6/25/90

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMO:

TO: George LaRocca
Insecticide Branch
Registration Division

FROM: James Akerman, Chief
Ecological Effects Branch
Environmental Fate and Effects Division

SUBJECT: Data submission for Diazinon

The following studies have been reviewed by the Ecological Effects Branch:

1) Marselas, G. 1989. A one-generation reproduction study with the northern bobwhite quail (Colinus virginianus). Conducted by Wildlife International, Easton, MD for Ciba-Geigy Corporation, Greensboro, NC. MIRD No. 413229-02.

This study is scientifically sound and fulfills guideline requirements for an avian reproduction study. The no-observed effect concentration for reproductive parameters for diazinon in this study was 32.0 ppm, the highest concentration tested. The no-observed-effect concentration for cholinesterase inhibition was less than 8.3 ppm, the lowest concentration tested.

2) Marselas, G. 1989. A one-generation reproduction study with the mallard duck (Anas platyrhyncos) using parental reproduction. Conducted by Wildlife International, Easton, MD for Ciba-Geigy Corporation, Greensboro, NC. MIRD No. 413229-01.

This study is scientifically sound and fulfills guideline requirements for an avian reproduction study. The no-observed-effect concentration for reproductive parameters in this study was 8.3 ppm. The no-observed-effects concentration for cholinesterase inhibition was less than 4.02 ppm, the lowest concentration tested.

259292
Record No.

Review No.

057801
Shaughnessey No.

EEB REVIEW

DATE: IN 12-13-89 OUT 6-25-90

FILE OR REG. NO. 100-524

PETITION OR EXP. NO.

DATE OF SUBMISSION 12-13-89

DATE RECEIVED BY EFED 02-08-90

RD REQUESTED COMPLETION DATA 06-08-90

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RD ACTION CODE/TYPE OF REVIEW 660

TYPE PRODUCTS(S): I, D, H, F, N, R, S Insecticide

DATA ACCESSION NO(S). 413229-01 and 413229-02

PRODUCT MANAGER NO. G. LaRocca (15)

PRODUCT NAME(S) Diazinon MG 87%

COMPANY NAME Ciba-Geigy

SUBMISSION PURPOSE Review Avian Reproduction Studies

SHAUGHNESSEY NO.	CHEMICAL AND FORMULATION	% A.I.
	Diazinon	

Because this study represents the first time that the Ecological Effects Branch requested an avian reproduction involving parental incubation, the final results were discussed with numerous biologists and staticians inside and outside the Agency. The comments of two of these reviewers are attached. Particular attention should be paid to Dr. Richard Bennett's concerns about the variability in key reproductive parameters and the resulting lack of statistical power. Given this variability, much larger sample sizes would be required to provide definitive answers to questions about possible effects of diazinon on mallard reproduction.

If you should have questions about either of these reviews, please contact Clyde Houseknecht at 557-4372.

MIRD No. 413229-01

DATA EVALUATION RECORD

1. **CHEMICAL:** Diazinon
Shaughnessey No. 057801

2. **TEST MATERIAL:** Diazinon Technical 100%

3. **STUDY TYPE:** Avian Reproduction Test

4. **STUDY ID:** Marselas, G. 1989. A one-generation reproduction study with the mallard (Anas platyrhyncos) using parental incubation. Conducted by Wildlife International, Easton, Maryland for Ciba-Geigy Corporation, Greensboro, North Carolina. MIRD No. 413229-01.

5. **REVIEWED BY:**

Clyde R. Houseknecht
Wildlife Biologist
EEB/EFED

Signature: Clyde R. Houseknecht
Date: June 20, 1990

6. **APPROVED BY:**

Henry T. Craven, Head
Review Section #4
EEB/EFED

Signature: Henry T. Craven
Date: 6/20/90

7. **CONCLUSIONS:**

The no-observed-effect concentration for reproductive parameters for diazinon in this study was 8.3 ppm. The no-observed-effect level for cholinesterase inhibition was less than 4.02 ppm, the lowest concentration tested.

8. **RECOMMENDATIONS:** N/A

LOEC = 16.3 ppm ?

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If you should have questions about either of these reviews, please contact Clyde Houseknecht at 557-4372.

9. **BACKGROUND:** N/A

10. **DISCUSSION OF INDIVIDUAL TESTS:** N/A

11. **MATERIALS AND METHODS:**

A. Test Animals: Pen-reared mallards that were apparently healthy and phenotypically indistinguishable from wild birds were purchased from a commercial hatchery. All birds were from the same hatch and were 25 weeks of age at test initiation (first day of exposure to test diet). Sex of the birds was determined by examination of the plumage.

B. Test System: For the reproductive segment, 160 mallards (80 males and 80 females) were randomly distributed into four groups, each containing 20 pairs of birds. For the cholinesterase (ChE) segment, 32 mallards (16 males and 16 females) were randomly distributed into four groups.

Diets contained 28% protein minimum, 2.5% fat minimum and 5% fiber maximum. Five percent (wet weight) limestone was added to the diet to provide additional dietary calcium necessary for egg production. Water and feed were provided ad libitum during acclimation and during the study period.

