26a) OECD 206-OPPTS 850.2300 Protocol

PROTOCOL

T-7485: A REPRODUCTION STUDY WITH THE NORTHERN BOBWHITE

FIFRA Subdivision E, Section 71-4

OECD Guideline 206

3M Environmental Lab Project No. E03-0583

Submitted to

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T-7485: A REPRODUCTION STUDY
WITH THE NORTHERN BOBWHITE

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SPONSOR'S REPRESENTATIVE:	Dr. John Newsted Entrix East Lansing Office 4295 Okemos Road, Suite 101 Okemos, MI 48864
TESTING FACILITY:	Wildlife International, Ltd. 8598 Commerce Drive Easton, Maryland 21601
STUDY DIRECTOR:	Sean P. Gallagher Senior Avian Biologist
LABORATORY MANAGEMENT:	Joann B. Beavers Director of Avian Toxicology

FOR LABORATORY USE ONLY

Proposed Dates:
Experimental Start Date: _____ Experimental Termination Date: _____
Project No.: _____ Study Room: _____
Test Concentrations: _____
Test Substance No.: _____ Reference Substance No. (if applicable): _____

PROTOCOL APPROVAL

_____ _____
STUDY DIRECTOR DATE

_____ _____
LABORATORY MANAGEMENT DATE

_____ _____
SPONSOR'S REPRESENTATIVE DATE

OBJECTIVE

The objective of this study is to evaluate the effects of dietary exposure to T-7485 upon adult northern bobwhite (*Colinus virginianus*) over a five-month period. Effects on adult health, weight gain and feed consumption will be evaluated. In addition, the effects of adult exposure to the test substance on the number of eggs laid, normal development of eggs, viability of the embryos, percent hatchability, offspring survival and egg shell thickness will be evaluated.

SUMMARY

Three treatment groups, each containing 16 pairs of Northern bobwhite, will be fed diets containing T-7485 at selected concentrations. A control group containing 16 pairs will be maintained concurrently. The bobwhite will be 16 to 36 weeks old and approaching their first breeding season. The birds will be fed the test diets for approximately ten weeks prior to the expected onset of egg laying, as well as during an egg laying period of at least 8 weeks. During the study, all birds will be monitored for toxicological responses. In addition, each of the treatment groups will be compared to the controls in order to detect statistically significant differences in body weight, feed consumption, egg production, fertility, hatching success, shell thickness and survivorship of offspring. Samples of sera, liver, and egg contents will be collected for potential analysis. Additionally, samples of liver, kidney and gonad will be collected for histopathological examination. The approximate duration of the study is six months.

MATERIALS AND METHODS

The methods, species used and route of administration described in this protocol are based upon procedures specified in Section 71-4 of the Environmental Protection Agency's Registration Guidelines, Pesticide Assessment Guidelines, FIFRA Subdivision E Hazard Evaluation: Wildlife and Aquatic Organisms (1); ASTM "Standard Practice for Conducting Reproductive Studies with Avian Species" (2); and upon OECD Guideline 206, Avian Reproduction Test (3). In order to control bias, adult birds will be randomized to pens. No other potential sources of bias are expected to affect the results of the study.

The test substance will be administered in the diet. This route of administration was selected because it represents the most likely route of exposure to avian species in the environment.

Test Substance

The test substance will be T-7485. Information on the characterization of test, control or reference substances is required by Good Laboratory Practice (GLP) Standards and Principles. The Sponsor is responsible for providing Wildlife International, Ltd. verification that the test substance has been characterized according to GLPs prior to its use in the study. If verification of GLP test substance characterization is not provided to Wildlife International, Ltd., it will be noted in the compliance statement of the final report.

The Sponsor is responsible for all information related to the test substance and agrees to accept any unused test substance and/or test substance containers remaining at the end of the study.

Treatment Groups

There will be at least three treatment groups and a control group. Each treatment and the control group will consist of 16 pairs of birds, housed with one male and one female per pen. Control birds will receive diet identical to the treated birds, but without the addition of the test substance. The test concentrations were established by first conducting a pilot reproduction study. . Wildlife International, Ltd. consulted with the Sponsor prior to establishing the test concentrations.

Experimental Group (ppm a.i.)	Number of Pens Per Concentration	BIRDS PER PEN	
		MALES	FEMALES
Control (0)	16	1	1
100	16	1	1
300	16	1	1
900	16	1	1

Duration of Test

The primary phases of the test and their approximate durations are:

1. Acclimation - At least 2 weeks.
2. Pre-photostimulation - 7 to 10 weeks.

3. Pre-egg laying (with photostimulation) - Generally 1 to 4 weeks depending on response of birds.
4. Egg laying - At least 8 weeks.
5. Post-adult termination (final incubation, hatching, and 14-day offspring rearing period) - Approximately 6 weeks.

