AR226-1836

PFOS:
A REPRODUCTION STUDY WITH THE MALLARD

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454-109

3M LAB REQUEST NO. U2723

FIFRA Guideline 71-4

OECD Guideline 206

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STUDY INITIATION DATE: January 22, 2001

STUDY COMPLETION DATE: August 21, 2003

Submitted to

3M Corporation
Environmental Laboratory
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St. Paul, Minnesota 55106

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: 3M Corporation

TITLE: PFOS: A Reproduction Study with the Mallard

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454-109

STUDY COMPLETION: August 21, 2003

The study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, 40 CFR Part 160, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF, 59 NohSan Notification No. 3850, Agricultural Production Bureau, 10 August 1984, with the following exceptions:

Complete signed and dated reports for blood and tissue analyses conducted by Exygen Research and 3M Corporation are not included in the report. Summary tables are included as appendices and full reports will be submitted separately.

The study was conducted under multiple protocols. The in-life and 3M analytical portions were conducted under one protocol (Wildlife International, Ltd. study number 454-109), and the Exygen Research analytical portions were conducted under two additional separate protocols (Exygen Research study number 023-066 and 023-070).

Analyses of egg contents conducted under Exygen Research study number 023-070 were not conducted in accordance with Good Laboratory Practice Standards, and results of these analyses are reported separately.

The analytical phase performed by Exygen Research under study number 023-066 was performed in compliance with EPA TSCA GLP (40 CFR Part 792).

STUDY DIRECTOR:

Sean P. Gallagher — 3/23/04
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Senior Biologist, Avian Toxicology

SPONSOR'S REPRESENTATIVE:

John L. Newsted — 3/24/04
John L. Newsted — DATE

QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, 40 CFR part 160, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984. The dates of all audits and inspections and the dates any findings were reported to the Study Director and Laboratory Management were as follows:

ACTIVITY	DATE CONDUCTED	DATE REPORTED TO: STUDY DIRECTOR	MANAGEMENT
Test substance preparation	January 22, 2001	January 22, 2001	January 23, 2001
Matrix fortification	January 30, 2001	January 31, 2001	January 31, 2001
Body weight and environmental conditions	February 6, 2001	February 7, 2001	February 9, 2001
Hatchling body weight	May 24, 2001	May 24, 2001	May 25, 2001
14-day old body weight	May 31, 2001	May 31, 2001	May 31, 2001
Egg shell thickness measurements	July 5, 2001	July 5, 2001	July 6, 2001
Candling	July 6, 2001	July 6, 2001	July 25, 2001
ARDS data entry check	August 14 & 15, 2001	August 15, 2001	August 15, 2001
Analytical data and draft report	October 29, November 2 & 5, 2001	November 5, 2001	November 9, 2001
Biological data and draft report	December 5, 7, 10-12, 2001	December 12, 2001	March 11, 2002
Final report	August 20, 2003	August 20, 2003	August 21, 2003

Robert N. McGee
Quality Assurance Representative

August 21, 2003
DATE

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A Reproduction Study with the Mallard

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454-109

3M LAB REQUEST NO. U2723

STUDY DIRECTOR:

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TABLE OF CONTENTS

TABLE OF CONTENTS
PAGE 2

TABLES AND FIGURES

TABLE OF CONTENTS
PAGE 3

APPENDICES

TABLE OF CONTENTS
PAGE 4

SUMMARY

STUDY: PFOS: A Reproduction Study with the Mallard

SPONSOR: 3M Corporation

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454-109

TEST DATES: Study Initiation – January 22, 2001
Experimental Start – January 23, 2001
Photostimulation – April 4, 2001
First Eggs Set – April 13, 2001
Adult Termination – June 20-21, 2001
Analytical Termination – April 11, 2002
Biological Termination – July 26, 2001
Experimental Termination – April 11, 2002

TEST ANIMALS: Mallard (*Anas platyrhynchos*)

AGE TEST ANIMALS: 24 weeks of age at the initiation of the test

SOURCE TEST ANIMALS: Whistling Wings, Inc.
113 Washington Street
Hanover, IL 61041-0509
U.S.A.

NOMINAL TEST CONCENTRATIONS: 0, 10, 50, and 150 ppm a.i.

RESULTS: There were treatment-related mortalities, overt signs of toxicity and treatment-related effects upon adult body weight at the 50 and 150 ppm a.i. test concentrations. There were also treatment-related effects upon feed consumption at the 150 ppm a.i. test concentration. The 50 and 150 ppm a.i. test concentrations were terminated prior to the reproductive phase of the test. There were no treatment-related mortalities, overt signs of toxicity, or treatment-related effects upon body weight, feed consumption or reproductive performance at the 10 ppm a.i. test concentration. However, there was an increased incidence of small testes for males in the 10 ppm a.i. test concentration that may have been related to treatment. Based on the effects observed at the 10 ppm a.i test concentration, the no-observed-effect concentration for mallards exposed to PFOS in the diet was determined to be less than 10 ppm a.i., the lowest concentration tested during this study.

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for 3M Corporation at the Wildlife International, Ltd. avian toxicology facility in Easton, Maryland 21601. The biological portion of the test was conducted from January 23, 2001 until July 26, 2001. Raw data generated at Wildlife International, Ltd. and a copy of the final report are filed under Project Number 454-109 in archives located on the Wildlife International, Ltd. site. Biological specimens are stored at 3M Corporation, St. Paul, MN 55133.

OBJECTIVES

The objective of this study was to evaluate the effects upon the adult mallard (*Anas platyrhynchos*) of dietary exposure to the potassium salt of Perfluorooctane Sulfonic Acid (hereafter referred to as PFOS) over a period of approximately 21 weeks. Effects on adult health, body weight, and feed consumption were evaluated. In addition, the effects of adult exposure to PFOS on the number of eggs laid, fertility, embryo viability, hatchability, offspring survival, and egg shell thickness were evaluated. Histopathological examination of selected tissues and analyses of blood and tissue samples were also used to evaluate the effects upon adults exposed to PFOS and then offspring.

EXPERIMENTAL DESIGN

Mallard (80 males and 80 females) were randomly distributed into one control group and three treatment groups. The test concentrations were selected in consultation with the Sponsor, based upon the results of a pilot reproduction study (Wildlife International, Ltd. Project Number 454-105) and additional toxicity information provided by the Sponsor.

PFOS Treatment Groups

Group	Nominal Concentration (ppm a.i.)	Pens per Group	Extra Pens - Hematology	Birds per Pen	
				Males	Females
1	(Control) 0	16	4	1	1
2	10	16	4	1	1
3	50	16	4	1	1
4	150	16	4	1	1

Each treatment and control group contained 16 pairs of birds with one male and one female per pen. Four additional pairs of birds were added to each test level (for a total of 20 pairs per level) and maintained for collection of blood samples during the test. Three treatment groups were fed diets containing either 10, 50, or 150 ppm a.i. of PFOS. The control group was fed diet comparable to the treatment groups, but without the addition of the test substance.

Because overt signs of toxicity and treatment-related mortalities were noted at the 150 ppm a.i. test concentration, the dietary concentration for the 150 ppm a.i. treatment level was reduced to 20 ppm a.i. of PFOS at the beginning of Week 3 of the test. The 20 ppm a.i. level was terminated at the beginning of Week 5 of the test due to the limited recovery of birds at this test concentration. For reporting purposes, the 150/20 ppm a.i. test concentration will be referred to as the 150 ppm a.i. concentration. Adults in the 50 ppm a.i. test concentrations were euthanized at the end of Week 7 of the test due to treatment-related mortality and overt signs of toxicity. Therefore, the test was completed with the control group and the 10 ppm a.i. test concentration only.