All mallards were acclimated to the test facilities for 8 weeks prior to the initiation of the study. All adult birds were observed at least once each day throughout the study period for signs of toxicity or abnormal behavior. Birds that died during the study were necropsied. At the conclusion of the adult exposure period birds were sacrificed and necropsied. Brain samples were collected at necropsy from six randomly selected pairs of birds from the control group and each of the treatment groups to be analyzed for ChE activity.

Adult mallards were housed in pairs in floor pens measuring approximately 1 x 1.2 meters. The average temperature of the study room during the study was 18.8°C $\pm$ 2.9°C with an average relative humidity of 80%. During the 8 week acclimation period, the birds were maintained under a photoperiod of 8 hours of light per day. Upon initiation of the study, the photoperiod was decreased to 7 hours of light per day to delay the onset of egg production. At the end of week 2, the photoperiod was increased to 17 hours of light per day and was maintained at that level until adult sacrifice.

At the time photoperiod was increased to induce egg laying, nest boxes measuring approximately 37 x 28 x 34 cm were placed in each pen. Once the hens began incubation, the males were removed from the pen. Hatchlings remained with the hen until they reached 14 days of age and were fed the same diet as the hen.

Adult body weights were measured at study initiation, at the

end of Week 2 and at terminal sacrifice. Feed consumption was measured for each pen over a seven day period every week until adult sacrifice. No attempt was made to quantify the amount of feed wasted by the birds.

Following photostimulation and placement of nest boxes, pens were examined daily for egg production. Eggs found were recorded, marked according to pen, dated and returned to the nest. Eggs found to be cracked or broken were recorded and discarded. When the nests were examined an attempt was made to account for all eggs previously recorded. Eggs not found were recorded as missing. If the hen continued to lay after having reached 20 eggs in the clutch, the oldest eggs were recorded and discarded according to the methods of Smith and Anders. Hens that did not lay eggs or hens that laid eggs but did not attempt to incubate were sacrificed late in the study.

Daily egg production was recorded for each pen. Daily notations were made as to whether the hen was on or off the nest. The start of incubation was determined to be at the end of egg laying and when the hen was consistently observed on the nest. Approximately 5 to 7 days after the last egg was laid the drake was removed from the pen.

If the hen destroyed all eggs in the nest, abandoned the nest, or if the eggs failed to hatch after 35 days of incubation, the remaining eggs were removed, examined and recorded. If this occurred and the drake was still in breeding plumage, he was returned to the pen and the hen was allowed to attempt to renest. If the second clutch was destroyed, abandoned or failed to hatch, the hen and drake were sacrificed and weighed.

The date hatchlings were first observed was recorded and the hatchlings were weighed as a group the following day. Any unhatched eggs were recorded and removed from the nest. The unhatched eggs were opened and examined for stage of development. hatchlings were housed with the hen until 14 days of age and received the same diet as the hen. At 14 days of age all surviving hatchlings were weighed as a group.

The second and fourth eggs laid (when available or suitable) were removed from the nest, weighed, candled for defects and measured for shell thickness and strength.

In the ChE segment of the study housing and husbandry were identical to the reproductive segment. Blood samples were collected from the adult birds on Day 7, at the start of egg production, at approximately mid-incubation, at hatch and at 14 days post hatch. Serum and brain samples were refrigerated (serum) or frozen (brain) immediately after collection. Samples were analyzed for ChE activity by a commercial analytical laboratory.

C. **Dosage:** Treatment levels were based upon known toxicity data, results from the pilot reproduction study conducted by Wildlife International, and consultation with Ciba-Geigy. Three treatment groups were fed diets containing either 5 ppm, 10 ppm or 20 ppm diazinon. A control group was maintained concurrently.

D. **Design:** One-generation avian reproduction study with parental reproduction and ancillary measurement of serum and brain cholinesterase levels.

E. **Statistics:** Dunnett's method was used to determine statistically significant differences between the control group and each of the treatment groups. Sample units were the individual pens within each experimental group. The following parameters were analyzed statistically:

1. Adult body weight - Individual body weight was measured at initiation, at the end of Week 2 and at termination of the study.

2. Adult feed consumption - feed consumption expressed as grams of feed per bird per day by pen for each seven day period during the study.

3. Eggs laid of maximum laid - The number of eggs laid per hen divided by the largest number of eggs laid by any one hen.

