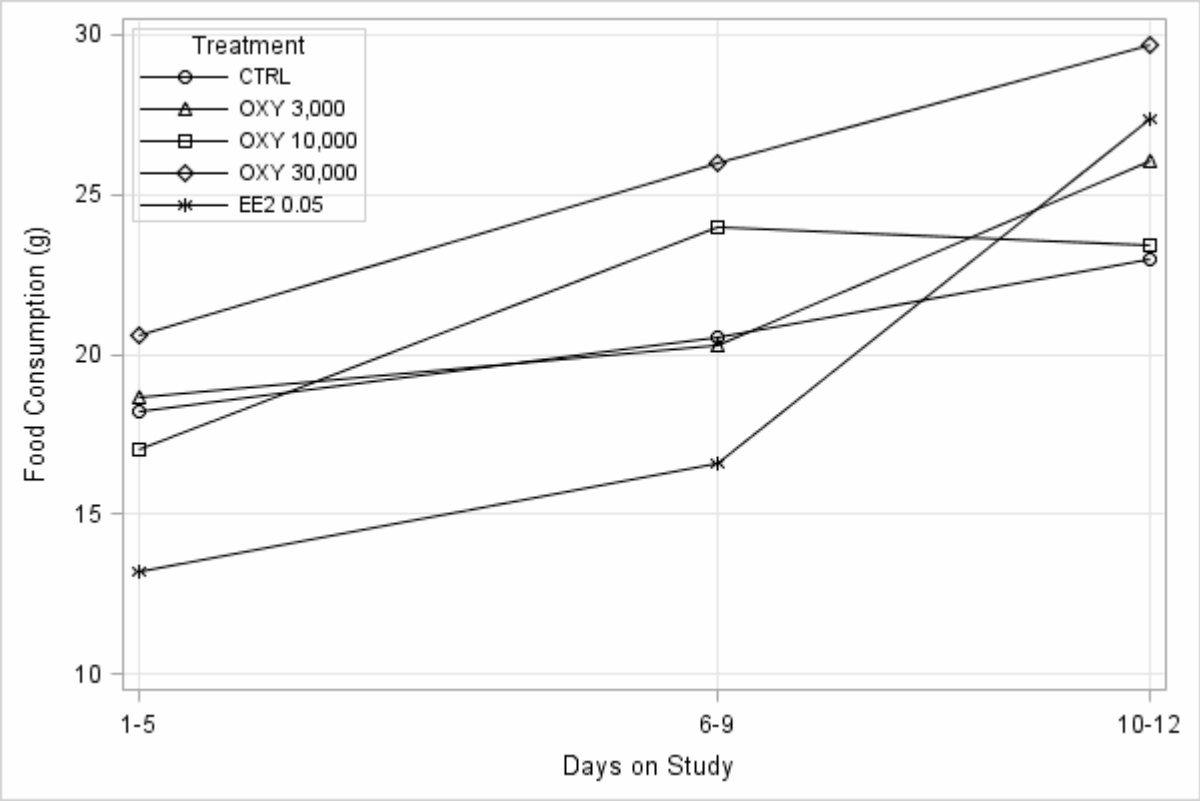
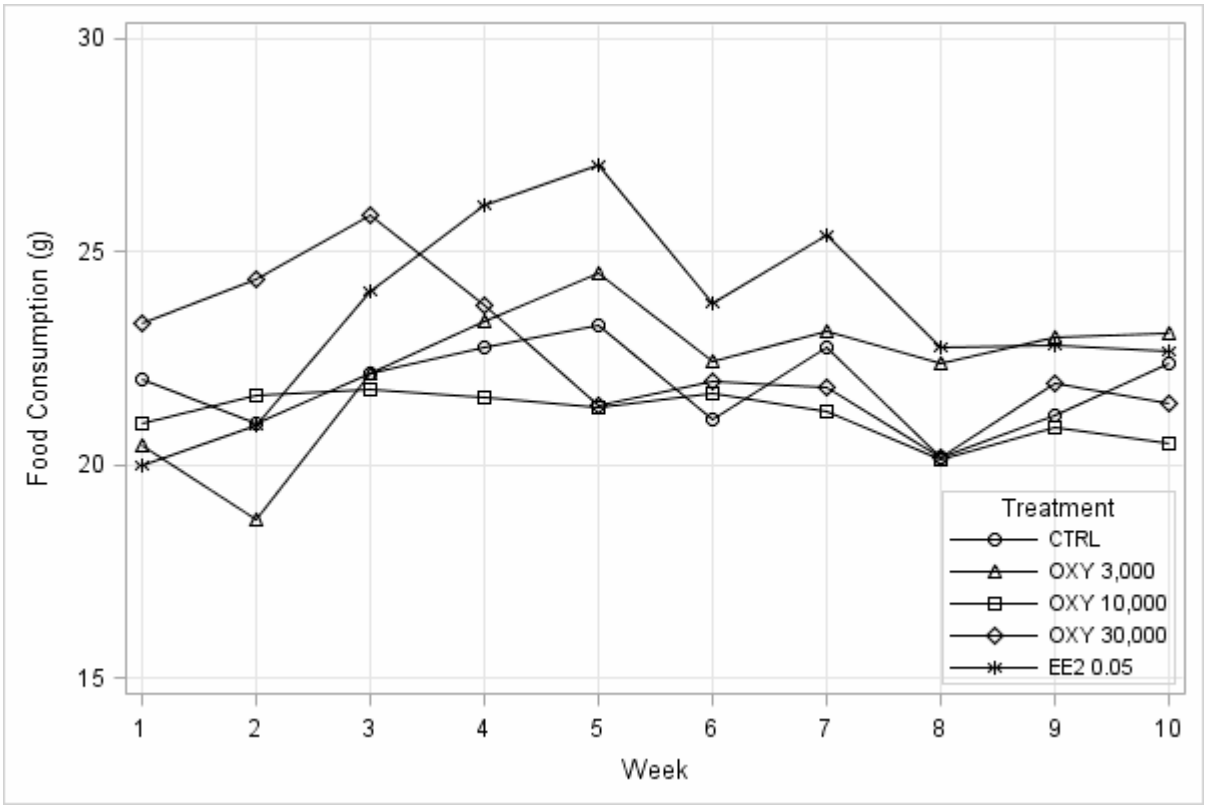


Figure 2. Food Consumption by Week for Males



A3. Data

Pre-breeding food consumption data were extracted from the Genesis database using SAS Proc SQL, utilizing the Vortex ODBC driver.

Statistical Analysis of Pre-Breeding Food Consumption 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 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.