DRAFT
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OECD GUIDELINE FOR TESTING OF CHEMICALS

PROPOSAL FOR A NEW TEST GUIDELINE

Avian Two-generation Toxicity Test in the Japanese Quail

INTRODUCTION

1. The purpose of this guideline is to describe a laboratory testing procedure that can be used to assess the impact of pesticides and other chemicals upon avian health and reproduction. Data collected from such test may be used in the assessment of risks to birds exposed to pesticides and other chemicals under field conditions. These data from a two-generation test are principally intended to evaluate the adverse consequence of exposure to a "possible" endocrine disruptor to the reproductive viability of F1 birds, but may also provide information to confirm a chemical as an endocrine disruptor.

2. The test is designed for dietary exposure, but also may include exposure through drinking water. This guideline describes a two-generation test. The test monitors health and reproductive parameters in Japanese quail (*Coturnix japonica*) adults and their progeny, including viability of second generation offspring.

3. The number of second generation (F2) 14-day old survivors per F1 generation hen is the primary biological endpoint of this test. Additional hormone and morphological endpoints are included to assess and confirm potential endocrine disruption.

PRINCIPLE OF THE TEST

4. Tests performed under this guideline begin with 4 week old birds. Parameters for adult (P and F1), egg production (P and F1), and offspring health (F1 and F2) are evaluated by making statistical comparisons between treated groups and the control group. The no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) are determined for these adult health and fitness parameters.

VALIDITY CRITERIA

5. The test substance concentration in the diet to which birds are exposed should be satisfactorily maintained and reported. Losses of 20% or less relative to initial concentrations are generally considered acceptable. Higher rates of loss must be investigated and explained.

6. Parental mortality during the last two weeks of acclimation should not exceed 3%. When possible, all mortalities in the control group should be explained. At least eight breeding F1 pairs that have produced eggs must be available in the control group at the end of the test period.

DESCRIPTION OF THE METHOD

Test animals

7. The test species is the Japanese quail (*Coturnix japonica*). Birds should be approaching their first breeding season.

8. Japanese quail generally start laying eggs at or above six weeks of age. Some strains require photostimulation to initiate egg laying, while others do not. Once egglaying has begun it will take about two to three weeks for birds to reach full egg production. Depending on the strain, eggs will be fertile when birds are about eight to ten weeks of age.

9. Birds used in the test should appear healthy and be free of abnormalities or injuries that may affect test results. Birds should not receive any dietary medications beginning one week prior to the start of the test and continuing until the test is terminated. All birds used in a test should be from the same hatch.

Housing

10. Temperature, ventilation, and light controlled facilities are needed throughout the test. Recommended ventilation is about 8 to 15 air changes per hour.

11. Artificial lighting should approximate the daylight visual spectrum and be automatically controlled. The photoperiod for adult birds, from the start of acclimation onwards, and the chicks is 16 or 17 hours light and 7 or 8 hours of darkness. Birds should be exposed to light intensity of a t least 10 lux, measured at the level of the feeder. The light intensity is dictated by the amount required for daily observations. Light intensity that is too high will encourage aggressive behaviour.

12. Incubators and hatchers. preferably with automatic temperature and humidity controls and an egg-turning device, are necessary. In addition, suitable equipment is required to maintain stored eggs within the temperature and humidity ranges specified.

13. Suitable pens of appropriate size for adults and for chicks are required. Wire pens with slanting floors and egg-catchers or other measures to prevent breakage of eggs, are recommended for adults. Pens for both adults and chicks should preferably be of stainless or galvanized steel or other inert materials. When using wire meshing for the floors, the wire mesh should be of a size sufficient to prevent foot injury but large enough to allow excreta to drop through. Measures should be taken to minimize spillage of diet, e.g., by covering the food troughs with a wire grid.

14. Adult birds should be housed in pairs (one male/one female). The parental cages should be distributed in such a way that cross-contamination and positional effects are avoided. Pen mate aggression can be a problem and measures must be taken to minimize injuries, stress and mortality resulting from such aggression. An example might be to separate pairs and place them together often enough to maintain fertility of eggs.

15. For rearing chicks, the use of rearing pens with thermostatically controlled warm compartments or cages, which are free of draughts and have a radiator (e.g., ceramics), are recommended. Sufficient space for feeding and drinking must be provided, especially during the first week after hatching, to reduce the problem of the weaker animals not getting access to the feed and water facilities. Chicks should be identified individually or by pen of origin. They may be housed together, in groups of approximately equal number, preferably by treatment group. The optimal number of chicks per pen depends on pen size. F1 birds must be separated into pairs (one male/one female) when 4 weeks old or as soon as sex can be discriminated.

