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Standard Evaluation Procedure

Avian Reproduction Test

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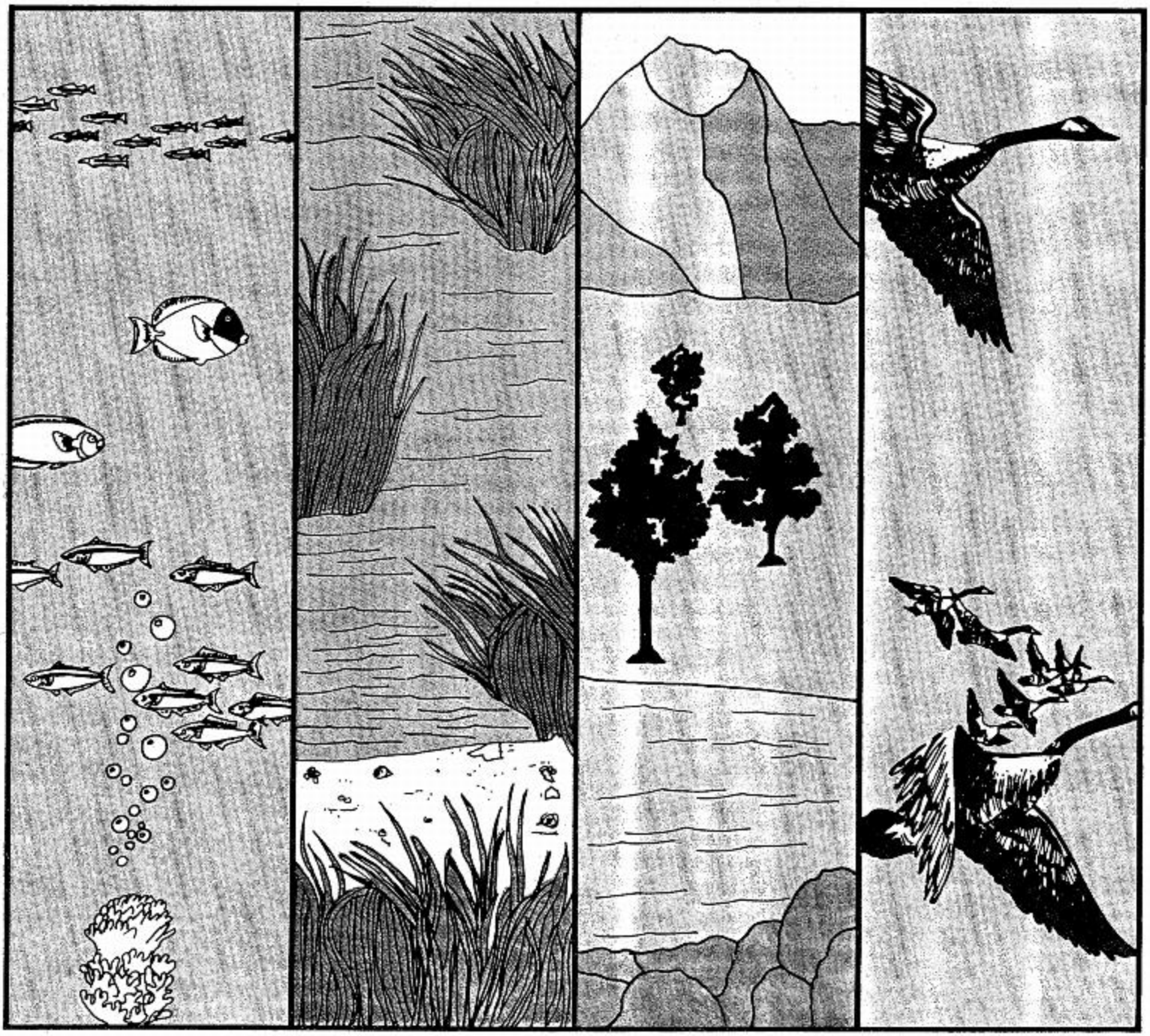

HAZARD EVALUATION DIVISION

STANDARD EVALUATION PROCEDURE

AVIAN REPRODUCTION TEST

Prepared by

Dennis J. McLane, B.S.