Test Species

The Northern bobwhite represents an ecologically significant and widely distributed species in the United States. The bobwhite has demonstrated sensitivity to the effects produced by known toxic chemicals and has proven to be a good laboratory species from which a large amount of baseline data has been gathered.

All bobwhite will be 16-36 weeks of age and apparently healthy at initiation of the test (first day of exposure to diets containing the test substance). All test birds will be from the same hatch. Selection of the appropriate weight range will be in accordance with Wildlife International, Ltd. Standard Operating Procedures. Test birds will be approaching their first breeding season at the time of test initiation. Sex of the birds will be determined by a visual examination of the plumage. Birds will be obtained from K & L Quail Farm, Oroville, California or from another reputable supplier.

All test birds will be acclimated to the caging and facilities for at least fourteen days prior to initiation of the test. Extra birds from the same lot will be acclimated and may be used as replacements prior to the initiation of administration of test diets. Birds will be replaced if a pair appears to be incompatible or if a bird is deemed unsuitable due to injury, poor health or abnormal behavior.

Identification

Adult birds will be identified by individual leg bands and will be randomly assigned to a pen and treatment group. Each pen will be identified by a unique number, and groups of pens will be identified by project number and concentration. All eggs laid during the study will be marked with a soft lead pencil or permanent ink for identification. Hatchlings will be identified by leg banding.

Animal Diet

All adult test birds will be fed a game bird ration formulated to Wildlife International, Ltd. specifications (Table 1). Five percent (w/w) limestone will be added to the ration of adult birds to

provide a calcium source. Water will be provided by the town of Easton public water supply. Feed and water will be analyzed periodically according to Wildlife International, Ltd. Standard Operating Procedures. Neither the adults nor offspring will receive any form of antibiotic medication in the diet during the test without prior approval from the Sponsor.

Specifications for acceptable levels of contaminants in game bird ration for avian species have not been established. However, there are no known levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the study.

The adult birds will receive the appropriate treated or control diet for at least ten weeks prior to the anticipated onset of egg production. Exposure will continue until the adult termination. Appropriate feed and water will be provided *ad libitum* during acclimation and during the test.

All offspring also will receive game bird ration (Table 1), but without the addition of limestone. The test substance will not be mixed into the diet of the offspring. Offspring will receive a water-soluble vitamin mix in their water. Feed and water will be provided to the offspring *ad libitum* during rearing.

Diet Preparation

Test diets will be prepared by mixing the test substance directly into the feed. . Diet premixes will be prepared in a Hobart mixer (Model Number AS200T). If not used immediately, the premix will be frozen until used. Premixes will be prepared as frequently as necessary to assure stability of the test substance. Once each week, aliquots of the premix will be blended into bulk quantities of the ration to give the desired dietary concentrations of the test substance. Bulk diet mixing will be done in a Hobart mixer or a Patterson-Kelley twin shell dry blender. Excessive feed consumption may necessitate more frequent preparation of the diet.

All test substance calculations will be based on the purity of the test substance as received or corrected to 100% active ingredient based on the information provided by the Sponsor.

Diet Sampling

Samples of the experimental diets will be collected for chemical analysis to determine the homogeneity and stability of the test substance in the avian diet and to verify/measure test concentrations. All samples will be placed in uniquely identified polypropylene jars.

Samples will be collected on Day 0 of Week 1, at diet preparation, to determine homogeneity, measure test concentrations, and establish Day 0 values for evaluating stability. On Day 7 of Week 1, samples will be collected from the feeders containing the experimental diets to assess stability of the test substance under test conditions. Additionally, verification samples will be collected at specified intervals to measure/verify test concentrations. If a separate study is conducted with a different species (e.g., mallard), at the same test concentrations, diets that are prepared may be divided between the two studies. Therefore, samples collected for homogeneity and verification will be common to both studies. However, stability samples (e.g., Week 1 - Day 7) will be unique to that respective study. Stability samples will be collected by compositing diet from feeders presented to the birds, and taking samples from the composited feed. The diet sampling scheme is summarized below:

ESTIMATED NUMBER OF SAMPLES

Experimental Group	Week 1		Week	Week	Week	Week	Week
	Day 0	Day 7	4	8	12	16	20
Control	1	1	1	1	1	1	1
100 ppm	62	2	2	2	2	2	2
300 ppm	62	2	2	2	2	2	2
900 ppm	62	2	2	2	2	2	2
Totals	19	7	7	7	7	7	7
							GRAND TOTAL = 61

[1]Samples collected during Weeks 4 through 20 will be taken on Day 0 of the corresponding week.

