

# Test of the Impact on Reproductive Potential and Future Generations of Mammals and Test of the Impact on Reproduction of Birds

## I.

This paper sets forth standard methods of tests concerning the impact on reproductive potential and future generations of mammals and tests of the impact on reproduction of birds.

## II. Test of the Impact on Reproductive Potential and Future Generations of Mammals

Regarding tests of the impact on reproductive potential and future generations of mammals, these tests should be conducted in accordance with methods stipulated in "IV. Tests of the Impact on Reproductive Potential and Later Generations" in "Chronic Toxicity Test, Test of the Impact on Reproductive Potential and Future Generations, Teratogenicity Test, Mutagenicity Test, Carcinogenicity Test, Test on *in vivo* fate, and Pharmacological Test."

## III. Test of the Impact on Reproduction of Birds

### Objective

The objective of this test is to demonstrate the toxicity of the test substance for the reproduction of birds by administering the test substance on parent birds and by observing the effects on the fatality rate of the parent bird, the number of eggs laid, the number of eggs with cracked shells, thickness of egg shells, the rate of embryonic growth, the proportion of hatched eggs and baby birds.

#### 1. Definition

The meaning of the terms used in this testing method is as follows.

NOEC (No Observed Effect Concentration): of the various levels of concentration used in the test, the maximum concentration of the test substance that produces no adverse effect

Basic feed: feed during reproduction period for parent birds and initial feed for baby birds, which are suitable for respective kinds for testing and contain all necessary nutritional elements

Egg group: eggs hatched at a time, or all eggs laid excluding eggs with cracked shells and eggs to be used for measuring the thickness of egg shells

## 2. **Physico-chemical characteristics of the test substance**

In order to conduct the test, a reliable method of analysis is needed to measure water solubility and vapor pressure of the test substance and to determine the quantity of the test substance within the feed. It is also necessary to collect as much information as possible on structural formulas, purity, stability against water and light, and stability within the feed of the test substance, which are closely related to testing techniques.

## 3. **Preliminary tests**

In order to know the rough toxicity of the test substance beforehand, toxicity tests on feed intake for birds are conducted in accordance with the methods stipulated under the OECD Test Guidelines 205.

## 4. **Test organisms**

One or more species of birds are used. The use of *Coturnix japonica* is recommended, but *Anas platyrhynchos* or *Colinus virginianus* can also be used. When species other than the above are used, the reasoning for the use of such species should be described in a report. Tests are conducted with birds purchased or birds being kept and raised at test-related facilities. When birds are brought in, they should be examined to confirm that they are not sick or hurt in any way. Birds to be used in tests should belong to the same group as the known breed. *Anas platyrhynchos* and *Colinus virginianus* should be similar to wild species in appearance.

## 5. **Test method**

### 5-1. Test facilities and equipment

#### (1) Test facilities

Adequate test facilities should be used, and it is desirable to raise birds indoors. Test facilities need to have mechanisms to control ventilation, temperature, humidity and lighting at appropriate levels. Artificial lighting used should be automatically controlled and approximate to sunlight in terms of the visible spectrum. It is desirable to have a lighting transition period of 15 to 30 minutes between lights-on and lights-out (it is desirable to gradually light up or black out over 15 to 30 minutes).

#### (2) Equipment

The following equipment should be used.

- Bird cages or other closures (hereinafter "bird cages") with enough space to raise parent birds and baby birds. Clean bedding may also be used. Brooders for baby birds must have temperature controls.

- It is desirable to use incubators that can control temperature and humidity automatically and have egg-turning mechanisms.
- Equipment or facilities that can store eggs at a stable temperature and levels of humidity.

### 5-2. Acclimatization

Parent birds are allocated at random to test concentration groups and control groups. Condition birds in test concentration groups and control groups to test facilities and basic feed for at least two weeks. Birds that are found to be unable to live together during the first week of acclimatization may be reallocated.

In case 3% of either males or females die or become weak during the period of acclimatization, birds in the groups concerned should not be used in tests.

### 5-3. Implementation of tests

#### 5-3-1. Test conditions

##### (1) Environmental conditions

Parent birds should be kept in an environment with temperatures of  $22\pm 5^{\circ}\text{C}$ , humidity of 50-75%, and good ventilation. Table 1 lists other unique conditions necessary for respective species of birds.

Apart from the fact that the test substances are not added during acclimatization, the environmental conditions are identical for both the acclimatization and exposure periods. Use of chemical substances or administration of drugs should be avoided as much as possible. If and when they are used, they should be described in a report.

Environmental disruptions that could significantly affect behaviors of birds should be avoided. (Any significant disruption to the environment and influence on the birds' behavior should be avoided as much as possible.)

Environmental conditions necessary for eggs and baby birds are shown in [Table 2](#).

Temperature and humidity levels indicated in the table are conditions applicable when incubators with forcible ventilators are used. When forced ventilation is not applied, temperature levels should be set  $1.5\text{-}2^{\circ}\text{C}$  higher and humidity levels about 10% higher. Temperatures in brooders should be measured at a height 2.5-4 cm above the floor.