4. Eggs cracked of eggs laid.

5. Time to onset of egg production.

6. Duration of egg laying.

7. Normal hatchlings of eggs incubated.

8. Normal hatchlings per pen.

9. Fourteen-day old survivors of eggs incubated.

10. Fourteen-day old survivors of normal hatchlings.

11. Fourteen-day old survivors per pen.

12. Hatchling body weights -

13. Egg weight - The weight of the second and fourth eggs laid by each hen.

14. Egg shell strength - The breaking strength of the second and fourth eggs laid by each hen.

15. Egg shell thickness.

16. Offspring's body weight - The group body weights of hatchlings and 14-day survivors was measured by pen group.

17. Serum cholinesterase.

18. Brain cholinesterase.

12. REPORTED RESULTS:

Reproductive Segment

Samples of control and test diets fed to mallards were analyzed for diazinon. Nominal and mean concentrations were as follows:

Diazinon (ppm)	
Nominal	Measured
0	< 0.50
5	4.02 ± 0.15
10	8.30 ± 0.48
20	16.33 ± 0.97

There were no treatment related mortalities during the course of the study. No overt signs of toxicity or incidental clinical signs were observed at any of the concentrations tested. All birds appeared normal throughout the course of the study.

There were no apparent treatment related effects upon body weight of adult birds at any of the concentrations tested. Feed consumption was highly variable due to wastage by some adult birds. there were no apparent treatment related effects upon feed consumption among birds at 5 ppm or 10 ppm. In the 20 ppm treatment group, while not statistically significant, there appeared to be a slight treatment related reduction in mean feed consumption during the first two weeks of the study.

Mean length of time from photostimulation until the start of egg production was comparable between the control and all treatment groups. There was no statistically significant difference in reproductive parameters between the control group and the 5 ppm and 10 ppm treatment groups. For the 20 ppm treatment group, the mean length of incubation and the number of eggs laid per hen per day were comparable to the control group. However, the mean length of egg production in the 20 ppm group was significantly longer than that for the control group. While not statistically significant, the number of eggs laid per hen was greater in the 20 ppm group than in the control group. These differences appeared to be related to treatment and were due to the increased number of hens in the 20 ppm group that continued to lay eggs but did not start incubation.

Of those hens laying eggs, most ultimately attempted to build a nest and incubate eggs. The number of hens successfully incubating eggs was comparable between the control group and the 5 ppm and 10 ppm treatment groups. In the 20 ppm treatment group, due to fewer hens successfully incubating eggs, there was a significant reduction in the number of eggs incubated as a percentage of eggs laid.

The number of hatchlings per pen and the number of hatchlings as a percentage of eggs incubated were examined for each treatment group. There were no statistically significant differences between the control group and the 5 ppm and 10 ppm treatment groups. At 20 ppm there was a treatment related reduction in the number of hatchlings per pen.

When compared to the control group, there were no statistically significant differences in the number of 14-day old survivors per hen or 14-day old survivors as a percentage of hatchlings at any treatment group.

There were no apparent treatment related effects upon egg weight, shell thickness or shell strength at any of the concentrations tested.

Cholinesterase Segment

There was a statistically significant reduction in serum cholinesterase activity in males at all treatment levels for all sampling periods. In females, all serum cholinesterase activities were significantly lower in all sampling periods except at the 5 ppm level at sampling periods 3 (mid-incubation) and 4 (hatching). Cholinesterase inhibition was dose responsive at all five sampling periods and was consistent between sexes.

Brain cholinesterase was significantly inhibited in mallards receiving 20 ppm diazinon and in males receiving 5 ppm diazinon.

13. **STUDY AUTHOR'S CONCLUSION/QUALITY ASSURANCE MEASURES:** Ciba-Geigy certifies that this study complies with Good Laboratory Practice regulations as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160.

14. **REVIEWER'S DISCUSSION AND INTERPRETATION OF STUDY RESULTS:**

A. Test Procedures: EPA required that parental incubation be used in this study. Therefore, there were many deviations from the protocol that is specified for the typical avian reproduction test. There were only two minor deviations not related to parental incubation.

o Eggs were not candled and, therefore, no data were

reported for numbers of viable embryos or numbers of live three week embryos.

o The SEP recommends that the test be conducted at 21 degrees C with 55% relative humidity. Temperatures during this study averaged 18.8 degrees C and the relative humidity averaged 80%.

Neither minor deviation should have affected the results of the study.

B. Statistical Analysis:

The EEB reviewer recalculated all of the statistics reported by the author. Only minor variations from reported results were noted. Parametric statistical procedures (ANOVA, Duncan's Multiple Range test) were employed in the analysis of normally distributed data. Tke Kruskal-Wallis test was used for data which were not normally distributed.

The author found no significant differences in body weights at any treatment levels. EEB attempted to reduce experimental variability by considering only body weight changes that occurred between the beginning and the end of the study. With this restriction there was a significant reduction in weight by males in the highest (20 ppm) treatment group.

It was reported that the number of eggs laid per hen did not differ significantly in any of the treatment groups. Using Duncan's Multiple Range test this reviewer found that the group fed 20 ppm diazinon laid significantly more eggs than did the control group. This was due to the increased number of hens in the 20 ppm group that continued to lay eggs but did not start incubation.

Contrary to the report submitted, EEB found a significant reduction in the numbers of 14-day survivors in the 20 ppm treatment group. This was undoubtedly related to the fact that, because of the experimental design mandated by the requirement for parental incubation, hatchlings consumed the same diet as their mothers. Thus, hatchlings in the 20 ppm group suffered mortality due to the toxicity of the diet.