[2]Samples collected from the left and right sides of the top, middle and bottom layers of feed in the mixing vessel to determine homogeneity.

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study. If the number of treatment groups is increased, the number of samples collected will increase proportionately.

Diet Analyses

Samples of the experimental diets will be stored in a freezer until prepared and/or extracted and analyzed. Chemical analyses of diets will be performed by Wildlife International, Ltd using liquid chromatography-mass spectrometry. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Housing and Environmental Conditions

The adult birds will be housed in battery breeding pens manufactured by GQF Manufacturing Company (Model No. 206 or 0330) or equivalent. Each pen has floor space that measures approximately 25 X 51 cm. Floors are sloping so that ceiling height will range from approximately 20 to 26 cm. External walls, ceilings and floors of each breeder pen are constructed of wire mesh while side walls are constructed of galvanized sheeting. Each pen will house one male and one female. During the test all birds will be maintained at an ambient temperature of approximately 15 to 30°C. Ambient temperature and relative humidity will be recorded at least twice per day during the test.

During the first seven weeks, the photoperiod for the adults will be 8 hours of light per day. The photoperiod will then be increased to 17 hours of light per day to induce egg laying. The average light intensity for each test concentration will be at least sixty-five lux. Light intensity will be measured at least once during the test in accordance with Wildlife International, Ltd. Standard Operating Procedures. The average will be based upon light intensity measurements in each pen at bird height. Lighting will be provided by fluorescent lights which closely approximate noon-day sunlight (noon-day sun - 4870°, Chroma 50 or equivalent - 5000° Kelvin).

Hatchlings will be placed in batteries of thermostatically controlled brooding pens manufactured by the Beacon Steel Company (Model B735Q) or equivalent. Each pen measures approximately 72 X 90 X 23 cm high. The external walls and ceilings of each pen are constructed of wire mesh and galvanized sheeting. Floors are of wire mesh. The brooding compartment temperature will be maintained at approximately 38°C and will be recorded once per day. Acceptable ranges for brooding compartment temperatures will be specified in Wildlife International, Ltd. Standard Operating Procedures. Ambient room temperature will be maintained at approximately 16 to 32°C. Ambient temperature and relative humidity will be recorded at least twice daily throughout the test. Photoperiod for the offspring will be 16 hours of light per day.

Housing and husbandry practices will be conducted so as to adhere to the guidelines established by the National Research Council (4).

Incubation and Hatching

Eggs will be collected daily, and marked with a soft lead pencil or permanent ink according to the pen from which they were collected. Eggs then will be stored at an average temperature of approximately 10 to 16°C and an overall relative humidity of approximately 40 to 95%. Temperature and relative humidity in the cold room will be recorded twice per day during egg storage.

All eggs collected will be placed in order by pen number and counted each week. Eggs selected for egg shell thickness measurements then will be removed. All remaining eggs will be candled to detect egg shell cracks or internal abnormalities. Cracked or abnormal eggs will be recorded and discarded. All eggs not selected for egg shell thickness measurement or discarded as cracked or abnormal will be fumigated with formaldehyde gas to reduce the possibility of pathogen contamination.

Following fumigation the eggs will be incubated. Temperature and relative humidity in the incubator will average approximately 37.2-37.8°C and 50-60%, respectively. The eggs will be candled between Days 10-12 for embryonation, and between Days 20-22 for embryo survival. Between Days 20-22 of incubation the eggs will be removed from the incubator and placed in a hatcher. Temperature and humidity in the hatcher will average approximately 36.9-37.5°C and 60-80%, respectively. Dividers will be placed in the hatching trays to separate hatchlings by parental pen. Wet and dry bulb temperatures in the incubator and hatcher will be recorded at least twice daily during incubation and hatching.

Hatchlings will be removed from the hatcher over an approximately 24 hour period beginning on approximately Day 25. Hatchlings will be leg banded so that they can be identified by parental pen. Offspring will be observed over a 14 day period beginning when birds are first removed from the hatcher. The observation period will be extended if late mortality occurs which appears to be treatment related.

Observations

All adults and offspring will be observed at least once daily for mortality, general condition, overt signs of toxicity and abnormal behavior.

Animal Body Weights/Feed Consumption

Individual body weights of the adults will be taken at the initiation of the test, at the end of test weeks 2, 4, 6, 8 and at adult termination. Group body weights of offspring by parental pen will be recorded at hatching and on Day 14 post-hatch.