Standard Evaluation Procedures Project Manager
Stephen L. Johnson
Hazard Evaluation Division
Office of Pesticide Programs

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Washington, D.C. 20460

STANDARD EVALUATION PROCEDURE

PREAMBLE

This Standard Evaluation Procedure (SEP) is one of a set of guidance documents which explain the procedures used to evaluate environmental and human health effects data submitted to the Office of Pesticide Programs. The SEPs are designed to ensure comprehensive and consistent treatment of major scientific topics in these reviews and to provide interpretive policy guidance where appropriate. The Standard Evaluation Procedures will be used in conjunction with the appropriate Pesticide Assessment Guidelines and other Agency Guidelines. While the documents were developed to explain specifically the principles of scientific evaluation within the Office of Pesticide Programs, they may also be used by other offices in the Agency in the evaluation of studies and scientific data. The Standard Evaluation Procedures will also serve as valuable internal reference documents and will inform the public and regulated community of important considerations in the evaluation of test data for determining chemical hazards. I believe the SEPs will improve both the quality of science within EPA and, in conjunction with the Pesticide Assessment Guidelines, will lead to more effective use of both public and private resources.

John W. Melone, Director
Hazard Evaluation Division

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AVIAN REPRODUCTION TEST

I. INTRODUCTION

A. When Required

Avian reproductive effects are required to support the registration of an end-use product which meets one or more of the following criteria:

- Its labeling contains directions for using the product under conditions where birds may be subject to repeated or continuous exposure to the pesticide or any of its major metabolites or degradation products, especially preceding or during the breeding season;
- The pesticide or any of its major metabolites or degradation products are stable in the environment to the extent that potentially toxic amounts may persist in avian feed;
- The pesticide or any of its major metabolites or degradation products is stored or accumulated in plant or animal tissues, as indicated by the partition coefficient of lipophilic pesticides, metabolic release and retention studies, or as indicated by structural similarity to known bioaccumulative chemicals; and/or
- Any other information, such as that derived from mammalian reproduction studies, that indicates reproduction in terrestrial vertebrates may be adversely affected by the anticipated use of the pesticide product.

B. Purpose

The data from this study are used to:

- Establish the effects of the active ingredient on bird reproduction;
- Determine the stage of the reproductive cycle affected for the tested species;
- Compare toxicity information with measured or estimated pesticide residues in the terrestrial environment in order to assess potential impact on avian wildlife;
- Determine susceptibility differences between waterfowl and upland game;

° Provide support for precautionary label statements to minimize adverse effects to avian wildlife; and

° Indicate the need for further testing and/or field studies.

C. Test Substance

1. Technical Grade

This study must be conducted with the technical grade active ingredient (a.i.). If more than one a.i. constitutes a technical product, then the technical grade of each a.i. must be tested separately.

II. MATERIALS AND METHODS: TESTING STANDARDS/RECOMMENDATIONS

A. Acceptable Protocols

Because avian reproduction testing is an established technique for assessing toxicity of a chemical to avian wildlife, much of the methodology for performing these studies, as well as the procedures for statistical analysis of results, have been carefully outlined and documented in the published literature. Notably, the information to be discussed in this Standard Evaluation Procedure (SEP) is presented in greater detail in the following reference.

Ecological Effects Branch 1982. Pesticide Assessment Guidelines Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms, EPA-540/9-82-024. pp. 48-57.

B. Test Organisms

1. Acceptable Species/Age/Physical Condition

Testing must be done on both a wild waterfowl species, preferably mallard duck (Anas platyrhynchos) and an upland game species, preferably bobwhite quail (Colinus virginianus). Birds approaching their first breeding season should be used.

Test birds should be pen-reared and phenotypically indistinguishable from wild birds. It is recommended that the birds to be used are selected only from colonies that have had breeding histories maintained for them. This history should include lighting practices during rearing, disease record, drugs and any other medication administered, and exact age. If shipped, all birds should be examined following shipment for possible physical injury that may have been encountered in transit. If deemed necessary, several birds may be randomly selected for pretreatment necropsy at a diagnostic laboratory to assess the state of health upon arrival.

Sickness, injuries, excessive mortality of hatchlings or any abnormal observations of birds are all signs that the affected lot may not be adequate for testing.