**Table 1. Conditions recommended for parent birds**

Species	Age at the start of exposure	Age range	Minimum bird cage floor space per brace <sup>*1</sup>
<i>Coturnix japonica</i>	<sup>*2</sup>	±1/2 week	0.15m <sup>2</sup>
<i>Anas platyrhynchos</i>	9-12 months	±2 weeks	1m <sup>2</sup>
<i>Colinus virginianus</i>	20-24 weeks	±1 week	0.25m <sup>2</sup>

<sup>\*1</sup>: When the number of birds increases, the floor space should be widened accordingly.

<sup>\*2</sup>: As for *Coturnix japonica*, it is recommended that birds used are confirmed to be capable of reproduction beforehand in order to minimize the margin of fluctuation often seen for this species.

**Table 2. Conditions recommended for eggs and baby birds**

	Temperature (°C)	Humidity (%)	Egg turning
<i>Coturnix japonica</i>			
Egg storage	15-16	55-75	optional
Egg incubation	37.5	50-70	yes
Hatching	37.5	70-75	no
Baby birds (1 week)	35-38	50-75	-
Baby birds (2 weeks)	30-32	50-75	-
<i>Anas platyrhynchos</i>			
Egg storage	14-16	60-85	optional
Egg incubation	37.5	60-75	yes
Hatching	37.5	75-85	no
Baby birds (1 week)	32-35	60-85	-
Baby birds (2 weeks)	28-32	60-85	-
<i>Colinus virginianus</i>			
Egg storage	15-16	55-75	optional
Egg incubation	37.5	50-65	yes
Hatching	37.5	70-75	no
Baby birds (1 week)	35-38	50-75	-
Baby birds (2 weeks)	30-32	50-75	-

(2) Administration of the test substance

The test requires at least three concentration groups for the test substance.

The in-feed concentration of the test substance is set on the basis of results of feed toxicity tests for birds. The maximum concentration is set at about half of LC<sub>10</sub> (the concentration of a test article in feed that is assumed to have killed 10% of birds in the test). Other concentration levels are set geometrically against the maximum concentration (for example, 1/6 and 1/36 of the maximum concentration). The recommended maximum concentration is 1,000mg/kg.

Feed with the necessary amount of test substance is prepared by mixing the necessary amount of the test substance with basic feed to raise parent birds. The test substance should be dispersed evenly in feed. In order to ensure that the test substance is evenly dispersed, auxiliary agents with low-toxicity to birds may be used. Auxiliary agents should not exceed 2% of the weight of feed.

When auxiliary agents are used, the same auxiliary agents should be used in feed for birds in control groups. Water, corn oil or other auxiliary agents that are proven not to alter the toxicity of the test substance may be used. When auxiliary agents that are not proven to not alter the toxicity of the test substance, the reasoning for their use should be explained in a report.

The test substance or auxiliary agents should not be added to the feed for baby birds.

**Table 3. Normal value for items related to reproduction <sup>\*3</sup>**

Item	<i>Coturnix japonica</i>	<i>Anas platyrhynchos</i>	<i>Colinus virginianus</i>
No. of eggs laid/female birds (10 weeks)	40-65	28-38	28-38
Incidence of eggs with cracked shells (%)	-	0.6-6	0.6-2
Incidence of embryonic growth (surviving embryo per egg group, %)	80-92	85-98	75-90
Rate of hatching (hatched eggs per egg group, %)	65-80	50-90	50-90
Rate of baby birds that lived for 14 days (%)	93	94-99	75-90
No. of baby birds that lived	28-38	16-30	14-25

for 14 days/female birds			
Thickness of egg shells (mm)	0.19-0.23	0.35-0.39	0.19-0.24

<sup>\*3</sup> Normal values in the table can be said to be typical, but do not necessarily represent values obtained by all testing organizations. When values obtained for birds in control groups do not correspond to values in the table, test methods and test conditions must be reexamined.

### (3) Test operations

A brace of birds or a group of one male and two females (for *Coturnix japonica* and *Colinus virginianus*), or a group of one male and three females (*Anas platyrhynchos*) are raised in bird cages. When deemed appropriate, other combinations of male and female birds may be applied. Birds in test concentration groups and control groups are raised under the identical test conditions. When a brace of birds are used, at least 12 bird cages should be used for each test concentration group and control group. When a group of birds are used, at least eight bird cages for *Anas platyrhynchos* and at least 12 bird cages for *Colinus virginianus* and *Coturnix japonica* are used for each test concentration group and control group.

Tests begin with giving feed containing the test substance to parent birds, and parent birds should continue to be fed with such feed throughout the exposure period. Baby birds are given feed that does not contain the test substance or auxiliary agents. They should be allowed to drink clean water at will.