Data on reproductive parameters, body weight changes and cholinesterase inhibition are summarized in Tables 1 through 3.

C. Discussion/Results:

This study breaks new ground in that it is the first time that EEB required parental incubation. The rationale for requiring this approach is well-founded. Diazinon inhibits cholinesterase production and that in turn might be expected to affect the ability of females to incubate and the resulting

numbers of offspring produced.

The results of this study suggest that (at least at the levels tested) diazinon does not significantly affect the major outcome of interest, namely the number of ducklings produced. From a purely scientific standpoint it would be interesting to have information on how diazinon or any other cholinesterase inhibitor affects nesting and incubation behavior (as opposed to outcome) but this would require significant modifications to the experimental design to allow for nearly continuous observation of incubating females. Given the results of this study and the fact that egg laying, incubation and hatching are inherently very variable processes (as evidenced by the control birds in this study), this reviewer feels that future studies should rely on the widely accepted practice of artificial incubation.

The requirement for measuring food consumption presents additional difficulties. The is no effective way to measure food wastage and therefore data on food consumption are suspect. This is particularly true when hatchlings are allowed to remain with the mother. Furthermore, little information can be gained about possible effects of maternal consumption of a toxic substance and subsequent survival of offspring when the offspring are allowed to consume the toxin.

In conclusion, dietary diazinon at nominal concentrations of 5 ppm, 10 ppm and 20 ppm did not result in treatment related mortalities or overt signs of toxicity. The no-observed-effect concentration for reproductive parameters for diazinon in this study was 8.3 ppm, while the no-observed-effects level for cholinesterase inhibition was less than 4.02 ppm, the lowest concentration tested.

D. **Adequacy of the Study:**

(1) **Classification:** Core

(2) **Rationale:** N.A.

(3) **Repairability:** N.A.

15. **COMPLETION OF ONE-LINER:** Yes, March 1, 1990.

Table 1 Summary of Statistical Analyses of Various Reproductive Parameters tested with Diazinon

Nominal Concentration of Parameter	Diazinon Technical (ppm) 0	5	10	20
Egg shell thickness (mm)	0.37	0.36	0.38	0.36
Female body weight change (x)	-39 g	0 g	0 g	-36 g
Male body weight change (x)	+57 g	+24 g	+67 g	-17 g*
Hatchling weight (x)	+37 g	+36 g	+37 g	+36 g
14 day survivor weight (x)	220 g	213 g	223 g	148 g*
Eggs laid/hen (EL)	24.2	29.4	27.6	38.5*
Eggs cracked/hen (EC)	1.1	1.1	1.3	2.7
Eggs set/hen (ES)	11.2	12.8	9.7	6.7
Number hatchlings/hen (NH)	6.8	8.7	6.6	4.8
14-day-old survivors/hen	6.1	8.3	6.2	3.6*
ES/EL#	40.3	35.4	30.9	16.4
NH/EL#	25.1	25.8	21.9	13.2
Day14/NH#	75.4	82.8	80.5	65.2
Day14/ES#	45.9	56.1	54.0	48.6
EC/EL#	9.1	8.2	8.5	11.7
NH/ES#	51.6	58.8	57.1	61.8
Days to production (x)	20	17	20	18
Days in production (x)	29	29	34	46*
Length of incubation (days)	23	23	23	24

*Significantly different from the control value at $p < 0.05$.

#Arcsine transformed before analysis of variance. Reported as arcsine transformed data for statistical comparison.

Table 2: Serum cholinesterase levels (international units/l) in mallard ducks fed diazinon technical.

Serum cholinesterase (females)

	Sampling Period 1	2	3	4	5
Control	1039	1160	1141	1237	1152
5 ppm	668*	650*	831	726	674*
10 ppm	455*	595*	543*	527*	500*
20 ppm	354*	269*	436*	380*	384*

Serum cholinesterase (males)

	Sampling Period 1	2	3	4	5
Control	1152	1556	1396	1180	1249
5 ppm	768*	830*	737*	464*	811*
10 ppm	355*	526*	363*	404*	391*
20 ppm	323*	339*	228*	297*	324*

* Statistically different from the control at $p < 0.05$.

Table 3. Brain cholinesterase levels (international units/gram wet weight brain tissue) in mallard ducks fed diazinon technical.

	Males	Females
Control	7.06	6.56
5 ppm	3.33*	8.84
10 ppm	6.07	4.10
20 ppm	2.09*	2.08*

* Statistically different from the control at $p < 0.05$

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
ENVIRONMENTAL RESEARCH LABORATORY
200 S.W. 35TH STREET
CORVALLIS, OREGON 97333

15 May 1990

Clyde Houseknecht H7507C
Ecological Effects Branch
Environmental Fate and Effects Division
Office of Pesticide Programs
U. S. Environmental Protection Agency
401 M. Street S.W.
Washington, D.C. 20460

Dear Mr. Houseknecht:

I have reviewed the project report on "A one-generation reproduction study with the mallard (Anas platyrynchos) using parental incubation" by Wildlife International. I am interested in the investigator's use of parental incubation because our research has shown that dietary exposure of organophosphorus insecticides can affect mallard incubation behaviors and nesting success. However, I am disappointed that the investigators gave no rationale for the use of parental incubation or for why they felt it was appropriate in this particular case. Test results could be quite different using parental incubation versus artificial incubation, so the rationale for choosing parental incubation should be made clear.