These species were chosen because of their commercial availability, ecological significance, broad geographical distribution, commercial and recreational importance, ease of handling in the laboratory, and their past use in toxicity testing and known susceptibility to chemical exposure. The test species should be verified by its scientific name.

2. Source/Acclimation

Within a given test all organisms must be from the same source (this includes laboratory or commercial stocks). It is desirable to have a two- to six-week health observation period prior to selection of birds for treatment.

C. Test Conditions

1. Number Per Level

For either bobwhite quail or mallard ducks a minimum of three test groups should be used. One group should serve as a control and two groups as treated birds. For bobwhite quail one male and two females per pen by random distribution, replicated by a minimum of 12 pens, should be used per group.

For mallards two males and five females per pen by random distribution, replicated by five or more pens, should be used per group. For either species if individual pairs (one male and one female) are to be used per pen, considerably more pens, greater than 12 per test group, should be used to provide similar sensitivity to the group testing design. Control and treated birds should be kept under the same experimental conditions.

When other test data reveal bioaccumulative potential, the number of test animals in the test group should be increased sufficiently to partly offset animal deaths or data-gathering problems associated with morbidity or with tissue residue determinations.

2. Pen Facilities

Pen conditions should conform to good husbandry practices. This implies clean pens, adequate room, clean food and water, heated areas for young birds, and protection from excessive disturbance. Pens should also be arranged to prevent cross-contamination. Pen construction materials should not be toxic, capable of excessively adsorbing test substance, dissolve in water, or loosened by pecking. For example, stainless steel, galvanized steel and materials coated with perfluorocarbon plastics can be used. The temperature and relative humidity should be controlled throughout the reproductive test and should be recorded. Recommended levels are 21°C and 55 percent relative humidity. Ventilation is necessary. It is desirable to offer mallards water in which to bathe.

3. Photoperiod

Since light is extremely important, both during rearing and during the egg laying period, all birds should be maintained for the first eight weeks under a regime of seven hours of light per day for maximum egg production.

The photoperiod should then be increased to 16-17 hours of light per day and either maintained at this level or increased by 15 minutes per week for the following 12 weeks. (The 12-week period may vary depending upon the time required for the onset of egg production.) An illumination intensity of six footcandles at the bird level during the lighting phase of the reproductive study is adequate. Avoid the use of shorter wavelength "cool white" fluorescent lights which do not emit the daylight spectrum.

4. Body Weights

Body weights should be recorded at test initiation and at biweekly intervals up to week eight or up to the onset of egg laying and at termination. During egg laying, body weight recording is discouraged because of the adverse effects that handling may have on egg production.

5. Food Consumption

Food consumption should be recorded at least at biweekly intervals throughout the study.

Estimates of average consumption for each concentration level must be reported (grams per day). Provisions for minimizing food spillage and preventing air contamination by volatile chemicals should be reported.

6. Dose Preparation

Concentrations for the test substance should be based on measured or calculated residues expected in the diet from the proposed use pattern(s). The concentrations should include an actual or expected field residue exposure level and a multiple level such as five. The highest nonlethal level may be estimated from data developed from the avian dietary LC_{50}.

The test material should be added to table grade corn oil or another appropriate vehicle and premixed with an aliquot of basal diet, utilizing a mortar and pestle or mechanical blender. It is recommended that the aliquot of basal diet used for the premix be screened to remove large particles of diet before blending in the corn oil and test material. The final diet should be a uniformly mixed composition consisting of 98 to 99 parts by weight of basal diet and one or two parts by weight of corn oil. The basal diet

should be a commercial game bird breeder ration (or its equivalent) that is treated with an equivalent amount of vehicle. The premix should be stored under conditions which maintain stability. Test diets should be analyzed for pesticide concentrations at intervals during the tests. If other long-term animal tests have demonstrated a propensity for the test chemical to persist or bioaccumulate, the degree of bioaccumlation in birds should be determined by measurement of tissue residues in the birds from an extra pen group put through the reproduction test. Two or three tissues should be selected for residue analysis at the end of the exposure period, based on tissues known from other studies to hold highest residues.

Control diets must contain the maximum amount of vehicle available to treatment birds.

7. Feeding and Husbandry

All birds should receive the appropriate diet ad libitum for the duration of the study. Water is to be provided ad libitum. The test chemical should be administered for at least ten weeks prior to the onset of egg laying.