All adult birds were observed daily throughout the test for signs of toxicity or abnormal behavior. Adult body weights were measured at test initiation, on Weeks 2, 4, 6, 8, and at adult termination and feed consumption was measured weekly throughout the test. At the beginning of Week 11, the photoperiod was increased to induce egg production. Following the start of egg production, eggs were set weekly for incubation. Weekly, eggs were selected by indiscriminate draw for egg shell thickness measurement and all remaining eggs were candled prior to incubation to detect egg shell cracks or abnormal eggs. Eggs were also candled twice during incubation to detect infertile eggs or embryo mortality. On Day 24 of incubation, the eggs were placed in a hatcher and allowed to hatch. Once hatching was completed, hatchlings were removed from the hatcher and the group body weight of the hatchlings by pen was determined. At 14 days of age, the average body weight by parental pen of all surviving offspring was determined. Upon completion of the test, statistical analyses were performed to determine statistically significant differences between groups.

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "PFOS: A Reproduction Study with the Mallard". The protocol was based on procedures outlined in the Environmental Protection Agency's Registration Guidelines *Pesticide Assessment Guidelines, FIFRA Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms,* Subsection 71-4; OECD Guideline 206; and the ASTM "*Standard Practice for Conducting Reproductive Studies with Avian Species*" (1,2,3).

Test Substance

The test substance, PFOS, was received from 3M Corporation on October 29, 1998 and was assigned Wildlife International, Ltd. identification number 4675 upon receipt. The test substance was a white powder and was identified as: FC-95; Lot 217. The test material had a reported purity of 86.9% and expiration date of August 31, 2006 (Appendix I). The test substance was held under ambient conditions in locked storage at the Wildlife International, Ltd. facilities in Easton, Maryland. Concentrations of the test substance in the diet were adjusted to 100% active ingredient. Therefore, dietary concentrations are expressed as parts per million active ingredient (ppm a.i.) in the diet.

Test Organisms

Approximately 84 pairs of pen-reared mallard were purchased from Whistling Wings, Inc., 113 Washington Street, Hanover, IL 61041-0509, U.S.A. At the start of acclimation, the mallard were apparently healthy and phenotypically indistinguishable from wild type. The birds were from the same hatch, approaching their first breeding season and had not been used in any previous testing. At the start of acclimation, a random number generating function in a spreadsheet program was used to randomize pen assignment for each bird. Immediately prior to test initiation, all potential study birds were examined for physical injuries and general health. Birds that did not appear healthy, either due to injury or inability to acclimate to laboratory conditions, or were outside the weight range for the test, were excluded from the study. All birds were 24 weeks of age at test initiation (first day of exposure to test diet) and ranged in weight from 781 to 1444 grams at test initiation. Sex of the birds was determined by a visual examination of the plumage.

Identification

Adult birds were identified by individual leg bands, each pen was identified with a unique number, and groups of pens were identified by project number and concentration. All eggs laid during the study

were marked with the pen number using a permanent ink marking pen for identification. Hatchlings were identified by wing bands so that they could be traced to their parental pen of origin.

Avian Feed and Water

All adult birds and their offspring were given feed and water *ad libitum* during acclimation and testing. The basal diet fed to both adults and offspring was formulated to Wildlife International, Ltd. specifications by Agway Inc. (Appendix II). The basal ration contained at least 27% protein and 2.5% fat, and no more than 5% fiber.

The basal diet contained approximately 1.1% calcium, derived from feedstuffs and the 0.9% limestone used in the formulation of the basal diet by Agway. While this level of calcium is sufficient for growth and maintenance rations, additional calcium is required in the ration of breeding birds for egg shell formation. Therefore, an additional 5% (w/w) of limestone (approximately 38.5% Ca) was added to the basal diet for the adults. This raised the calcium level in the diet for the breeding birds to approximately 3%, slightly above the minimum recommended for quail (2.3%) and mallard (2.75%) (4). Offspring received basal diet without test substance and without the addition of 5% supplemental limestone.

Water was supplied by the town of Easton public water supply. Neither the adults nor offspring received any form of medication in the feed during the test. Feed and water were analyzed periodically in accordance with Wildlife International, Ltd. Standard Operating Procedures.

Diet Preparation

Test diets were prepared by mixing PFOS into a premix that was used for weekly preparation of the final diet. Control diet and each of the three treated diets were prepared weekly beginning on January 23, 2001 and presented to the birds on Tuesday of each week. Dietary concentrations were adjusted for purity of the test substance and are presented as parts per million active ingredient (ppm a.i.). Details of the weekly preparation of test and control diets are shown in Appendix III.

Diet Sampling

Homogeneity of the test substance in the diet was evaluated by collecting six samples from each of the treated diets and one sample from the control diet on Day 0 of Week 1. Samples were collected from

the top, middle and bottom of the left and right sections of the mixing vessel. Control and treatment group diet samples were also collected from the feed troughs on Day 7 of Week 1 to assess stability of the test substance under actual test conditions. Additionally, a sample was collected from the control and treatment group diets, when available, during Weeks 2, 3, 4, 8, 12, 16 and 20 of the test to measure/verify test concentrations. The diet samples were stored frozen or transferred immediately to the Wildlife International, Ltd. analytical chemistry facility for analysis.

Analytical Method

The method used for the analysis of PFOS in avian diet was based upon methodology developed at Wildlife International, Ltd. and entitled "Analytical Method Verification for the Determination of PFOS in Avian Diet" (Wildlife International, Ltd. Project No. 454C-110). Three modifications from the cited methodology were incorporated into the present study. These changes were (1) the use of high performance liquid chromatography with triple quadrupole mass detection (HPLC/MS/MS) rather than single quadrupole mass detection to reduce potential matrix interferences, (2) the elimination of the internal standardization procedure because an appropriate internal standard could not be identified, and (3) the use of a shorter (50 mm vs.100 mm) analytical chromatography column to expedite analysis time.

Samples were extracted with methanol, vacuum filtered, diluted in methanol, and then diluted in a 50:50 methanol: NANOpure® water dilution solvent so that they fell within the calibration range of the PFOS methodology. A method flow chart is provided in Appendix IV, Figure 1. Concentrations of PFOS in the standards and extracts of the samples were determined by reverse-phase HPLC/MS/MS using a Hewlett-Packard Model 1100 High Performance Liquid Chromatograph interfaced with a Perkin-Elmer API 3000 Mass Spectrometer operated in multiple reaction monitoring (MRM) detection mode. The mass spectrometer was equipped with a Perkin-Elmer TurboIonSpray ion source. HPLC separations were achieved using a Keystone Betasil C_{18} analytical column (50 mm × 2 mm I.D., 3-µm particle size) fitted with a Keystone Javelin C_{18} Guard Cartridge (20 mm × 2 mm I.D.). The instrument parameters are summarized in Appendix IV, Table 1.

Calibration standards were prepared in 50:50 methanol: NANOpure® water by appropriate dilutions of a 1.00 mg a.i./L stock solution of PFOS in methanol. The calibration standards, ranging in concentration from 0.00100 to 0.0100 mg a.i./L, were analyzed with each sample set. The same and most prominent peak response for PFOS was utilized to monitor PFOS in all calibration, quality control, and

study samples. No attempt was made to quantify PFOS on the basis of individual isomeric components. Linear regression equations were generated using the peak area responses versus the respective concentrations of the calibration standards. An example of a calibration curve is presented in Appendix IV, Figure 2. The concentration of PFOS in avian feed samples was determined by substituting the peak areas into the applicable linear regression equation. Typical ion chromatograms of low and high calibration standards are shown in Appendix IV, Figures 3 and 4, respectively. Examples of equations used in calculations are presented in Appendix IV, Table 2.