16. Guidance for housing conditions is provided in Table 1.

Table 1: Housing conditions

Age (week)	Temperature (^{o}C)	Relative humidity (%)	Minimum floor area (cm^2/bird)

1	35-38	40-80	50
2	30-35	40-80	75
3-4	23-27	40-80	100
>4	16-27	40-80	625

Note: The acceptability of housing conditions of adults will be evaluated on the basis of results of reproductive performance.

Feeding

17. Diet and drinking water are provided *ad libitum*. The diet should be described and should meet the specific nutrient requirements of Japanese quail. The caloric contents of the diet must be reported. If the same diet is used for chicks and adults, extra calcium should be added for the adult diet. During the treatment period, birds are fed basal diet mixed with the test substance at specified concentrations.

18. Guidance for recommended nutritional values is provided in Table 2.

Table 2: Recommended nutritional values

Nutrient	Adults (> 4weeks) Recommended range (%)	Chicks Recommended range (%)
Crude protein	27 to 29	27 to 30
Crude fibre	3.5 to 5.0	3.0 to 6.0
Crude fat	2.5 to 7.0	5.5 to 7.5
Calcium	2.6 to 3.6	0.75 to 1.2
Phosphorous	0.9 to 1.1	0.6 to 1.0

TEST DESIGN

19. An acclimation period of at least two weeks is required prior to the start of the test. The test begins with the start of treated diet. Treated diet begins with 4 week (28 day) old birds. If necessary, birds may be photostimulated during the acclimation period. Onset of egglaying can take place during the acclimation period.

20. Prior to the start of treatment, individual birds are weighed, genetically sexed, and randomly allocated to pens and treatment groups. Birds are paired , preferably by known lineage such that males from one line are paired with females from another line. Pairs should be observed for signs of excessive aggression, and if present the sexes separated except for periodic conjugal visits. Otherwise, the pairs will be housed together in the same pen. The number of pairs allocated to each group (for example 12) should be sufficient to ensure that there are 8 breeding pairs in the parental generation (P) control group at the end of the treatment period. Parental (P) birds are held and data collected until the end of the 7^{th} week post-treatment. Brood from the 6^{th} and 7^{th} week are used to establish the breeding first generation offspring (F1) group. Optimally, 6 eggs are set for each parental pair, but a minimum of 40 eggs must be set for each test level. If a treatment level produces insufficient eggs to set the minimum, that level is dropped from further analysis in the test. Within control and treatment (F1) groups at week 4, birds are weighed, genetically sexed, and randomly allocated to pens by pairs with odd numbered pen males being paired with even numbered females and even numbered pen males being paired with odd numbered females. The F1 generation breeding birds are maintained until 6 weeks post-fertility. The test is terminated when observations of the 14-day survival of the 6^{th} week offspring (F2) are made.

Dietary concentrations

21. The concentration of the test substance in the diet is expressed as weight of the test substance per unit weight of diet with a specific water content: mg/kg of diet (or weight of test substance per weight of water when birds are exposed through drinking water). The concentration should also be expressed as mg/kg body weight per bird per day.

22. Dietary concentrations of the test substance should be chosen on the basis of toxicological data from a range-finding study or other preceding avian tests. Information may also be gained from tests with rodents or other mammals. The highest concentration should be chosen at a level that is expected to reveal significant effects on adult health or reproductive parameters. If no significant effects are expected, then the highest concentration to be tested should be the expected concentration of the chemical in the environment with the addition of a safety factor (e.g., 5X). However, the highest dose administered should not cause mortality or other severe signs of parental toxicity that will preclude the evaluation of reproductive parameters. The lowest concentration tested should be chosen so that no effects are seen on adult health or reproductive parameters. Intermediate concentrations should be geometrically spaced between the highest and lowest doses. A minimum of four concentrations and a control are required for the definitive test.

23. A range finding test can be performed before initiating the main study to aid in establishing test concentrations and to evaluate any potential avoidance effects.

Preparation and monitoring of the test diet

24. To prepare the test diets, the appropriate amounts of test substance are mixed into the diet. The mixing method should be developed so as to obtain a homogeneous distribution of test substance in the diet. The use of a premix is advisable.

25. If necessary, a vehicle of negligible toxicity (e.g., food grade corn oil, water, etc.) may be used to ensure a uniform distribution. Acetone may be used to dissolve a test substance, provided it is allowed to evaporate before feeding the diet to the birds. The amount of vehicle used should be as low as possible and should not exceed two per cent by weight of the diet. When used, a constant amount of vehicle should be added to each test group and the control group diet, in order to keep the caloric value of the diet equal between dosage groups.