8. Egg Collection, Storage, and Incubation

All eggs should be collected daily, marked according to pen from which collected, and stored at 16°C and 65 percent relative humidity. Eggs should be set at weekly intervals for incubation in a commercial incubator. All eggs should be candled on day 0 for eggshell cracks; on approximately day 11 for bobwhites and day 14 for mallards to measure fertility and early death of embryos; and on day 18 for bobwhite and day 21 for mallards to measure embryo survival. For hatching, transfer of the eggs to a separate commercial incubator or hatcher should be made on day 21 for bobwhites and day 23 for mallards.

Recommended temperatures and relative humidity during hatching phase are 39°C and 70 percent, respectively.

9. Observations

a. Bobwhite Chicks

On day 24 of incubation, the hatched bobwhite chicks should be removed, hatchability recorded, chicks housed according to the appropriate parental grouping, and maintained on control diet for 14 days. The time period should be extended if mortality occurs appreciably late. The diet should be a commercial bobwhite starter diet or its equivalent.

b. Mallard Ducklings

On day 27 of incubation, the hatched mallard ducklings should be removed, hatchability recorded, ducklings housed according to

the appropriate parental grouping, and maintained on control diet for 14 days. The time period should be extended if mortality occurs appreciably late. The diet should be commercial mallard starter diet or its equivalent.

10. Eggshell Thickness

One day every two weeks newly laid eggs should be collected and measured for eggshell thickness. For consistency, the eggs used for thickness determinations should be collected during weeks one, three, five, seven, and nine of the egglaying period. An accepted procedure is to crack open the eggs at the widest portion (girth or waist), wash out all egg contents, air-dry the shells for at least 48 hours, and then measure the thickness of the dried shell plus the membranes at three or four points around the girth using a micrometer calibrated to 0.01 mm units.

11. Withdrawal

If the test substance is toxic (reduced reproduction evident), then a withdrawal study period should be added to the test phase. The withdrawal period need not exceed three weeks. Continued observations should be made on egg production, fertility, hatchability, and hatching survival.

12. Definitions

a. Eggs Laid

The total egg production during a breeding season (which is approximately ten weeks).

b. Eggs Cracked

Eggs determined to have cracked shells when inspected with a candling lamp; fine cracks cannot be detected without utilizing a candling lamp and if undetected will bias data by adversely affecting embryo development.

c. Eggs Set

All eggs placed under incubation, i.e., total eggs laid minus cracked eggs and those selected for eggshell thickness analysis.

d. Viable Embryos (Fertility)

Eggs in which fertilization has occurred and embryonic development has begun. This is determined by candling the eggs six to 14 days after incubation has begun. It is difficult to distinguish between the absence of fertilization and early embryonic death. This distinction can be made by breaking out eggs that appear infertile and examining further. This is especially important when a test compound induces early embryo mortality.

e. Live Three-Week Embryos

Embryo that is developing normally after three weeks of incubation. This is determined by candling the egg.

f. Hatchability

The percentage of embryos that mature, pip the shell, and liberate themselves from their eggs as computed from the number of fertile eggs. For quail this generally occurs on day 23 or 24 of incubation, and for mallard on day 25, 26, or 27.

g. Fourteen-Day-Old Survivors

Birds that survive for 2 weeks following hatch.

h. Eggshell Thickness

The thickness of the shell and the membrane of the egg at the girth after the egg has been opened, washed out, and dried for at least 48 hours at room temperature.

III. REPORTING REQUIREMENTS

A. Test Material

The purity of the test material must be stated and should generally be "technical" grade unless otherwise required. The percent a.i. must be stated.

B. Observable Effects Criteria

The criteria for determining effects must be defined. In addition to any deaths and other acute effects, the following reproductive parameters, as defined, must be reported:

- Eggs laid/hen;
- Eggs cracked (%);
- Viable embryos of egg set (%);
- Live three-week embryos (%);
- Normal hatchlings of live three-week embryos (%);
- 14-day-old survivors per hen; and
- 14-day-old survivors of normal hatchlings (%).

C. Statistically Significant Differences

Statistical methods must be presented which calculate the differences between reproductive parameters.