When tests are conducted under artificial room conditions, birds are raised under short-day conditions (7-8 hours of lighting per day) for eight weeks after the start of the exposure period. During this period, lighting should not be halted in the dark period. After that, lighting hours are extended to 16-18 hours a day to put birds in a breeding condition. Birds start laying eggs 2-4 weeks after lighting hours are extended.

When tests are conducted under open-air conditions, they should be carried out in times corresponding to the egg-laying seasons of species used in given testing locations. Feed containing testing articles should be given to birds for at least 10 weeks before they begin laying eggs.

Under either test conditions, tests should continue at least 8 weeks, and 10 weeks if possible, after birds start laying eggs.

The concentration of the test substance in feed one week after the start of exposure should be kept at least at 80% of the prescribed concentration. When the stability of the test substance in feed is not fully proven, during the first week, feed containing the maximum and minimum test concentration levels of the test substance should be analyzed immediately after the mixing and within four days of feed replacement. If all analysis values exceed 80% of the prescribed concentration levels, no new analysis is required and feed under test should be replaced sufficiently often in order to maintain the concentration levels of the test substance.

When the concentration levels of the test substance in feed are found to be below 80% of the prescribed concentration level, adjustments should be made to maintain actual concentration levels by either raising the initial level of concentration or by replacing feed frequently. Another analysis should be conducted in the second week of exposure in order to ascertain that these adjustments are effective in keeping the actual concentration levels at least at 80% of the present level of concentration.

Even when the test substance in feed remains stable, feed in bird cages should be replaced at least once a week. If the stable administration of the test substance is possible only when feed is replaced every day, the very effectiveness of such tests cannot be ensured.

After birds start laying eggs, eggs are collected every day and marked with signs corresponding to bird cages where they are collected. Eggs are put into incubators every week or every other week for storage and hatching (see [Table 2](#)). Hold up eggs to the light to detect any dent before placing them in incubators. Dented eggs should not be used for tests. Six to 11 days later, eggs intended for hatching should be held up to the light to see if the generation is in progress.

At least two eggs from each bird cage are collected according to preset schedules (for example, 3<sup>rd</sup> eggs and 10<sup>th</sup> eggs, or all eggs collected on the 5<sup>th</sup>, 20<sup>th</sup> and 35<sup>th</sup> day) to measure the thickness of egg shells. Dented eggs are counted to record their numbers, but are not measured for their shell thickness. Subject eggs are broken, cleaned, dried with membranes attached, and then measured for the thickness of shells at three or four points around shells.

Eggs are moved from incubation to hatching conditions on the 16<sup>th</sup> day for *Coturnix japonica*, on the 23<sup>rd</sup> day for *Anas platyrhynchos*, and on the 21<sup>st</sup> day

for *Colinus virginianus*. Hatching should be completed by the 17<sup>th</sup> or 18<sup>th</sup> day for *Coturnix japonica*, by the 25<sup>th</sup> or 26<sup>th</sup> day for *Anas platyrhynchos*, and by the 23<sup>rd</sup> or 24<sup>th</sup> day for *Colinus virginianus*.

Baby birds are housed either as groups corresponding to original bird cages or with individual signs marked on them. Baby birds are raised for 14 days with appropriate feed that does not contain the test substance. Temperature and humidity levels for baby birds are shown in [Table 2](#). Preferably, baby birds are raised under conditions with light and dark cycles (for example, 14 hours of light and 10 hours of darkness), with 15-30 minutes of transition time between lights-on and lights-out.

#### 5-3-2. Observation

During the test period, the following items should be observed.

- Death and symptoms of poisoning – every day
- Body weight of parent birds – at the start of feeding, before the start of laying eggs, and upon completion of tests
- Body weight of baby birds – at the age of 14 days
- Feed intake of parent birds – every week or every other week during the test period
- Feed intake of baby birds – the first week and second week after hatching
- Pathological examination with the naked eye – all parent birds

#### 5-4. Validity of tests

If tests are to be valid, the following conditions must be satisfied.

- The fatality of parent birds in control groups should be no higher than 10% at the completion of tests.
- The average survival rate of 14-day-old baby birds per parent female bird in control groups should be at least 24, 14 and 12 birds, respectively, for *Coturnix japonica*, *Anas platyrhynchos* and *Colinus virginianus*.
- The average thickness of egg shells for control groups should be at least 0.19, 0.34 and 0.19 mm, respectively, for *Coturnix japonica*, *Anas platyrhynchos* and *Colinus virginianus*.
- It has to be proven that the concentration levels of the test substance in feed are adequately maintained (at least 80% of the prescribed concentration levels).



When the recommended concentration levels are used with no impact on reproduction observed, NOEC (No Observed Effect Concentration) can be reported as above the maximum concentration level tested.

**6. Processing of test results**

Using appropriate statistical methods, including the analysis of variance, data collected for test concentration groups are individually compared with data from control groups. Items for analysis are those shown in [Table 3](#) and, if possible, the ratio of egg-laying birds (%), the body weight of parent birds and the body weight of surviving 14-day-old baby birds.