The instrument limit of detection (LOD) for these analyses was 10.0 pg on-column, calculated from the product of the injection volume (10.0 µL) and the lowest standard concentration 1.00 µg a.i./L = 1.00 pg a.i./µL. The method limit of quantitation (LOQ) for these analyses was 2.00 ppm a.i., calculated as the product of the lowest calibration standard analyzed (0.001 mg a.i./L) and the overall dilution factor of the matrix blank sample (2000 L/Kg). Examples of calculations are presented in Appendix IV, Table 2.

Along with the sample analyses, seven matrix blanks were analyzed to determine possible interferences. No interferences were observed at or above the LOQ during the sample analyses (Appendix IV, Table 3). A typical ion chromatogram of a matrix blank is presented in Appendix IV, Figure 5.

Avian diet samples were fortified at the following levels and analyzed concurrently with the samples to determine the mean procedural recovery. The fortification levels were 5.00, 50.0, and 200 ppm a.i. for sampling intervals Week 1, Day 0; Week 1, Day 7; Week 2, Day 0; and Week 3, Day 0. The fortification levels were 5.00, 25.0, and 100 ppm a.i. for sampling interval Week 4, Day 0. The fortification levels were 5.00 and 25.0 ppm a.i. for sampling intervals Week 8, Day 0; Week 12, Day 0; Week 16, Day 0; and Week 20, Day 0. The method yielded mean procedural recoveries of 101%, 101%, 102%, 100% and 99%, respectively, for the 5.00, 25.0, 50.0, 100, and 200 ppm a.i. fortification levels (Appendix IV, Table 3). Sample measured concentrations were not corrected for the mean procedural recoveries. A typical ion chromatogram of a matrix fortification is presented in Appendix IV, Figure 6.

Study Phases

The primary phases of the study and their approximate durations were:

1. Acclimation - 3 weeks.
2. Pre-photostimulation - 10 weeks.
3. Pre-egg laying (with photostimulation) - 0 weeks.
4. Egg laying - Approximately 10 weeks.
5. Post-adult termination (final incubation, hatching, and 14-day offspring rearing period) - 5 weeks.

Housing and Environmental Conditions

Housing and husbandry practices were conducted so as to adhere to the guidelines established by the National Research Council (5). The adult birds were housed indoors in batteries of pens manufactured by Safeguard Products, Inc. (Model No. 5355), measuring approximately 75 X 90 X 45 cm high. The pens were constructed of vinyl-coated wire mesh. A diagram of the test layout is presented in Appendix V.

Each pen was equipped with a feed trough. Weekly, sufficient feed for the feeding period was placed in the trough for each pen and presented to the birds. During the feeding period additional feed was weighed and added to the troughs as needed. Water was supplied by nipple-type waterers.

Only birds associated with this study were maintained in the study room in order to avoid excessive disturbances. The average temperature in the adult mallard study room during the course of the test was 21.7°C ± 1.1°C (SD) with an average relative humidity of 40% ± 19% (SD). The air handling system in the study room was designed to vent up to 15 room air volumes every hour and replace them with fresh air.

The photoperiod in the adult mallard room was maintained by a time clock. The photoperiod during acclimation and the first 10 weeks of the test was eight hours or less of light per day. The photoperiod was increased to 17 hours of light per day at the beginning of Week 11 to induce egg laying and was maintained at that length until the adult birds were euthanized. Throughout the test, the birds received a mean of approximately 188 lux (~ 17 ft. candles) of illumination provided by fluorescent lights that closely approximated noon-day sunlight.

Observations

The test birds were acclimated to the facilities and study pens for approximately 3 weeks prior to initiation of the test. During acclimation, all birds were observed daily. Birds exhibiting abnormal behavior or debilitating physical injuries were not used for the test. During the study, all adult birds were observed daily for signs of toxicity or abnormal behavior. Additionally, all offspring were observed daily from hatching until 14 days of age. A record was maintained of all mortalities and clinical observations.

Adult Body Weight and Feed Consumption

Adult body weights were measured at test initiation, on Weeks 2, 4, 6, 8, and at adult termination. Body weights were not measured during egg laying because of the possible adverse effects handling may have on egg production.

Feed consumption for each pen was measured weekly throughout the test. Feed consumption was determined by weighing the freshly filled feeder on Day 0, recording the amount of any additional diet added during the week, and weighing the feeder and remaining feed at the end of the feeding period (Day 7). The amount of feed wasted by the birds was not quantified, since the wasted feed was normally scattered and mixed with water and excreta. Therefore, feed consumption is presented as an estimate of total feed consumption.

Adult Blood Collection

Four additional pairs of adult birds were added to each test level to be maintained for collection of blood samples during the test. These pairs received the same diet and were handled in the same manner as other pairs in their test group. However, because of the potential stress of the blood collection procedures, data from the four additional pairs were not used to evaluate treatment-related effects. Blood samples were collected from birds in these designated pairs, when possible, at five week intervals throughout the test. At the time of adult termination, blood samples were collected from all surviving birds, when possible, prior to euthanasia. All blood samples were separated into serum and hemacytes/platelets, stored frozen and shipped to the sponsor or sponsor's designate for possible analysis.

Adult Necropsy and Tissue Collection

When the remaining birds from 150/20 pm a.i. treatment group were euthanized at the end of the Week 4, no necropsies were performed. All other adult birds that died or were euthanized during the

course of the study were subjected to a gross necropsy. At the conclusion of the exposure period, all surviving adult birds were euthanized by cervical dislocation, necropsied, and disposed of by incineration. At the time of necropsy, tissues were collected for histopathological examination and possible analyses. When available, samples of gall bladder, liver, proventriculus, kidneys, brain, gonads, bursa of Fabricius and adipose tissue were fixed in 10% buffered formalin and shipped to EPL in Herndon, VA for histopathology. When available, samples of bile, liver, proventriculus, kidneys, brain, reproductive organs and adipose tissue were stored frozen and shipped to the Sponsor for possible analysis. Fecal samples were also collected, when available, from the lower digestive tract and stored frozen for possible analysis.

Egg Collection and Storage

Eggs were collected daily from all pens, when available. Eggs to be incubated were washed to reduce the possibility of pathogen contamination before storing them in the cold room. Eggs were washed in a commercial egg washer (Kuhl Egg Washer) with a chlorine based detergent (Kuhl Super CD). Water in the washer was warmed to approximately 45°C. The eggs were placed in the wash water and soaked for approximately 15 seconds. The washer's circulation motor was then turned on for approximately three minutes. The eggs were removed from the washer, allowed to cool to approximately room temperature and rinsed with fresh water. The eggs were then stored in a cold room until incubation. The cold room was maintained at a mean temperature of 13.5°C ± 0.2°C (SD) with a mean relative humidity of approximately 69% ± 6% (SD). Groups of eggs were identified by an alphabetic lot code. All eggs laid in a weekly interval were considered as one lot.

Candling and Incubation

At the end of the weekly interval, all eggs were removed from the cold room, counted and eggs selected by indiscriminate draw for egg shell thickness measurement. The remaining eggs were candled with a Speed King (Model No. 32) egg-candling lamp to detect egg shell cracks or abnormal eggs. Cracked or abnormal eggs were recorded and discarded.

All eggs not discarded or used for egg shell thickness measurements were placed in a Petersime Incubator (Model No. SP20). In the incubator the temperature was maintained at an average 37.5°C ± 0.0°C (SD) with an average wet bulb temperature of 30.5°C ± 0.3°C (SD) (relative humidity of approximately 60%). The incubator was equipped with a pulsator fan and blades that produced a mild

breathing air movement designed to eliminate intracabinet temperature and humidity variation during incubation. In order to prevent adhesion of the embryo to the shell membrane, the incubator was also equipped with an automatic egg rotation device, designed to rotate the eggs from 50° off of vertical in one direction to 50° off of vertical in the opposite direction (total arc of rotation was 100°) every two hours through Day 24 of incubation. Eggs were candled on Day 13 or 14 of incubation to determine embryo viability and on Day 21 to determine embryo survival.