D. Results of Chemical Analysis

If the concentration of the test material was measured, the results should be reported. It is particularly important that concentrations of pesticide in the diet be measured and results reported when:

- The test material was a dry powder and no vehicle was used;
- The test material was volatile; or
- The test material was administered in the feed or water and data from other subdivision(s) suggest that the actual concentrations over the length of the test may decrease by 30 percent or more.

E. Body Weight and Food Consumption

Body weights and food consumption should be recorded at test initiation. Both should be recorded biweekly thereafter except for body weights. Weighing should be discontinued after week eight or after the onset of egg laying. The average for each test group should be provided.

F. Raw Mortality Data

Raw mortality data or percentage of death/effects must be reported. The data must indicate the numbers of birds dying at each dose or control level. The day of death/effects must be reported; it is preferable to also report the time of death.

G. Gross Necropsy

Gross necropsies are preferred. When performed, all dead birds should be examined, as well as a sufficient number of survivors in order to provide a characterization of gross lesions. Inspections of the GI tract, liver, kidneys, heart, reproductive organs, and spleen should be made. Also, subcutaneous fat and muscles should be examined for evidence of deterioration.

H. Other Observations

Signs of intoxication should be described as to what was observed, when, and for how long. Technical terminology used to name signs of intoxication should be adequately defined if these terms are not in general use. The report should indicate whether deaths are associated with specific signs of intoxication, and the time of onset and remission of toxic signs. If affected birds

recover, the time interval to recovery should be reported. Marked observation of fecal material and urine should be reported.

I. Statistical Analysis

A statistical analysis of the mortality data is required. Reproductive data consist of continuous variables (e.g., shell thickness, and body weight data) and discrete variables (e.g., number of eggs laid or 14-day-old survivors). For continuous variables, experimental groups should be compared to controls by analysis of variance. For the following discrete variables, survival percentages should be computed: eggs cracked, viable embryos of eggs set, live three-week embryos, normal hatchling of live three-week embryos, 14-day old survivors per hen, and 14-day old survivors of normal hatchlings. These values should then be arcsine transformed prior to analysis of variance. Alternately, a chi square analysis of survival (contingency tables) may be used for discrete variables. Analyses should include body weight, food consumption, eggs laid, eggshell thickness, eggs cracked, viable eggs, fertility, live three-week ducklings, 14-day old survivors (per number of eggs hatched, per hen, and per number of eggs laid). Sample units are generally the pens within each group.

In order that the concentration causing an adverse effect can be identified, a test such as the Duncan's Multiple Range Test should be performed if the analysis of variance indicates statistical differences.

J. Acceptable Protocols

EEB does not endorse any one protocol. It is sometimes necessary and desirable to alter the procedures presented in published protocols to meet the needs of the chemical or test organisms used. However, EEB does recommend some protocols as guidance for developing avian dietary toxicity tests. These protocols include:

> Ecological Effects Branch, 1982. Pesticide Assessment Guidelines Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms, EPA-540/9-82-024. pp. 48-57.

This referenced protocol provides flexible guidance to help researchers design scientific protocols and to assist the reviewer in validating studies. It is important to recognize that avian reproduction studies are validated based upon whether they fulfill guideline requirements and whether they provide scientifically sound information on the reproductive toxicity of the test material to avian wildlife that is useful in hazard assessments. This is more important than whether they follow a referenced protocol step-by-step.

IV. REVIEWER EVALUATION

The reviewer should identify each aspect of the reported procedure that is inconsistent with recommended protocol. The significance of these deviations must be determined. The number of

deviations and their severity will determine the validity of the study and the interpretation of the results.

A. Review of Materials and Methods

1. Test Substance

When the technical grade is greater than 80% a.i., the reviewer may elect not to adjust the concentration levels at which statistically significant adverse effects occurred to reflect actual amount of a.i.. Reviewers should check the accuracy of the reported effect levels by recalculating the doses in ppm a.i. in treated diets.

2. Diet

A review of the diet composition should be made specifically to check for unfamilar ingredients or medications. Any such unfamiliar ingredient or suspected additive should be verified by contacting the testing laboratory prior to classifying the study.

3. Body Weight and Food Consumption

Body weight and food consumption data can be used to evaluate effects, if any, on feeding and/or weight change.

Reduced food consumption and body weight may affect the ability of a bird to reproduce. If reduced food consumption or body weight occurs, the reproductive parameters should be viewed with this in mind as a possible explanation for adverse effects.