With the exception of the last interval, feed consumption of the adults will be recorded by pen for a seven-day period each week throughout the test. The length of the last feed consumption interval will be determined by the scheduling of adult termination. Feed consumption will be measured by weighing the freshly filled feeder on Day 0 of each week, recording any additional diet added during the week, and weighing the feeder and remaining feed at the end of the seven day period. The accuracy of feed consumption values may be affected by unavoidable wastage of feed by birds. No attempt will be made to quantify the amount of wasted feed as it is normally scattered and mixed with water and excreta.

Reproductive Parameters

The following reproductive parameters will be measured and recorded:

1. eggs laid
2. eggs cracked
3. eggs incubated
4. infertile or clear eggs
5. dead embryos (to Day 10-12)
6. viable embryos
7. dead embryos (to Day 20-22)
8. live three week embryos
9. unhatched eggs
10. hatchlings
11. 14-day old offspring survival

12. offspring body weights, day 0 and 14
13. egg shell thickness

Egg Shell Thickness Measurements

Each week eggs will be selected from those eggs laid during that week for egg shell thickness measurement. Normally, one egg will be collected from each of the odd numbered pens during odd numbered weeks (1,3,5, etc.), and one egg will be collected from each of the even numbered pens during even numbered weeks (2,4,6, etc.). Each egg will be cut open, the contents will be placed in an appropriate labeled container and stored frozen for potential analysis. The empty shell will be rinsed with tap water, and the shell air dried for at least one week. The thickness of the dried shell, including membranes, will be measured to the nearest 0.002 mm at five points around the equator of the egg with a micrometer. Egg contents that are collected will be shipped to the Sponsor's designate and analyzed at the Sponsor's discretion.

Necropsy and Tissue Collection

All adult test birds that die during the course of the test and all adults remaining at the termination of the adult portion of the test will be subjected to a gross necropsy. Adult birds that are not found dead during the course of the study will have blood drawn prior to euthanasia. The blood will be separated and the serum collected for potential analysis. Livers from each bird will be weighed and collected. A portion of the liver will be collected for histopathological examination, and the remaining liver will be stored frozen for potential analysis. Samples of kidney and gonad also will be collected for histopathological examination. All histopathological samples will be fixed in 10% buffered formalin and sent to EPL in Herndon, Virginia for histopathology. Additionally, similar samples will be collected from a single indiscriminately selected offspring from each pen (if available) at 14 days of age. Offspring will be sampled from the last lot of offspring produced. All samples collected for potential analysis will be placed in labeled polypropylene jars or other suitable containers and stored frozen until shipped to the Sponsor's designate. Blood and tissue samples will be analyzed at the Sponsor's discretion.

Disposition of Test Birds

At test termination, test birds will be euthanized by using carbon dioxide gas, cervical dislocation or other appropriate methods. The method used will be documented in the raw data. All birds will be disposed of by incineration or other appropriate methods.

Statistical Analyses

Each of the treatment groups will be compared to the control group using Dunnett's Multiple Comparison Procedure (5,6). The sample unit will be the individual pens within each experimental group, except for adult body weights where the sample unit will be the individual bird. Data expressed as a percentage will be arcsine transformed prior to using Dunnett's method. Nonparametric statistics may be used, if appropriate. Any pen in which an adult mortality occurs, including the on the day the study is terminated, will not be used in statistical comparisons of the reproductive data Analyses will be performed on each of the following parameters:

1. adult body weight
2. adult feed consumption
3. eggs laid per pen per day (eggs laid/maximum laid)
4. eggs cracked of eggs laid
5. viable embryos of eggs incubated
6. live 3-week embryos of viable embryos
7. hatchability of 3-week embryos
8. fourteen-day old survivors of normal hatchlings
9. normal hatchlings of eggs incubated
10. normal hatchlings per pen per day (hatchlings/maximum set)
11. fourteen-day old survivors of eggs incubated
12. fourteen-day old survivors per pen per day (14-day olds/maximum set)
13. hatchling body weights
14. fourteen-day old survivor body weights
15. egg shell thickness

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International, Ltd. will include:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Date of initiation, critical phases, and termination of the test.
4. Animal history.
5. Husbandry and environmental conditions.
6. Dietary concentration calculations and diet preparation.
7. Individual body weight measurements of adults and group body weight measurements of offspring.
8. Feed consumption measurements of adults.
9. Daily observations.
10. Necropsy findings.
11. Records of all reproductive parameters detailed in this protocol.
12. Analytical chemistry methods, results and chromatograms, if applicable.
13. Statistical calculations, if applicable.
14. A copy of the final report.