The use of parental incubation is not described in EPA's Guidelines for Avian Reproduction Tests, but is an option mentioned in the ASTM "Standard Practice for Conducting Avian Reproduction Tests". The greatest concern I have with the use of parental incubation in avian reproduction tests in support of registration is the lack of standardized methods. Also, there is the potential, as I think was seen in this study, for greater variability among experimental units in some parameters and, consequently, reduced power in the statistical analysis.

Some of the lack of standardization is seen in the differences between the ASTM standard practices for parental incubation and this study. ASTM states that "eggs that are laid or rolled outside of the nest should not be returned", while this report states that "eggs laid outside of the nest were placed in the nest". Also, ASTM states that "cracked, infertile, or dead eggs should not be removed from the nest unless the entire clutch is affected", while this report states that cracked, broken, and abnormal eggs were discarded. Our experience has been that the way these egg issues are handled can make a large difference in the ultimate interpretation of the data.

One other point of confusion relates to the clutch size. The report describes (page 19) that when the clutch exceeded 20 eggs, "the oldest were recorded and discarded". This would lead one to believe that 1) the maximum number incubated was 20 and 2) that there would be several birds that incubated clutches of 20 eggs because so many birds laid more eggs than that. However, Appendix VII indicates that all birds incubated clutches of $\leq$18 eggs, except for two (clutches of 24 and 29). I do not disagree with their criteria of a maximum of 20 eggs, because it is unlikely that a hen can adequately incubate more than 20 eggs. As clutches increase above 20 eggs there would be an increase in the number of eggs incubated none or part of the time, thus biasing hatchability data. However, I do not understand why no clutches contained 20 eggs or why two nests were allowed to keep greater that 20 eggs.

I have several concerns about the cholinesterase segment of the study. Many of these concerns can not be answered from the report because so little information is provided on methodology. The primary reason for concern is the extreme variability in the brain cholinesterase data (see Appendix XI, page 111). The control groups have coefficients of variation of 53% for males and 80% for females. With a properly functioning assay it is normal to achieve a CV of 10 to 15% for mallard brains. Consequently, I have serious concerns about the validity of the cholinesterase assays and the interpretation of the data. I do not understand how a data set that ranges from 1.93 to 19.82 IU/g in females and 2.54 to 12.07 IU/g in males could be considered acceptable. The specific methods used by the Maryland Medical Laboratory need to be explored to determine if they are adequate for avian serum and brain cholinesterase. Human testing laboratories usually use different substrates than are used by wildlife toxicologists. There are a multitude of factors that can affect the quality of brain and plasma cholinesterase activity data, but some of the obvious factors should be further explored by the authors to attempt to explain this variability.

One other point related to ChE assays-- On page 24, there is a discussion about the death of a hen in the 20 ppm group, with a conclusion that the necropsy findings "were not considered to be related to treatment". The brain ChE activity in the hen was stated to be 1.98 IU/g, which is 33% of the male in that pen and 30% of the control mean for females. Making the assumption that the ChE values are valid, this represents a significant ChE depression. Although the findings of the necropsy are summarized, there is no mention of the body weights at death, which could be very important for interpreting cause of death. Achronic exposure to an OP insecticide can lead to a rapid loss of weight that results in death without overt signs of OP poisoning. Based on the information presented, I do not believe it can be concluded that the death was unrelated to treatment.

On page 27, egg production is discussed. Due to the nature of the experimental design, the use of total numbers of eggs laid per hen or eggs laid per hen-day are of less value for detecting treatment effects than in a lab test with artificial incubation. Since these parameters are compromised by having variable laying periods, probably a more useful indicator of treatment effects is the duration of egg production prior to incubation. Birds behaving normally would complete a clutch of probably less than 20 eggs and begin incubating. Birds that keep laying large numbers of eggs are likely behaving abnormally because of the artificial testing situation or the dietary treatment. Either stress could result in keeping levels of the reproductive hormone prolactin below levels necessary to start incubation. The propensity to incubate can be used as an indication of the suitability of the test conditions. This test had a high incidence of extended laying periods and non-incubating birds, both of which contribute to the variability in productivity parameters.

On page 28, it is stated that "the apparent increase in cracked eggs at 20 ppm was not considered to be treatment related", but there is not further explanation of the conclusion. The two birds that laid soft shelled eggs were in the 20 ppm group, and OPs have been shown to reduce eggshell quality in laboratory studies, so how can this be dismissed as unrelated to treatment without further information and justification?