Hatching and Brooding

On Day 24 of incubation, the eggs were placed in a Petersime Hatcher (Model No. SP-6H) and allowed to hatch. Pedigree baskets constructed of galvanized steel wire mesh were used to keep hatchlings separated by parental pen of origin. Eggs were not rotated in the hatcher. The average temperature in the hatching compartment was 37.2°C ± 0.0°C (SD), and the average wet bulb temperature was raised to 33.8°C ± 0.2°C (SD) (relative humidity of approximately 80%).

All hatchlings, unhatched eggs, and egg shells were removed from the hatcher on Day 27 or 28 of incubation. The group body weight of the surviving hatchlings by pen was determined. Hatchlings were wing banded for identification by pen of origin and then routinely housed according to the appropriate parental concentration grouping in brooding pens until 14 days of age. The hatchlings were fed untreated diet without the addition of 5% supplemental limestone. At 14 days of age, the average body weight by parental pen of all surviving ducklings was determined. The ducklings were euthanized with carbon dioxide and disposed of by incineration.

Hatchlings were housed in batteries of brooding pens manufactured by Safeguard Products, Inc. Each pen measured approximately 62 X 92 X 25.5 cm high. The walls, floors and ceilings of each pen were constructed of vinyl-coated wire mesh. Thermostats in the brooding compartment of each pen were set to maintain a temperature of approximately 38°C from the time of hatching until the birds were five to seven days of age, when the temperature was adjusted maintain a temperature of approximately 29°C. The average ambient room temperature was 24.2°C ± 1.2°C (SD) with an average relative humidity of 77% ± 15% (SD). The photoperiod for the hatchlings was maintained by a time clock at 16 hours of light per day.

Offspring Blood and Tissue Collection

Prior to euthanasia of the last group of offspring (Lot J), blood samples were collected from 10 offspring in both the control group and 10 ppm a.i. treatment group. All blood samples were separated into serum and hemacytes/platelets, stored frozen and shipped to the sponsor for possible analyses. Additionally, tissues from the 10 offspring in each group were collected for histopathological examination. When available, samples of gall bladder, liver, proventriculus, kidneys, brain, gonads, bursa of Fabricius and adipose tissue were fixed in 10% buffered formalin and shipped to EPL in Herndon, VA for histopathology. When available, samples of bile, liver, proventriculus, kidneys, brain, reproductive organs and adipose tissue were stored frozen and shipped to the sponsor for possible analyses. Fecal samples were also collected from pans below the floor of brooders housing Lot J offspring and stored frozen for possible analysis. A single composite sample was collected for the control group and a single composite sample was collected for the 10 ppm a.i. treatment group.

Egg Shell Thickness Measurements and Egg Components Collection

Weekly throughout the egg laying period one egg was collected, when available, from each of the test pens. Eggs were collected from odd numbered pens during odd numbered weeks (1,3,5, etc.) and from the even numbered pens during the even numbered weeks (2,4,6, etc.). The eggs from weeks 1, 2 and 10 were separated into albumen, yolk, shell and membrane and all components were stored frozen. Eggs collected during weeks 3-9 were opened at the waist and their contents removed. The egg contents were separated into albumen and yolk and the yolk was stored frozen. All egg samples were transferred to the Sponsor for possible analysis. The shells from Weeks 3 through 9 were allowed to air dry for at least one week at room temperature prior to being measured for thickness. The average thickness of the dried shell plus the membrane was determined by measuring five points around the waist of the egg using a micrometer. Measurements were made to the nearest 0.002 mm.

Statistical Analyses

Upon completion of the test, an analysis of variance (ANOVA) was performed to determine statistically significant differences between groups. Dunnett's multiple comparison procedure (6,7) was used to compare the three treatment means with the control group mean and assess the statistical significance of the observed differences. Sample units were the individual pens within each experimental group, except adult body weights where the sample unit was the individual bird. Percentage data were examined using Dunnett's method following arcsine square root transformation (see Appendix VI for

reproductive parameters). With the exception of liver weight, statistical analysis of the data was performed using Avian Reproduction Data System (ARDS) Software; a validated software package developed by Wildlife International, Ltd. (8). Liver weights were compared by Student's t-test using Microsoft Excel. Each of the following parameters was analyzed statistically:

1. Adult Body Weight - Individual body weight was measured at test initiation, Weeks 2, 4, 6, 8 and at adult termination. Statistical comparisons were made between the control group and each treatment group at each weighing interval by sex.
2. Adult Feed Consumption - Feed consumption expressed as grams of feed per bird per day was examined by pen weekly during the test. Statistical comparisons were made between the control and each treatment group.
3. Eggs Laid of Maximum Laid - The number of eggs laid per female divided by the largest number of eggs laid by any one female. This transformation was used to convert the number of eggs laid to a percentile value less than or equal to 100.
4. Eggs Cracked of Eggs Laid - The number of eggs determined by candling to be cracked divided by the number of eggs laid, per pen.
5. Viable Embryos of Eggs Set - The number of viable embryos at the Day 14 candling was divided by the number of eggs set, per pen.
6. Live 3-Week Embryos of Viable Embryos - The number of live embryos at the Day 21 candling was divided by the number of viable embryos, per pen.
7. Hatchlings of 3-Week Embryos - The number of hatchlings removed from the hatcher was divided by the number of live 3-week embryos, per pen.
8. 14-Day Old Survivors of Hatchlings - The number of 14-day old survivors was divided by the number of hatchlings per week, by pen.
9. Hatchlings of Eggs Set - The number of hatchlings was divided by the number of eggs set per week, by pen.
10. 14-Day Old Survivors of Eggs Set - The number of 14-day old survivors was divided by the number of eggs set per week, by pen.
11. Hatchlings of Maximum Set - The number of hatchlings per female divided by the largest number of eggs set from any one female. This transformation was used to convert the number of hatchlings to a percentile value equal to or less than 100.

12. 14-Day Old Survivors of Maximum Set - The number of 14-day old survivors per pen divided by the largest number of eggs set.
13. Egg Shell Thickness - The average egg shell thickness of indiscriminately selected eggs per pen was measured.
14. Offspring's Body Weight - The group body weights of surviving hatchlings and 14-day old survivors were measured by parental pen group.
15. Liver Weight – Individual liver weights were measured at adult termination and at termination of selected Lot J offspring. Statistical comparisons were made by sex between the control group and 10 ppm a.i. group.

RESULTS AND DISCUSSION

Mature mallards received PFOS at nominal dietary concentrations of 10, 50, and 150 ppm a.i. Due to extensive overt signs of toxicity and treatment-related mortalities in the 50 and 150 ppm a.i. treatment groups, the experimental design was modified. Adults in the 50 ppm a.i. test concentrations were euthanized at the end of Week 7 of the test. The dietary concentration for the 150 ppm a.i. treatment level was reduced to 20 ppm a.i. of PFOS at the beginning of Week 3 of the test. The 20 ppm a.i. level was terminated at the beginning of Week 5 of the test due to the limited recovery of birds at this level. Therefore, the test was completed with the control group and 10 ppm a.i. test concentrations only. The control group was fed diet comparable to the treatment groups, but without the addition of the test substance.