26. The frequency of diet preparation should be chosen so that degradation or volatilization of the test substance in the diet does not allow the actual concentration to fall below 80% relative to initial concentration. The frequency of diet renewal should not be more than once a day and not less than once a week. All feed should be discarded from the feeders, before fresh feed is supplied to the birds. It may be desirable to keep all feed in freezing condition until use.

27. Stability analysis under typical conditions of the test, must be performed prior to the start of the test or in parallel with the range-finding study. Stability data from previous (mammalian) feeding studies may be used as guidance, if available. Stability should be verified during the main reproduction test. In order to check stability of the test substance under test conditions, feed should be sampled and analyzed from the feed hoppers at the end of the first feeding period, i.e., before diet in the feeding bowls is renewed, and again at the end of the last feeding period. A sufficient number of samples should be taken into account for variability.

28. Homogeneity of the test substance in the diet may be evaluated prior to the test or samples for homogeneity measurements may be taken at the first mix prepared for the study.

29. In order to verify test concentrations, samples of diets fed to the birds should be taken every time new diet is mixed during the treatment period to allow measurement of the actual concentration of the test substance.

Measurements and observations

Adults

30. During the treatment period the parental birds are observed daily to detect any overt signs of toxicity or other clinical signs. The following observations are to be performed on adults during the study:

- Toxic signs and health conditions should be evaluated at least once daily, during the acclimation, stabilization, and treatment periods. Observations should include mortality and clinical sign of toxicity such as lethargy, depression, wing droop, ruffled feathers, lacrimation, etc. Any injuries sustained and subsequent treatment should also be recorded. Clearly moribund individuals or those otherwise in severe distress should be immediately euthanized.
- Food consumption (per pair) should be recorded at least weekly as often as food is replaced in the feeders. Any apparent food spillage should be noted.
- Body weights should be determined at least at the start of treatment and at the end of the treatment period. There should be no significant difference in mean body weight between test groups at the start of the treatment period. If there is a significant difference between test groups, birds should be again randomized.

Offspring - Eggs

31. During the treatment period, all eggs, with the exception of those that are cracked, broken or abnormal or used for eggshell measurements, are set, artificially incubated and allowed to hatch. All F1 generation offspring are maintained on treated diet until test termination. All F2 offspring are maintained on untreated diet until 14 days after hatching.

32. Eggs are collected at least once daily, numbered according to pen of origin and stored; broken eggs are numbered, recorded and then discarded. The total number of eggs laid per pen should be accounted for at the end of the study.

33. The eggs are stored in a cold storage facility, for a maximum of one week, prior to setting in the incubator (see Table 3). Cool storage prevents embryo development and aids in synchronizing and development of embryos of eggs laid during that week. Before placing the eggs in the incubator, they are candled to check for abnormalities and fine cracks that can be identified only by candling. After removing all cracked and abnormal eggs, the remaining eggs are equilibrated to room temperature and set in the incubator. All cracked and abnormal eggs should be recorded.

Table 3: Conditions for egg storage, incubation and hatching

	Temperature (^{o}C)	Relative humidity (%)	Turning
Storage	13-16	55-75	Optional
Incubation	37.5-37.8	50-70	Yes
Hatching	37-37.5	70-75	no

34. During the treatment periods one egg per pen is collected from odd numbered pens in odd numbered weeks and from even numbered pens during even numbered weeks. These eggs are used for eggshell strength and eggshell thickness measurements. Eggshell strength is measured using a strength tester. The unit of measurement is Newtons. The egg is placed on its side on the test stand so that the compression head will contact the egg at the equator. To determine eggshell thickness, each egg will be cut open around the equator and washed out; subsequently the shells are left to dry with the membrane intact for at least 48 hours at room temperature. Shell thickness is measured at a minimum of four points around the girth using a calibrated micrometer. Eggshells are measured to at least 0.01 mm and the mean value calculated per egg.

35. Incubation is best performed when eggs are set in an incubator with temperature and humidity control and an automatic turning device. From the second day of incubation onwards, eggs should be turned at least three times per day. If turned by hand, eggs should be turned an odd number of times a day.

36. Fertility and embryo viability are checked by removing the eggs out of the incubator and candling them. Eggs are candled for fertility after 8 days of incubation and again for viability of embryos approximately 2 days before hatching and transferred to a hatcher. During candling, eggs should not be allowed to cool to room temperature, since this may delay embryonic development. After hatching, chicks should be dry before they are taken out of the hatcher. Those chicks that have not hatched within approximately 24 hours of the majority of chicks hatching, should be considered unhatched. No assistance should be given to chicks during hatching.