My greatest concern for this study, in particular, and the use of parental incubation, in general, is the increase in variability in key reproductive parameter that seriously reduces the potential for detecting statistically significant effects of treatment. The coefficients of variation in the hatchability and survival parameters were often greater than 50%, so that only major treatment effects could be detected. Also, the distributions of many of the parameters are far from being normally distributed, bringing the use of parametric statistics into question. This is especially true for the number of hatchlings and 14-day-old survivors that have somewhat of a bimodal distribution. In these cases, comparisons of arithmetic means can be very misleading. At a minimum, alternative statistical methods more appropriate to the data sets should be employed. However, given the nature of tests using parental incubation, in the future it may be necessary to develop new experimental designs more appropriate to the question being addressed.

I hope these brief comments have been of help in your review. If you have any question concerning my review, please give me a call.

Sincerely;

Richard S. Bennett

Richard S. Bennett
Research Ecologist
FTS 420-4582

4-6-90

Dear Skip,

Sorry for the delay in getting my comments to you.

I did not go over all the appendices with a fine-toothed comb, but I did carefully read the text and tables.

It looks to me that this study was carefully conducted using an acceptable procedure. Some people would argue that the typical EPA protocol requiring that treated diet be fed for weeks in advance of the reproductive season could miss a reproductive effect with a cholinesterase inhibiting pesticide. It is possible that ducks could acclimate to a steady diet of a cholinesterase inhibitor and not have their reproduction affected, but would show an effect if the pesticide was introduced into the diet just before egg laying or at some critical point in incubation. This is just conjecture; no one really knows.

However, at least under the standard EPA schedule of exposure, I have no reason to question the findings of the study. It looks to me that the results were properly interpreted and the conclusions are correct.

The implications, of course, depend upon the likelihood of waterfowl being exposed to 5, 10, or 20 ppm diazinon in nature. I am not able to comment on that.

One of the interesting findings, from a research point of view, was that significant cholinesterase inhibition in the brain in the 10 ppm group caused no measurable decline in reproduction.

I know EPA is thinking about different kinds of exposure periods for cholinesterase inhibitors. I encourage you to pursue this idea.

Here is one last point that has nothing to do with the validity of the study, but it is something you may want to call to the attention of testing laboratories. There is a published paper describing the aging of mallard embryos. I have enclosed a copy for you. On page 34, reference number 4 is to a paper for aging chicken embryos. The use of the mallard paper might be better.

Sincerely,

Gary Heinz

DATA EVALUATION RECORD

1. **CHEMICAL:** Diazinon
Shaughnessey No. 057801

2. **TEST MATERIAL:** Diazinon Technical 100%

3. **STUDY TYPE:** Avian Reproduction Test

4. **STUDY ID:** Marselas, G. 1989. A one-generation reproduction study with the northern bobwhite (Colinus virginianus). Conducted by Wildlife International, Easton, Maryland for Ciba-Geigy Corporation, Greensboro, North Carolina. MIRD No. 413229-02.

5. **REVIEWED BY:**

Clyde R. Houseknecht
Wildlife Biologist
EEB/EFED

Signature: Clyde Houseknecht
Date: June 20, 1990

6. **APPROVED BY:**

Henry T. Craven, Head
Review Section #4
EEB/EFED

Signature: Henry T. Craven
Date: 6/20/90

7. **CONCLUSIONS:**

The no-observed-effect concentration for reproductive parameters for diazinon in this study was 32.0 ppm, the highest concentration tested. The no-observed-effect level for cholinesterase inhibition was less than 8.3 ppm, the lowest concentration tested.

8. **RECOMMENDATIONS:** N/A

9. **BACKGROUND:** N/A

10. **DISCUSSION OF INDIVIDUAL TESTS:** N/A

11. **MATERIALS AND METHODS:**

A. Test Animals: Pen-reared northern bobwhite were purchased from a commercial quail farm in New Jersey. All birds were from the same hatch and were 24 weeks of age at test initiation. All birds appeared to be in good health and free from physical injuries. Sex was determined by visual examination of the plumage. All bobwhite were acclimated to the facilities for eight weeks prior to the initiation of the test.

B. Test System: For the reproductive segment of the test, 128 bobwhite (64 males and 64 hens) were randomly distributed into four groups, each containing 16 pairs of birds. For the ChE segment, 32 bobwhite (16 males and 16 females) were randomly distributed into four groups. For both segments of the study, each of three treatment groups were fed diets containing either 10, 20, or 40 parts per million (ppm) of diazinon.

During acclimation and upon initiation of the study, the birds were maintained under a photoperiod of eight hours of light per day. At the end of Week 2, the photoperiod was increased to seventeen hours of light to induce egg laying. The photoperiod was maintained at 17 hours of light until the completion of the test.

Adult body weights were measured at study initiation, at the end of Week 2 and at terminal sacrifice.