Diet Analytical Results

None of the control samples showed any indication of the presence of the test substance or of the presence of co-eluting substances at the characteristic retention time of the test substance. Diet samples were collected from the 10, 50 and 150 ppm a.i. test concentrations on Week 1, Day 0, and were analyzed to evaluate the homogeneity of the test substance in the diet and to verify test substance concentrations. Means and standard deviations for the three test concentrations were 9.75 ± 0.313 ppm a.i., 50.1 ± 0.981 ppm a.i. and 147 ± 4.31 ppm a.i., respectively. The coefficients of variation were 3.21%, 1.96% and 2.93%, respectively. These values represented 98%, 100%, and 98% of nominal concentrations (Appendix IV, Table 4).

Samples collected throughout the test to verify test substance concentrations for the 10, 20, 50, and 150 ppm a.i. diets had means and standard deviations of 10.2 ± 0.553 ppm a.i., 20.8 ± 2.14 ppm a.i., 50.9 ± 2.04 ppm a.i, and 161 ± 15.6 ppm a.i., respectively. The coefficients of variation were 5.44%, 10.3%, 4.01% and 9.66%, respectively. These values represented 102, 104, 102, and 108% of nominal concentrations (Appendix IV, Table 5). Analysis of diet samples collected from feeders after being held at ambient temperature for 7 days averaged 100%, 101% and 97% of the Day 0 values for the 10, 50 and 150 ppm a.i. test concentrations, respectively (Appendix IV, Table 6). A typical ion chromatogram of a test sample is shown in Appendix IV, Figure 7. Mean weekly measured concentrations for each test group are presented in Table 1.

Mortalities

While there was one incidental mortality in the control group, there were no mortalities in the 10 ppm a.i. treatment group. Three incidental mortalities and ten treatment-related mortalities occurred in the 50 ppm a.i. treatment group and four treatment-related mortalities occurred in the 150 ppm a.i. treatment group.

The single incidental mortality in the control group was a male (Pen 210) that died during Week 15 of the test. Gross necropsy of the drake revealed foot lesions on both feet. Internally, hemorrhages were noted in the myocardium, the abdominal air sacs were cloudy and the spleen was enlarged and mottled. Additionally, the liver was firm, mottled and pale, the kidneys were slightly pale and a small amount of clear fluid was noted in the abdominal cavity. Necropsy of the bird's penmate at termination of the adult portion of the study revealed a regressed ovary, but was otherwise unremarkable.

Three incidental and ten treatment-related mortalities occurred in the 50 ppm a.i. treatment group and four treatment-related moralities occurred in the 150 ppm a.i. treatment group. Necropsy findings for the treatment related mortalities in the 50 and 150 ppm a.i. test concentrations were similar. Common findings included thin or emaciated condition with a loss of muscle mass and prominent keel, small spleen and pale kidneys. All remaining birds at the 150 ppm a.i. test concentration were euthanized at the beginning of Week 5. Due to the shortened nature of the exposure period, necropsies were not performed. All remaining birds at the 50 ppm a.i. test concentration were euthanized at the beginning of Week 8 of the test, and necropsies were performed following euthanasia. Necropsy findings are summarized in Table 2.

Clinical Observations

No overt signs of toxicity were observed at the 10 ppm a.i. test concentration. With the exception of foot lesions and occasional lameness, all birds in the control group and 10 ppm a.i. treatment group were normal in appearance and behavior throughout the test.

In the 150 ppm a.i. and 50 ppm a.i. treatment groups, signs of toxicity were first noted at the end of Week 2. One male in the 50 ppm a.i. treatment group and several birds in the 150 pm a.i. treatment group exhibited signs of toxicity induced by the stress of handling during the Week 2 body weight measurements. In several cases, birds convulsed during body weight measurements. Signs of toxicity in both the 50 and 150 ppm a.i. treatment groups continued to be observed until the birds were euthanized. Clinical signs of toxicity observed included depression, reduced reaction to external stimuli (sound and movement), wing droop, loss of coordination, ruffled appearance, thin appearance, lower limb weakness, lethargy, lacrimation and convulsions.

Adult Body Weight

There were no apparent treatment-related effects upon adult body weight at the 10 ppm a.i. test concentration, and any differences between the control group and the 10 ppm a.i. test concentration were not statistically significant at any of the body weight intervals. However, there were marked treatment-related reductions in mean body weight at both the 50 and 150 ppm a.i. test concentrations.

With the exception of the mean male body weight at the Week 2 interval, all differences between the control group and birds in the 50 ppm a.i. treatment group were statistically significant at $p < 0.01$. At the 150 ppm a.i. test concentration, mean male and female body weights were statistically different from the control group at $p < 0.01$ at both the Week 2 and Week 4 body weight intervals. Between the Week 2 and Week 4 body weight intervals, a weight gain was noted for birds at the 150 ppm a.i. test concentration. However, the weight gain observed was a reflection of the reduction of the concentration of PFOS in the diet to 20 ppm a.i. during Week 3 of the test. Mean body weight measurements are presented in Table 3, and Figures 1 and 2. Individual body weight measurements are presented in Appendix VII.

Adult Feed Consumption

There were no apparent treatment-related effects upon feed consumption at the 10 or 50 ppm a.i. test concentrations. No statistically significant differences between the control group and 10 or 50 ppm a.i. treatment groups were observed at any of the feed consumption intervals. However, treatment-related reductions in feed consumption were observed at the 150 ppm a.i. test concentration during Weeks 1 and 2. The reductions were statistically significant at $p < 0.05$ during Week 1 and at $p < 0.01$ during Week 2. The dietary concentration for the 150 ppm a.i. treatment group was reduced to 20 ppm a.i. during Week 3 of the test. There was a statistically significant ($p < 0.01$) increase in feed consumption at the 150 (20) ppm a.i. test concentration during Weeks 3 and 4 of the test. Mean feed consumption measurements are shown in Table 4 and Figure 3. Feed consumption measurements by pen are presented in Appendix VIII.

Adult Necropsy and Liver Weights

All surviving adults in the control group and the 10 ppm a.i. treatment group were subjected to gross necropsy following adult termination. When compared to the control group, there was an increased incidence of males with small testes (approximate length < 3.5 cm) in the 10 ppm a.i. treatment group for which a treatment-related effect could not be precluded. Two males in the control group were noted with small testes, compared to seven males in the 10 ppm a.i. group. All other findings were considered unrelated to treatment. Necropsy findings are reported in Table 5 and Appendix IX.

When compared to the control group, there were no treatment-related effects upon the mean weights of adult livers at the 10 ppm a.i. test concentration. Any difference between the 10 ppm a.i. treatment group and the control group was not statistically significant. Mean adult liver weights are presented in Table 6, while individual adult liver weights are presented in Appendix X.

Histopathology

No lesions considered related to test article (PFOS) administration were noted in the liver, kidney, adipose tissue, brain, and proventriculus of adult and offspring males and females, bursa of Fabricius in offspring males and females, ovaries of adult and offspring females and testes of male offspring. The increase in the number of adult males in the 10 ppm a.i. treated group with reduced testicular size (with or without a corresponding microscopic observation), suggests that PFOS may have accelerated early post-reproductive phase regression, a normal physiological phenomenon. Any potential test-article effects on the bursa of Fabricius in adult males and females could not be determined since samples were not

submitted for evaluation. In addition, any potential test-article effects on the gallbladder from adults and offspring could not be definitively determined due to the extensive autolysis that precluded any accurate diagnosis. The few other changes in various tissues of adult and/or offspring mallards from control and 10 ppm a.i. treated groups were considered incidental and unrelated to test article (PFOS) administration. The full histopathology report is provided in Appendix XI.

Adult Liver and Sera

Adult blood samples (both red blood cells and sera) collected through Week 15 of the test were analyzed by Exygen Research. The results for analysis of samples collected for this study are reported together with results for the northern bobwhite study run concurrently with the same test concentrations. Summary results of blood analyses performed for this study are presented in Exygen report Tables X through XXIV located in Appendix XIIA The full report for analysis of blood samples by Exygen Research is located under Exygen Study Number 023-066. "Extraction of Potassium Perfluorooctanesulfonate from Red Blood Cells and Serum for Analysis Using HPLC-Electrospray/Mass Spectrometry".