Also, those items mentioned in the SEPs for avian LD_{50} and LC_{50} should be incorporated into the design of this study.

4. Raw Data

The raw data must be checked to insure consistency with the written report. In cases where the two do not agree, the discrepancies must be explained in writing prior to classifying the study.

The control data must be checked and should be consistent with other studies on like species.

B. Verification of Statistical Analysis

The reviewer should "validate" the statistical analysis of the data. This may be done using EEB's "SAS" program "Bigbird" which performs analysis of variance with and without arcsine transformed variables. The significant groups are then identified by Duncan's Multiple Range Test. Any differences from the control should be noted and an explanation required from the registrant.

Adjustments to the study's statistical parameters may be made at this time to reflect the reviewer's estimate of the parameters. These may be necessary to account for actual amount of a.i. consumed, e.g., in cases where the test material a.i. content was lower than 80% or insufficient diet was consumed, or to correct errors in the authors' calculations or methodology.

C. Discussion of Results

1. Reproductive Effect, Mortality, and Behavioral Observations

The reviewer should discuss the results in light of the observations of intoxication. Statistically and biologically significant observations should be discussed if possible. Reproductive effects and mortality which cannot be fully attributed to the effects of the test material may be better understood if assessed in light of behavioral observations. Also, signs of intoxication could aid in understanding potential sublethal effects which could affect the birds' ability to develop and survive in the wild.

2. Implications of Dose/Effect Response

Dose/effect response many times reveals important characteristics about the toxicity of the test material, such as whether the response is commensurate with the decline of the test level.

Pertinent data on dose/effect response should be included in data evaluation records.

3. Gross Necropsy

The results of gross necropsies, when performed, may be helpful in evaluating the study. Lesions of reproductive systems may confirm changes in the reproductive parameters.

4. General Comments

Reviewers should comment on the protocol used and the methods or other aspects of the study which are irregular, unfamiliar, or unacceptable. Suggestions for improvements to the protocol can be made, as well as giving rationale for rejecting certain methods or aspects of the study. Of particular importance are the reviewer's comments on any aspect of the study, such as condition of the birds, husbandry practices, dose administration or rejection, toxic symptoms, or environmental conditions, which could bear on the interpretation of the results.

D. Reviewer's Conclusions

1. Category

The reviewer should indicate under which of the three validation categories the study fits:

- Core: All essential information was reported and the study was performed according to recommended protocols. Minor inconsistencies with standard methodologies may be apparent; however, the deviations do not detract from the study's soundness or intent. Studies within this category fulfill the basic requirements of current guidelines and are acceptable for use in a risk assessment.

- Supplemental: Studies in this category are scientifically sound; however, they were performed under conditions that deviated substantially from recommended protocols. Results do not meet guideline requirements; however, the information may be useful in a risk assessment. Some of the conditions that may place a study in a supplemental category include:

  - Unacceptable test species;
  - Inappropriate test material;
  - Deviations from recommended diet preparation procedures; or
  - Dosage levels tested were below the expected concentration on avian food items when treated at recommended label rates.

- Invalid: These studies provide no useful information. They may not be scientifically sound, or they were performed under conditions that deviated so significantly from the recommended protocols, that the results will not be useful in a risk assessment.

  Examples of studies placed in this category include those in which there were problems with volatility of the test material or a dry chemical was mixed without the use of a vehicle. Unless acceptable chemical analyses of actual toxicant concentrations were performed in studies such as these, the reviewer cannot be sure that test organisms were actually exposed to nominally designated concentrations.

  A study where the test material was not properly identified can also be invalidated.

2. Rationale

Identify what makes the study supplemental or invalid. It may also be necessary to justify a higher category in spite of deviations. That is, a study may have been called core or supplemental even though there were substantial deviations from recommended protocol. While all deviations should be noted, it may be that the deviations did not actually alter the response of the test organism to the test material. The reviewer is expected to exercise judgment in this area.

3. Repairability

Indicate whether the study may be upgraded or given a higher validation category if certain conditions are met. Usually this would involve the registrant submitting more data about the study.

E. References

The reviewer should reference any information used in the validation procedure. This should include protocol documents, statistical methods, or information taken from files of other Divisions or Branches within HED.