Egg parameters

37. Prior to start of the treatment period, egg production of all pairs of birds available for the study (including potential replacements) should be recorded. These data will be used to exclude pairs that do not lay eggs from further study. These eggs will be discarded after candling.

38. During the treatment periods, the following information is recorded:

- Eggs laid
- Eggs cracked
- Eggs broken
- Description of all egg abnormalities
- Eggs set
- Eggshell strength
- Eggshell thickness
- Fertile eggs
- Infertile eggs
- Early viability
- Early embryonic deaths
- Late viability
- Late embryonic deaths
- Eggs that hatch
- Eggs that do not hatch

39. If a pair is removed during the study due to death, injury, etc., data from all preceding weeks are evaluated. If data are used from a pen in which a mortality occurred or a bird was injured, this should be justified.

40. Typical control values for reproductive parameters in Japanese quail are provided in Annex 2. If control values do not meet these values, the test procedure and husbandry conditions should be checked for potential problems.

Chicks

41. The following observations on chicks are recorded during the treatment periods:

- Normal hatchlings
- Clinical signs of toxicity, frequency of occurrence of abnormalities and mortality
- Number of 14-day-old surviving chicks
- Chick body weight at hatching and 14 days after hatching
- Sex of F1 chicks

Pathology

42. All adults that survive to the end of the treatment period are killed as humanely as possible. All adult birds are to undergo necropsy and gross pathology assessment. The wet weight of the liver, spleen and testes are recorded as soon as possible after death. Adult birds that die or are killed during the course of the treatment period,will be subjected to the same procedures.

43. Birds (adults or chicks) which are in severe distress will be killed *in extremis*. If one member of a pair dies or is killed *in extremis* during the treatment period, the other member of the pair is killed as well.

DATA COLLECTION AND REPORTING

Treatment of results

44. Numerical data should be presented in tabular form. All adult health data should be recorded per individual bird (food consumption per pair). Since the P1 parental pair (or pen) is the primary statistical unit and the F1 parental pair (or pen) is the secondary unit, all reproductive data should be related by lineage. The raw data values should be reported by pen to provide sufficient detail for an independent statistical analysis.

45. Measurements of endpoints made on adult birds will be evaluated by comparing values obtained from birds in treated groups with values obtained from control birds. The following adult health endpoints should be analyzed for both P1 and F1 birds, using appropriate statistical procedures:

- Food consumption
- Body weight
- Number of eggs per hen per day

46. Moreover, mortality, clinical observations or pathological findings may be evaluated statistically to verify any apparent relationship between adult and treatment.

47. The following reproductive data (both continuous and discrete variables) are to be evaluated statistically by comparison with the control group using appropriate methods:

- Number of fertile eggs as a percentage of eggs set
- Number of early viable embryos as a percentage of eggs fertile
- Number of late viable embryos as a percentage of early viable embryos
- Number of eggs hatched as a percentage of late viable embryos
- Number of 14-day old chicks as a percentage of eggs hatched
- Number of 14-day old chicks as a percentage of eggs set
- Number of 14-day old chicks per hen per day
- Number of cracked eggs as a percentage of eggs laid
- Number of abnormal eggs as a percentage of eggs laid
- Egg shell thickness and eggshell strength

- Mean hatchling body weight
- Mean 14-day old chick body weight
- Sex ratio of chicks

48. The following parameters may be useful to evaluate statistically, depending on the findings:

- Number of hatchlings per hen per day
- Number of normal hatchlings as a percentage of total hatchlings
- Number of infertile eggs as a percentage of eggs set
- Number of early embryonic deaths as a percentage of fertile eggs
- Number of late embryonic deaths as a percentage of viable eggs

49. The following endocrine and physiological endpoints (both continuous and discrete variables) are to be evaluated statistically by comparison with the control group using appropriate methods:

- Weight of testes, ovaries, thyroid, adrenals, oviduct, cloacal gland, liver
- Histology of thyroid, adrenals, gonads, brain
- Testicular spermatid counts and morphology
- Gross anomalies of the genital tract
- Feather dimorphism
- Cloacal gland size, 1st appearance of foam
- 1st egg laid
- Sexual behavior
- Fecal/urate steroid hormones (estradiol, testosterone)
- Egg steroid content (estradiol, testosterone)
- Tibiotarsus length (F1)

50. A “no observed effect concentration” (NOEC) expressed in mg/kg diet and mg/kg body weight per day should be determined for all health and reproductive parameters evaluated.