Blood and liver samples collected at the time of adult termination for the control group and 10 ppm a.i. test concentration were shipped to 3M Environmental Laboratory for analysis. Summary results of sera and liver analyses conducted by 3M Environmental Laboratory are presented in 3M report Tables 1, 2 and 10 through 15 located in Appendix XIIB. The full report for analysis of sera and liver samples collected at the time of adult termination is located in 3M Environmental Laboratory report E01-1256. "Analytical Phase Report for PFOS: A Reproduction Study with the Mallard".

Reproductive Results

There were no treatment-related effects upon reproductive performance at the 10 ppm a.i. test concentration as compared to the controls. When compared to the control group, there were no statistically significant differences in any of the reproductive parameters measured in the 10 ppm a.i. test concentration. Summaries of the reproductive data are presented in Tables 7 and 8, and in Figures 4 and 5. Reproductive parameters by pen are presented in Appendix XIII and XIV.

Egg Shell Thickness

There were no apparent treatment related effects upon egg shell thickness at the 10 ppm a.i. test concentration. When compared to the control group, there were no statistically significant differences in egg shell thickness in the 10 ppm a.i. treatment group. Egg shell thickness data are presented in Table 9 and Appendix XV.

Offspring Body Weights

There were no apparent treatment related effects upon offspring body weight at the 10 ppm a.i. test concentration. When compared to the control group, there were no statistically significant differences in the body weight of hatchlings or 14-day old survivors from the 10 ppm a.i. treatment group. Offspring body weight data are presented in Table 10, and Appendices XVI and XVII.

Offspring Liver Weights

There were no treatment-related effects upon the mean weights of offspring livers at the 10 ppm a.i. test concentration. Any difference between the 10 ppm a.i. treatment group and the control group was not statistically significant. Mean offspring liver weights are presented in Table 6, while individual offspring liver weights are presented in Appendix XVIII.

Offspring Observations

There were incidental observations of neck curl, weakened condition and foot/leg lesions and associated lameness, for offspring in both the control group and 10 ppm a.i. treatment group. At the 10 ppm a.i. test concentration, a general observation was made that the offspring from Lots C and D were sluggish, with a slight reduced reaction to external stimuli. None of the offspring in the Lots proceeding or following Lots C and D were noted as sluggish or with a reduced reaction to external stimuli. Due to the isolated nature of the clinical signs of toxicity noted, these observations were not considered treatment related. All other offspring were normal in appearance and behavior throughout the test.

Offspring Liver and Sera

Blood and liver samples collected at the time of offspring termination for the control group and 10 ppm a.i. test concentration were shipped to 3M Environmental Laboratory for analysis. Summary results of sera and liver analyses conducted by 3M Environmental Laboratory are presented in 3M report Tables 1, 2 and 10 through 15 located in Appendix XIIB. The full report for analysis of sera and liver samples

collected at the time of juvenile termination is located in 3M Environmental Laboratory report E01-1256. "Analytical Phase Report for PFOS: A Reproduction Study with the Mallard".

CONCLUSION

There were no treatment-related mortalities, overt signs of toxicity, or treatment-related effects upon body weight, feed consumption or reproductive performance at the 10 ppm a.i. test concentration. However, there was an increased incidence of small testes for males in the 10 ppm a.i. test concentration that may have been related to treatment. There were treatment-related mortalities, overt signs of toxicity and treatment-related effects upon adult body weight at the 50 and 150 ppm a.i. test concentrations. There were also treatment-related effects feed consumption at the 150 ppm a.i. test concentration. The 50 and 150 ppm a.i. test concentrations were terminated prior to the reproductive phase of the test. Based on the effect observed at the 10 ppm a.i test concentration, the no-observed-effect concentration for mallards exposed to PFOS in the diet was determined to be less than 10 ppm a.i., the lowest concentration tested during this study.

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Table 1

Mean Measured Concentrations (ppm a.i.) of PFOS
in Avian Diet from a Mallard Reproduction Study

Nominal Concentration (ppm a.i.)		Interval								
		W	k 1	Week 2	Week 3	Week 4	Week 8	Week 12	Week 16	Week 20
		Day 0	Day 7[1]	Day 0	Day 0	Day 0	Day 0	Day 0	Day 0	Day 0
0	Measured	<2.00 [2]	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
10	Mean Measured									
	% Nominal	98 [3]	100 [4]	103	101	97	109	101	101	99
50	Mean Measured									
	% Nominal	100 [3]	101 [4]	97	104	104	-	-	-	-
150	Mean Measured									
	% Nominal	98 [3]	97 [4]	108	96 [5]	112 [5]	-	-	-	-

[1] Feed samples were coll ed from feed troughs at the end of Week 1 to assess stability of the test substance under actual test conditions.
[2] <LOQ indicates the val was less than the limit of quantitation.
[3] The mean of homogene samples collected at Week 1, Day 0.
[4] The percent of the mea alues for Week 1, Day 0.
[5] The 150 ppm a.i. treate iet was reduced to 20 ppm a.i. during this interval.
- No values available, te roup terminated.

Table 2
Summary of Gross Pathological Observations
from a Mallard Reproduction Study with PFOS
Adult Birds Found Dead/Euthanized during the Test

	Males			Females	
	Control (0 ppm a.i.)	50 [1] ppm a.i.	150 [2] ppm a.i.	50 [1] ppm a.i.	150 [2] ppm a.i.
Number of birds	1	17	2	18	2
Abrasion on mandible	0	1	0	0	0
Foot/leg lesions	1	3	0	0	0
Thin	0	4	0	3	0
Emaciated	0	0	1	2	1
Loss of muscle mass	0	1	1	3	1
Prominent keel	0	0	1	2	1
Breast muscle - hematoma and loose blood	0	1	0	0	0
Heart - flaccid	0	0	0	1	0
Heart - pale	0	3	0	1	0
Heart - petechial to ecchymatic hemorrhages	1	0	0	0	0
Left lung - yellow plaques on margins	0	1	0	0	0
Lungs - pale	0	1	0	0	0
Abdominal air sacs - cloudy	1	0	0	0	0
Spleen - small and/or pale	0	7	0	5	0
Spleen - enlarged and mottled	1	0	0	0	0
Liver - firm, pale and mottled	1	0	0	0	0
Abdominal cavity - autolysis	0	1	0	0	2
Abdominal cavity - small amount of clear fluid	1	0	0	0	0
Abdominal cavity - blood in left thoracic air sac	0	1	0	0	0
Gizzard contents - bile-stained	0	3	0	2	1
Small intestines - small white ulcers (polyps)	0	1	0	0	0
Intestinal contents - pasty	0	0	0	0	1
Intestinal contents - firm	0	1	0	1	0
Kidneys - pale	1	4	0	0	1
Not remarkable	0	7	1	10	0

[1] Remaining birds in the 50 ppm a.i. test concentration were euthanized at the end of Week 7 of the test. Gross necropsy observations for birds from which blood samples were collected are not reported.

[2] Remaining birds in the 150 ppm a.i. test concentration were euthanized at the beginning of Week 5. Gross necropsies were not performed at the termination of the 150 ppm a.i. test concentration.