Feed consumption was measured for each pen weekly. Eggs were collected daily from all pens and were stored at a temperature of 10.3 $^{\circ}$ C. At weekly intervals, all eggs were removed from storage and counted. Samples were taken for eggshell strength and thickness measurements. The remaining eggs were candled to detect cracks or abnormalities. Eggs not used for shell thickness determinations were placed in a mechanical incubator. Eggs were candled again on Day 11 of incubation to determine embryo viability and on Day 21 to determine embryo survivability. On Day 21 eggs were placed in another incubator and allowed to hatch. Pedigree baskets were used to keep hatchlings separated by pen.

All hatchlings, unhatched eggs and eggshells were removed from the hatcher on Day 25 or 26 of incubation. The average body weight of the hatchlings by pen was determined. Hatchlings were housed in brooding pens until 14 days of age. At 14 days of age, the average body weight by parental pen of all surviving chicks was determined.

In the ChE segment of the study, 32 bobwhite (16 males and 16

females) were randomly distributed into one of four groups, receiving either a control diet or diets containing 10, 20 or 40 ppm of diazinon. Housing and husbandry were identical to the reproductive segment.

Blood samples were collected from the adult birds at Day 7, the start of egg production, Week 4 of egg production and at study termination. Brain samples were obtained at the conclusion of the adult exposure period. All blood and brain samples were tested for levels of cholinesterase.

C. **Dosage:** Nominal concentrations of diazinon were 10, 20 and 40 ppm. Mean measured concentrations were 8.3, 16.3 and 32.0, respectively.

D. **Design:** One-generation avian reproduction study with ancillary measurement of serum and brain cholinesterase levels.

E. **Statistics:** Dunnett's method was used to determine statistically significant differences between the control group and each of the treatment groups. Sample units were the individual pens within each experimental group. The following parameters were analyzed statistically:

1. Adult body weight - Individual body weight was measured at initiation, at the end of Week 2 and at termination of the study.

2. Adult feed consumption - feed consumption expressed as grams of feed per bird per day by pen for each seven day period during the study.

3. Eggs laid of maximum laid - The number of eggs laid per hen divided by the largest number of eggs laid by any one hen.

4. Eggs cracked of eggs laid.

5. Viable embryos of eggs set.

6. Live 3-week embryos of viable embryos.

7. Hatchlings of 3-week embryos.

8. Fourteen-day old survivors of hatchlings.

9. Hatchlings of eggs set.

10. Fourteen-day old survivors of eggs set.

11. Hatchlings of maximum set - The number of hatchlings per hen divided by the largest number of eggs set from any one hen.

12. Fourteen-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 weight - The weight of the second and fourth eggs laid by each hen.

14. Egg shell strength - The breaking strength of the second and fourth eggs laid by each hen.

15. Egg shell thickness.

16. Offspring's body weight - The group body weights of hatchlings and 14-day survivors was measured by parental pen group.

12. REPORTED RESULTS:

Reproductive Segment

There were no mortalities during the reproductive segment of the study. Clinical signs such as intermittent lameness, ruffled appearance, wing droop, lethargy, ventral head curl, coughing and swelling on the head were noted in several birds at various treatment levels throughout the study. They were considered to be incidental and not related to treatment.

There were no apparent treatment related effects upon body weight among adult birds at any of the concentrations tested.

Due to excessive wastage by some birds, feed consumption was highly variable between pens. There was no apparent treatment related effect upon feed consumption among birds at any of the concentrations tested.

There were no apparent treatment related effects upon reproductive parameters at any concentration tested. When compared to the control group, there was a statistically significant (< 0.05) reduction in eggs laid as a percentage of the maximum number of eggs laid in the 20 ppm treatment group. However, the reduction in the number of eggs was the result of one hen that did not lay any eggs, and two other hens that laid a combined total of five eggs. Because the difference in egg production was not dose related, the result was considered to be incidental to diazinon treatment.

There were no apparent treatment related effects upon egg weight, eggshell strength or thickness at any of the concentrations tested. Similarly, there was no statistically significant difference in body weights of offspring from birds in any of the treatment groups.

Cholinesterase Segment

There were no treatment related mortalities or overt signs of toxicity among birds in the cholinesterase segment of the study. Body weight and feed consumption measurements, reproductive results and necropsy findings were similar to that observed in the reproductive segment of the study.

There was a statistically significant reduction ($p < 0.01$) in serum cholinesterase activity in males at all test concentrations for all sampling intervals. In females, all serum cholinesterase levels were significantly lower ($p < 0.05$) in all treatment groups at all sampling intervals except at the 10 ppm concentration at sampling interval 2 (start of egg laying). There was a dose responsive, treatment related reduction in serum cholinesterase levels at all test concentrations.

Mean brain cholinesterase levels for males in all treatment groups were comparable to the control group. Mean brain cholinesterase levels of females were depressed ($p < 0.01$) in all treatment groups.

13. **STUDY AUTHOR'S CONCLUSION/QUALITY ASSURANCE MEASURES:** Ciba-Geigy certifies that this study complies with Good Laboratory Practice regulations as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160.

14. **REVIEWER'S DISCUSSION AND INTERPRETATION OF STUDY RESULTS:**

A. Test Procedures: Except for the minor deviation noted below, the procedures used in this study are in accord with those recommended in the EPA's SEP for the avian reproduction test.