Test report

51. The final report should include the following information:

Test substance:

- identification, including chemical name
- batch or lot number
- degree of purity
- chemical stability under the conditions of the test
- volatility

Test species:

- name of species tested (scientific name), strain or origin, source and age of birds at start of test (in weeks)

Test conditions:

- housing conditions (type, size and material of pens for adults and for chicks, additional floor covering, adjustments made to pen floors to facilitate egg collection and prevent breakage, temperature, humidity, ventilation, illumination intensity and photoperiod, water supply); any changes to these during the test; measures taken to minimize food spillage

- description of the untreated diet, manufacturer, composition, caloric content of diet and carrier, results of periodically performed contaminant and nutrient analysis of diet and drinking water contaminant analysis
- test groups (number of concentrations used, nominal concentrations); details of type of carrier used and concentration as percentage of diet; test substance concentrations must be reported as mg/kg diet and as mg/kg body weight
- specific analytical method used to determine the concentrations of test substance in the diet as well as actual values of homogeneity, stability and accuracy of preparation in diet under test conditions, as recorded in the study
- description of the kind and frequency of the procedure used to prepare the test diets; description of the manner of administrating the test diets; storage conditions
- description of acclimation, stabilization and pre-treatment procedures, and method of assigning pairs to test groups (P1 and F1); arrangement of pens; number of pairs per dose group; measures taken, if any, to reduce pen-mate aggression
- frequency, duration and methods of observation
- information on the period and conditions of storage of eggs and on the incubation method
- method of marking all birds and eggs

Results:

- description of incidence of mortality, indicating the number of dead animals and the time of death during the test
- description of all clinical signs of toxicity and other abnormal behaviour, including time of onset, duration and severity, and number of affected birds per test group and in the control group; any injuries sustained and subsequent treatment
- macroscopic pathological findings and organ weights and results of histopathological investigations
- mean body weights of P1 males and P1 females per pen at start of treatment and at the end of the P1 treatment period (end of week 6 of treatment); mean body weights of F1 males and F1 females per pen at pairing and at the end of the test; individual weights of the birds that died or were killed during the test
- weekly food consumption per test group during treatment periods and extrapolated mean food consumption per pair; and expressed as mg/kg body weight per bird per day; an indication of any apparent food spillage
- results of range finding test
- lowest observed effect concentration (LOEC)
- no observed effect concentration (NOEC)

Data on reproduction during treatment periods (tabulated separately for P1 and F1)

- number of eggs laid per hen and per day
- all eggs should be traceable to their pen of origin
- all egg abnormalities should be described and reported
- numbers of eggs cracked and the percentage of eggs cracked of eggs laid
- eggshell thickness, eggshell strength measurements
- number of fertile eggs and the percentage fertility related to eggs set
- number of infertile eggs and the percentage infertility related to eggs set
- number of early viable embryos and the percentage viability related to fertile eggs
- number of early embryonic dear and the percentage related to fertile eggs
- number of late embryonic dead and the percentage related to 8-day viable embryos
- number of normal hatchlings per hen per day and the percentage related to 15-day viable embryos
- number of normal hatchlings as a percentage of fertile eggs

- number of normal hatchlings as a percentage of eggs set
- number of normal hatchlings and the percentage related to total number of hatchlings
- number of 14-day old chicks per hen and per day
- number of 14-day old chicks as a percentage of (normal) hatchlings
- number of 14-day old chicks as a percentage of eggs set
- mean body weights of chicks on the day of hatching and after 14 days per pen, per test group
- description of abnormal behaviour of chicks, of severe birth defects and their general state of health, during the first 14 days after hatching, clinical signs of toxicity
- observations of hatchling mortality
- NOEC (and LOEC) per parameter that was evaluated statistically

A description of statistical methods used in the analysis of data.

LITERATURE

(1) Report of the SETAC/OECD Workshop on Avian Toxicity testing. (1996). Environment Health and Safety Publication - Series on Testing and Assessment: No. 5. OECD/GD (96)166, Paris.

(2) OECD Guidelines for the Testing of Chemicals. (1993). Section 2 - Effect on Biotic Systems: Test Guideline 206: Avian Reproduction Test (adopted April 1984).

(3) OECD Guidelines for Testing of Chemicals. Proposal for a new test guideline: Avian reproduction toxicity test in the Japanese quail or Northern bobwhite. (September 1999 draft)

(4) Endocrine Disruptor Screening and Testing Advisory Committee: Final Report. (1998)