Table 3

Mean Adult Body We ght (g) from a Mallard Reproduction Study with PFOS[1]

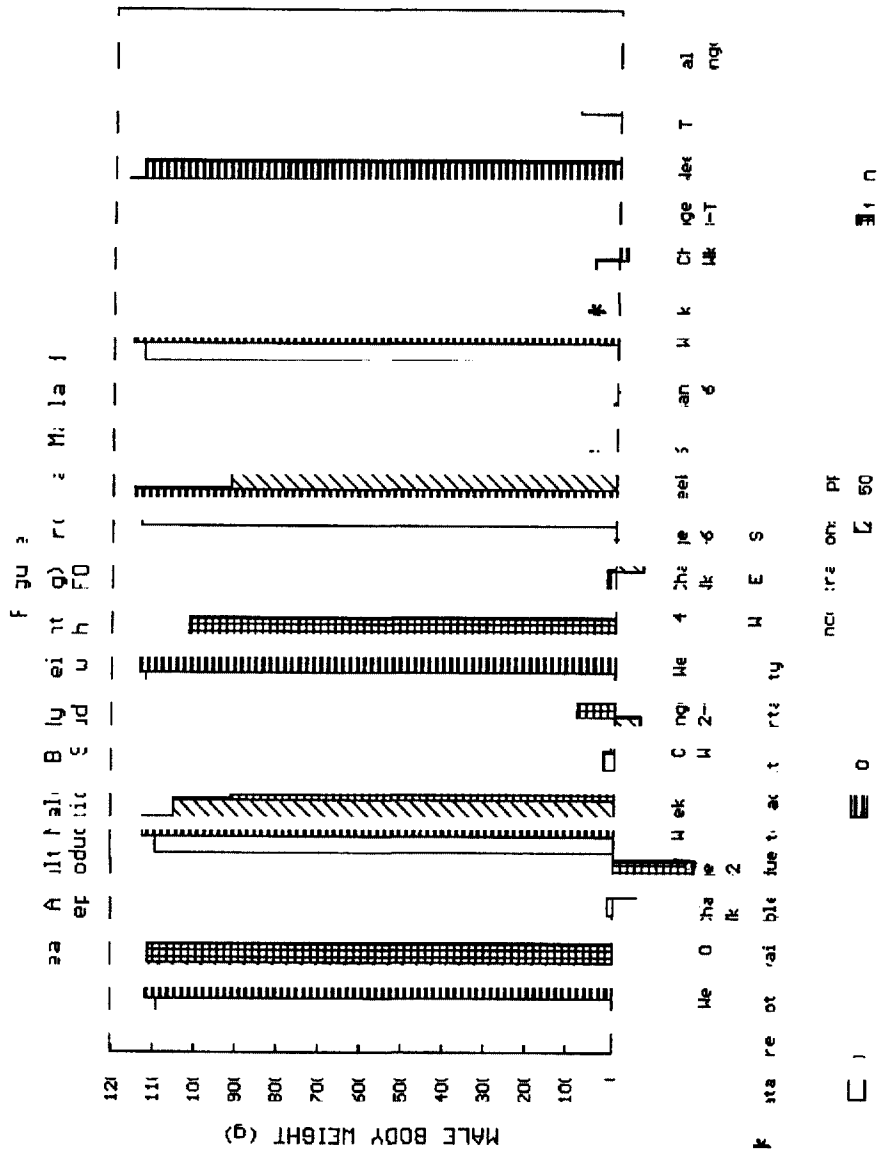
Experimental Group (ppm a.i.)	Sex	Week 0	Chan Week 0	Week 2	Change Week 2-4	Week 4	Change Week 4-6	Week 6	Change Week 6-8	Week 8	Change Week 8-Term	Test Term	Total Change
Control	Male	1084	7	1091	23	1114	13	1128	-3	1125	52	1164	92
	Female	984	4	988	14	1002	19	1020	12	1033	122	1155	171
10	Male	1111	10	1121	7	1127	17	1144	7	1151	-24	1127	17
	Female	982	22	1004	18	1022	19	1041	9	1049	95	1144	162
50	Male	1104	-57	1047	-64	983**	-66	916**	-	-	-	-	-
	Female	956	-83	873**	-60	813**	-31	781**	-	-	-	-	-
150	Male	1107	-195	912**	88	1016**	-	-	-	-	-	-	-
	Female	985	-206	780**	98	880**	-	-	-	-	-	-	-

The means for body weights and body weight changes are calculated and rounded separately.

- Data are not available due to adult mortality.

** Significantly different from the control at $p < 0.01$

[1] Only surviving birds were included in the calculations for each body weight interval.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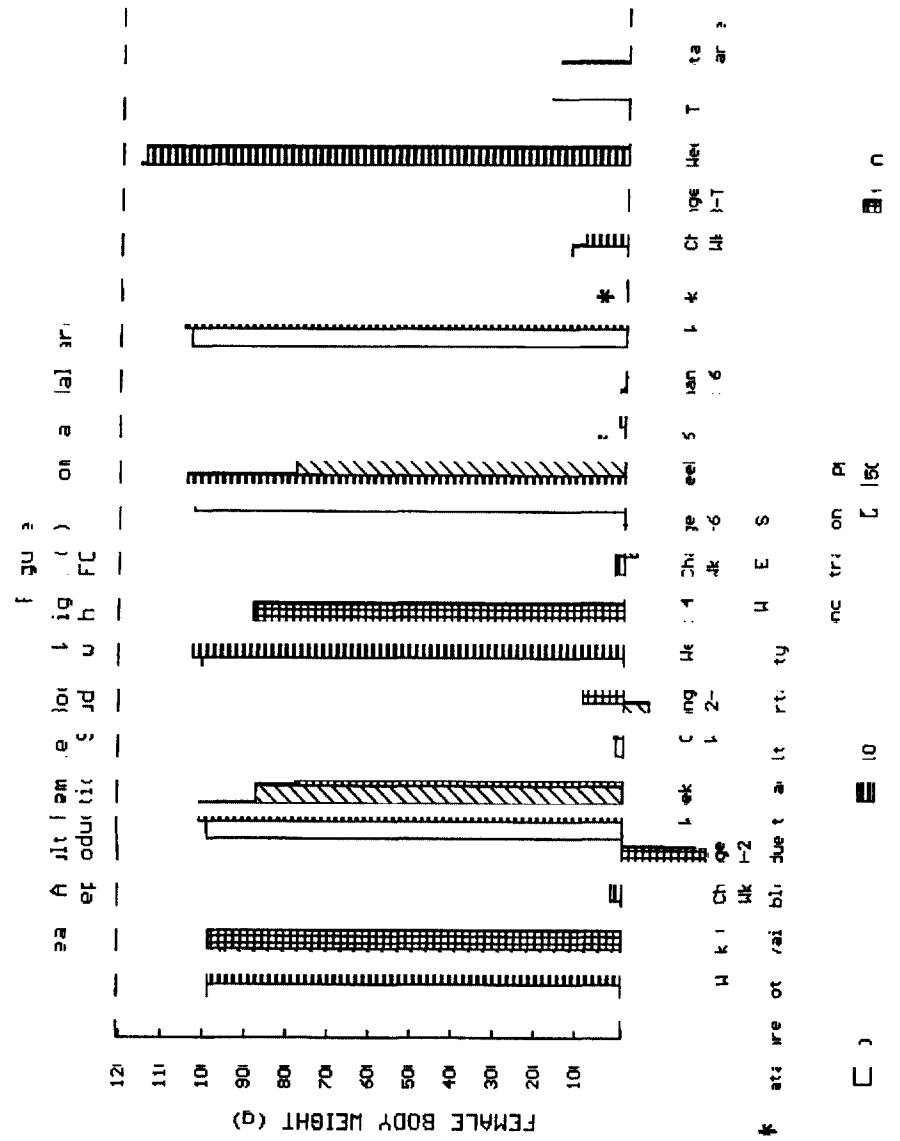
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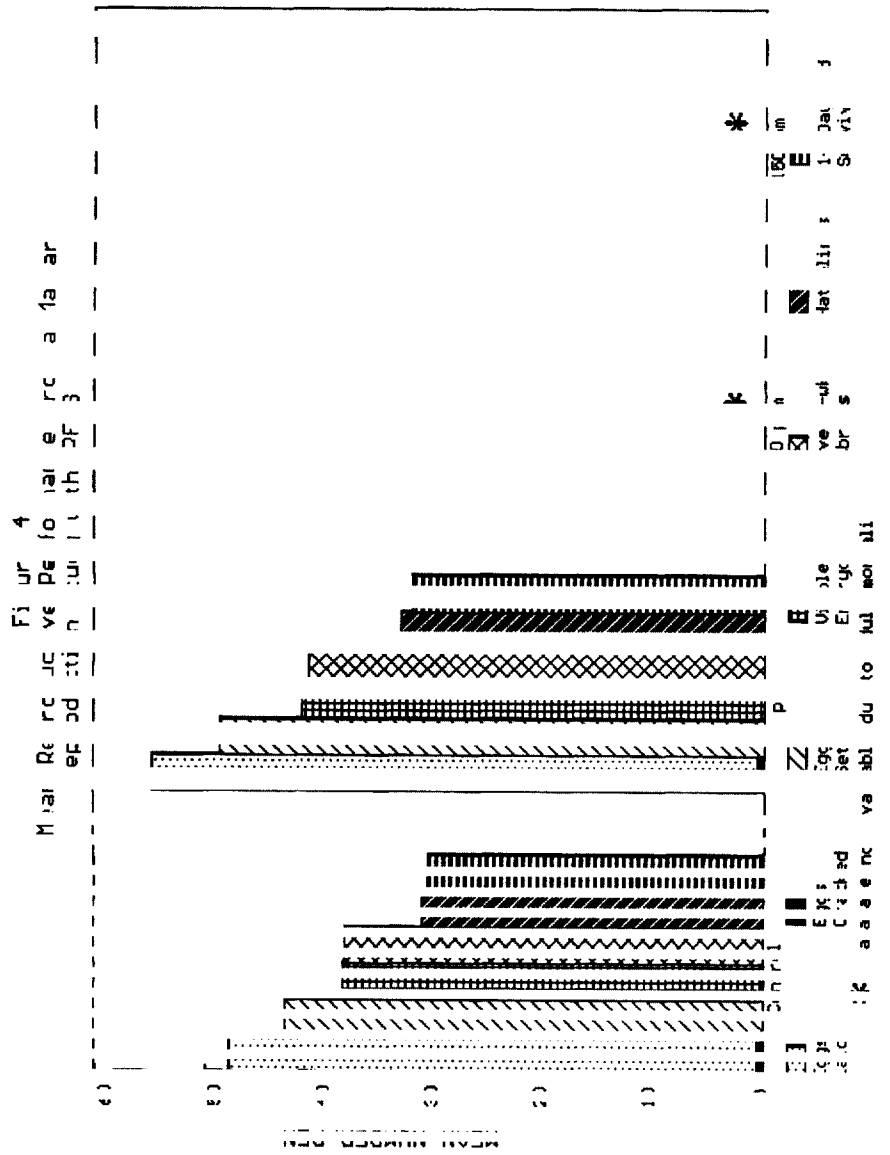
Table 7