- The SEP specifies that eggs should be stored at 16 degrees C. with 65% relative humidity. Those of this study were stored at 10.3 degrees C. with 75% relative humidity.

B. Statistical Analysis:

The EEB reviewer reanalyzed the raw data submitted with the report and found only minor deviations from the results presented by the author. It was stated that no significant differences were found in egg shell thickness in any of the treatment groups. Duncan's procedure shows a significant ($p < 0.05$) reduction in egg shell thickness in the group that was fed 40 ppm of diazinon. The mean difference in thickness between the treatment group and the control was small (0.01 mm) and probably of no biological significance.

Duncan's test showed significantly fewer viable embryos per hen and live three-week embryos per hen in the group fed 20 ppm of diazinon. As noted above, this difference is due to the

fact that one hen in the 20 ppm treatment group failed to lay any eggs and two other hens laid a combined total of five eggs. Due to the small number of hens with reduced egg production and because the reduction was not dose responsive, the difference is considered to be incidental to diazinon treatment.

Reproductive data, serum cholinesterase levels and brain cholinesterase levels are summarized in Tables 1-3, respectively.

C. **Discussion/Results:**

Dietary concentrations of diazinon at 10 ppm, 20 ppm and 40 ppm did not result in treatment related mortality, overt signs of toxicity, or effects upon body weight or feed consumption among adult bobwhite. There were no treatment related effects on reproductive parameters at any concentration tested.

Serum cholinesterase levels were inhibited at all diazinon test concentrations and was dose responsive. Brain cholinesterase levels of females was inhibited in all treatment groups; however, there was no apparent depression in brain cholinesterase activity among males.

The no-observed-effect concentration for reproductive parameters for diazinon in this study was 40 ppm, the highest concentration tested. The no-observed-effect level for cholinesterase inhibition was less than 10 ppm, the lowest concentration tested.

D. **Adequacy of the Study:**

(1) **Classification:** Core

(2) **Rationale:** N.A.

(3) **Repairability:** N.A.

15. **COMPLETION OF ONE-LINER:** Yes, February 27, 1990.

Table 1 Summary of Statistical Analyses of Various Reproductive Parameters tested with Diazinon

Parameter	Nominal Concentration of Diazinon Technical (ppm) 0	10	20	40
Egg shell thickness (mm)	0.20	0.20	0.20	0.19*
Female body weight change (%)	14.6	9.9	11.0	8.6
Male body weight change (%)	3.9	3.4	2.0	0.5
Hatchling weight (x)	5.6 g	5.6 g	5.4 g	5.6 g
14 day survivor weight	24 g	23 g	23 g	22 g
Eggs laid/hen (EL)	36.06	27.81	26.12*	28.50
Eggs cracked/hen (EC)	1.19	0.81	0.50	1.69
Eggs set/hen (ES)	31.00	23.69	22.81	22.94
Viable embryos/hen (VE)	27.75	20.62	19.06*	20.06
Number hatchlings/hen (NH)	24.12	18.88	17.00	18.50
Live 3-week embryos/hen (LE)	27.62	20.44	18.81*	19.88
14-day-old survivors/hen	21.81	16.06	14.81	16.25
ES/EL#	68.3	67.5	69.3	64.5
VE/ES#	73.0	72.3	68.3	74.0
VE/EL#	61.8	60.0	59.0	57.2
NH/VE#	70.2	74.6	73.4	78.0
NH/EL#	55.2	55.8	54.0	53.5
Day14/NH#	74.6	70.4	72.8	70.7
Day14/ES#	57.7	56.2	54.2	58.2
EC/EL#	7.7	7.1	5.9	10.3
NH/ES#	62.7	64.3	60.8	66.9
NH/LE#	70.9	75.9	74.4	79.3
LE/VE#	88.6	88.0	87.1	88.5

*Significantly different from the control value at $p < 0.05$.

"Arcsine transformed before analysis of variance. Reported as arcsine transformed data for statistical comparison.

Table 2: Serum cholinesterase levels (international units/l) in bobwhite quail fed diazinon technical.

Serum cholinesterase (females)

	Sampling Period 1	2	3	4
Control	2961	2425	2231	2076
10 ppm	1547*	1616	985**	1026*
20 ppm	848**	924*	569**	659**
40 ppm	485**	338**	264**	322**

Serum cholinesterase (males)

	Sampling Period 1	2	3	4
Control	3007	2458	2514	2184
10 ppm	1372**	1551**	1160**	1083**
20 ppm	1256**	767**	855**	750**
40 ppm	403**	319**	438**	353**

* Statistically different from the control at $p < 0.05$.
** Statistically different from the control at $p < 0.01$.

Table 3. Brain cholinesterase levels (international units/gram wet weight brain tissue) in bobwhite quail fed diazinon technical.

	Males	Females
Control	5.54	8.28
10 ppm	4.99	4.97*
20 ppm	5.40	5.38*
40 ppm	6.50	5.00*

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