FINAL REPORT

A final report of the results of the study will be prepared by Wildlife International, Ltd. The report will include, but not be limited to, the following:

1. Name and address of the facility performing the study.
2. Experimental start and experimental termination dates and study completion date. It is the responsibility of the Sponsor to provide the final date that data are recorded for chemistry, pathology and/or supporting evaluations that may be generated at other laboratories.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures stated in the approved protocol, including any changes in the original protocol.
5. Statistical methods employed for analyzing the data, when applicable.

6. The test, control and reference substances identified by name, chemical abstracts number or code number, strength, purity, and composition or other appropriate characteristics, if provided by the Sponsor.
7. Stability and, when relevant to the conduct of the study, the solubility of the test, control and reference substances under the conditions of administration, if provided by the Sponsor.
8. A description of the methods used.
9. A description of the test system used. Where applicable, the final report shall include the number of animals used, sex, body weight range, source of supply, species, age, and procedure used for identification.
10. A description of the dosage, dosage regimen, route of administration, and duration.
11. A description of all circumstances that may have affected the quality or integrity of the data.
12. The name of the Study Director, the names of other scientists or professionals, and the names of all supervisory personnel, involved in the study.
13. A description of the transformations, calculations, or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis.
14. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
15. The location where all specimens, raw data, and the final report are to be stored.
16. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.
17. If it is necessary to make corrections or additions to a final report after it has been accepted, such changes shall be in the form of amendment by the Study Director. The amendment should clearly identify the part of the final report that is being added to or corrected and the reasons for the correction or addition. Amendments shall be signed and dated by the Study Director.

CHANGING OF PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and approved by the Sponsor's Representative. Amendments will be considered as part of the protocol and will be attached to the final protocol. Any other changes will be in the form of written deviations signed by the Study Director and filed with the raw data. All changes to the protocol will be indicated in the final report.

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160); OECD Principles of Good Laboratory Practices (ENV/MC/CHEM (98) 17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International, Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

REFERENCES

1 **U.S. Environmental Protection Agency**. 1982. *Pesticide Assessment Guidelines, FIFRA Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms*, subsection 71-4, Environmental Protection Agency, Office of Pesticide Programs. Washington, D.C.

2 **American Society for Testing and Materials**. 1986. *Standard Practice for Conducting Reproductive Studies with Avian Species*. ASTM Standard E1062-86. Annual Book of ASTM Standards. Vol. 11.04. Philadelphia, PA. 15 pp.

3 **Organization for Economic Cooperation and Development.** 1984. *Avian Reproduction Test.* OECD Guideline for Testing of Chemicals. Guideline 206. Paris.

4 **National Research Council.** 1996. *Guide for the Care and Use of Laboratory Animals.* Washington, DC. National Academy Press. 125 pp.

5 **Dunnett, C.W.** 1955. A Multiple Comparison's Procedure for Comparing Several Treatments with a Control. *Jour. Amer. Statis. Assoc.* 50: 1096-1121.

6 **Dunnett, C.W.** 1964. New Tables for Multiple Comparisons with a Control. *Biometrics* 20: 482-491.

TABLE 1: DIET FORMULATION
WILDLIFE INTERNATIONAL, LTD. GAME BIRD RATION[1]

INGREDIENTS	PERCENT (%)
Fine Corn Meal	44.83
Soy Bean Meal, 48% Protein	30.65
Wheat Midds	6.50
Protein Base	6.00
Agway Special, 60% Protein	4.00
Alfalfa Meal, 20% Protein	3.00
Dried Whey	2.50
Ground Limestone	0.90
Eastman CalPhos	0.61
Methionine Premix + Liquid	0.34
Vitamin and Mineral Premix (see below)	0.32
GL Ferm (Fermatco)[2]	0.25
Salt Iodized	0.10
Total	100.00

VITAMIN AND MINERAL PREMIX	AMOUNT ADDED PER TON
Vitamin D_3	2,000,000 I.C.U.
Vitamin A	7,000,000 I.U.
Riboflavin	6 grams
Niacin	40 grams
Pantothenic Acid	10 grams
Vitamin B_{12}	8 mgs
Folic Acid	600 mgs
Biotin	64 mgs
Pyridoxine	1.2 grams
Thiamine	1.2 grams
Vitamin E	20,000 I.U.
Vitamin K (Menadione Dimethylpyrimidinol Bisulfite)	5.8 grams
Manganese	102 grams
Zinc	47 grams
Copper	6.8 grams
Iodine	1.5 grams
Iron	51 grams
Selenium	182 mgs

[1] The guaranteed analysis is a minimum of 27% protein, a minimum of 2.5% crude fat and a maximum of 5% crude fiber.
[2] Fermentation By-Products (Source of Unidentified Growth Factors)