Summary of Reproductive Performance from a Mallard Reproduction Study with PFOS

Reproductive Parameter	Experimental Group (ppm a.i.)			
	Control	10	50	150
Number of Replicates	15	16	-	-
Total Eggs Laid	719	877	-	-
Eggs Cracked	10	10	-	-
Eggs Set	644	781	-	-
Viable Embryos	567	664	-	-
Live 3-Week Embryos	564	653	-	-
Hatchlings	460	522	-	-
14-Day Old Survivors	454	507	-	-
Eggs Laid/Hen	48	55	-	-
Eggs Laid/Hen/Day[1]	0.67	0.76	-	-
14-Day Old Survivors/Hen	30	32	-	-

[1] Based on 72 days of eggs production.

- Data are not available due to adult mortality



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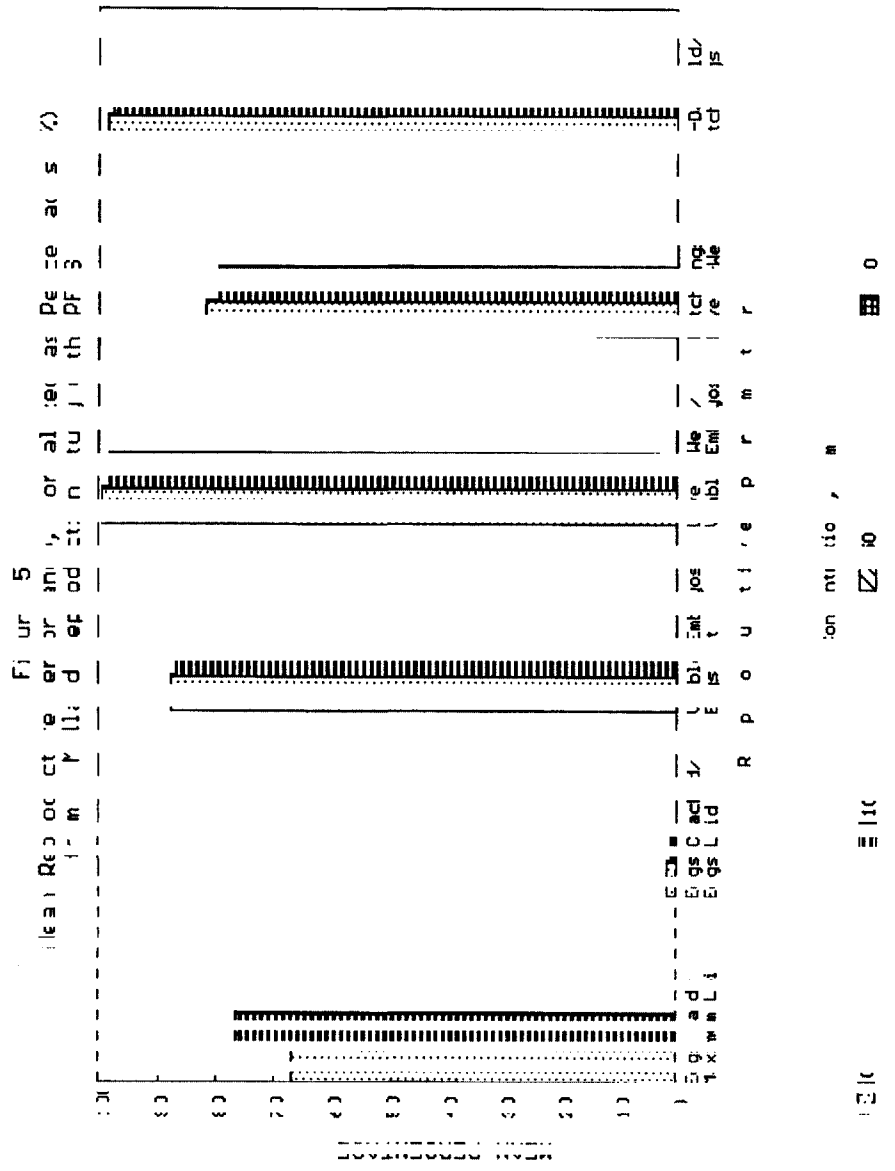

Table 9

Mean Eggshell Thickness Measurements (mm)
from a Mallard Reproduction Study with PFOS

Experimental Group (ppm a.i.)	No. Eggs Measured	Shell Thickness Mean (± SD)
Control	49	0.392 ± 0.034
10	54	0.396 ± 0.014
50	-	- -
150	-	- -

- Data are not available due to adult mortality

Differences between the control and each treatment groups were not significant ($p > 0.05$).

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