Appendix Q

Study Designs and Protocols for Recommended Tests in Tier 2 Testing Battery

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I. Two-Generation Mammalian Reproductive Toxicity Study Design

The two-generation reproductive toxicity study in rats (TSCA 799.9380, August 15, 1997; OPPTS 870.3800; Public Draft, February 1996; OECD no. 416 1983; FIFRA Subdivision F Guidelines - 83-4) is designed to comprehensively evaluate the effects of a chemical on gonadal function, estrous cycles, mating behavior, fertilization, implantation, pregnancy, parturition, lactation, weaning, and the offsprings' ability to achieve adulthood and successfully reproduce, through two generations, one litter per generation. Administration is usually oral (dosed feed, dosed water, or gavage) but other routes are acceptable with justification (e.g., inhalation). In addition, the study also provides information about neonatal survival, growth, development, and preliminary data on possible teratogenesis. The experimental design for a two-generation reproductive toxicity study is presented in Figure Q-1.

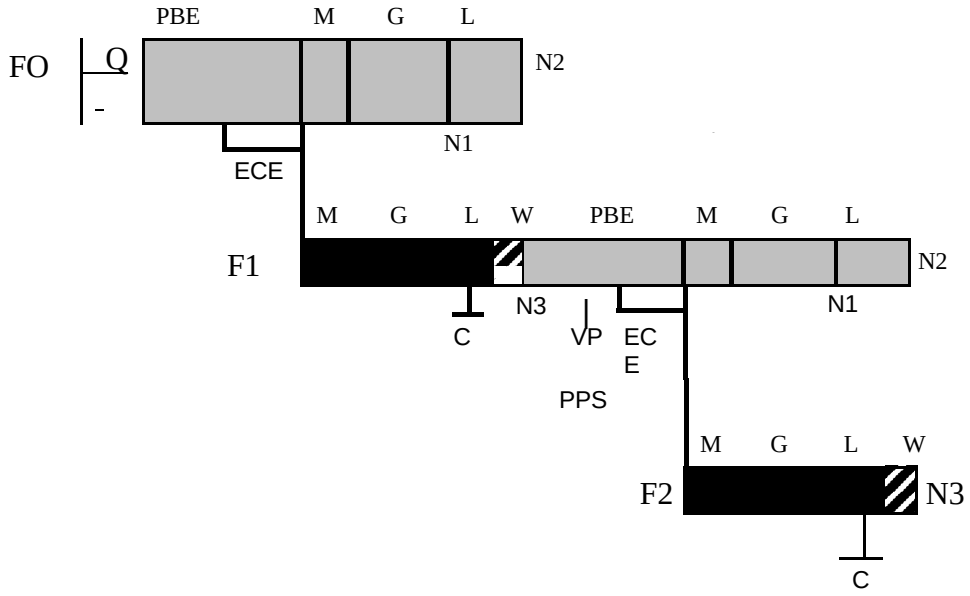
In the existing two-generation reproductive toxicity test, a minimum of three treatment levels and a concurrent control group are required. At least 20 males and sufficient females to produce 20 pregnant females must be used in each group as prescribed in this current guideline. The highest dose must induce toxicity but not to exceed 10% mortality. In this study, potential hormonal effects can be detected through behavioral changes, ability to become pregnant, duration of gestation, signs of difficult or prolonged parturition, apparent sex ratio (as ascertained by anogenital distances) of the offspring, feminization or masculinization of offspring, number of pups, stillbirths, gross pathology and histopathology of the vagina, uterus, ovaries, testis, epididymis, seminal vesicles, prostate, and any other identified target organs. Table 5.3 (Chapter Five, Section VI, B, provides a summary of the endpoints that are evaluated within the framework of the experimental design of the updated two-generation reproductive toxicity test (and some additions, still under consideration, to cover Estrogen, Androgen, and Thyroid concerns).

These observations are comprehensive and cover every phase of reproduction and development. Tests that measure only a single dimension or component of hormonal activity, (e.g., *in vitro* or short-term assays) provide supplementary and/or mechanistic information, but cannot provide the breadth of information listed in Table 5.3, which is critical for risk assessment.

Additionally, in this study type, hormonally-induced effects such as abortion, resorption, or premature delivery as well as abnormalities and anomalies such as masculinization of the female offspring or feminization of male offspring can be detected. Substances such as the phytoestrogen, coumesterol, and the anti-androgen, cyproterone acetate, which possess the potential to alter normal sexual differentiation, were similarly detected in this study test system (i.e., 1982 Guideline). The initial prebreed exposure period (ten weeks) of the two-generation reproductive toxicity test also provides information on subchronic exposures which can be used for other regulatory purposes.

Figure Q.1

Two-Generation Reproductive Toxicity Study Design



KEY

Q = Quarantine (one week)
PBE = Pre-Breed Exposure (ten weeks)
M = Mating (two weeks)
G = Gestation (three weeks)
L = Lactation (three weeks)
VP = Vaginal patency (evaluated in F1 females on postnatal day 22 to acquisition)
PPS = Preputial separation (evaluated in F1 males on postnatal day 35 to acquisition)
W = Weaning (postnatal day 21)
N1 = Necropsy of all paternal animals
N2 = Necropsy of all maternal animals
N3 = Necropsy of selected weanlings, three/sex/litter, if possible
ECE = Estrous Cyclicity Evaluations (three weeks)
C = Cull litters to ten pups (with equal sex ratio) on postnatal day four

Direct exposure via diet, drinking water, inhalation, etc.

Possible indirect exposure from transplacental and/or translactational exposure

Both direct and possible indirect exposure if in feed or water (nursing pups also self-feeding and drinking)

Study Design for the Alternative Mammalian Reproduction Test II.

Female P0 rats are quarantined for one week prior to mating. Sufficient numbers of animals are used in order to attain 20 pregnant dams per dosage group. During a two-week mating period females are removed from breeding cages when sperm positive smears are detected and they are randomly assigned to treatment groups. Chemicals are administered to pregnant female rats by oral gavage beginning on day seven of gestation (day one being the day sperm positive) and continued throughout gestation and lactation, up to weaning of the F1 generation. F1 pups are never exposed directly, but receive the chemical via transplacental and lactational exposures.

On the day of birth (postnatal day (PND) 0), anogenital distance (AGD) is measured in both male and female F1 pups. Animals with ambiguous gender should be uniquely identified such that sex can be identified at a later age. At about four days of age, litters should be standardized to eight pups (four males and four females where possible) by randomly removing excess pups (necropsy to confirm sex). At about twelve days of age, the pups are examined for the presence of nipples/aerolas, which should be clearly visible in female but not male pups. (These endpoints are sensitive to androgens and antiandrogens.)

After weaning at 21 days of age, pups (at least 20/sex/dose) are housed in unisexual pairs until mating ten weeks later to produce the F2. All surplus weanlings are necropsied and examined externally and internally for reproductive tract malformations. P0 parental females will be necropsied at the weaning of their F1 litters on PND 21. These dams will be subjected to a gross necropsy with organ weights: thyroid, ovaries, pituitary, uterus (count implants), kidneys, and liver.

After weaning, female F1 retained rats are examined daily, beginning on PND 22, for vaginal opening until complete. Both age and weight at vaginal opening are determined. For retained F1 male pups, the age of preputial separation is determined from PND 35 on, with age and weight at preputial separation recorded.

Three weeks prior to mating of the F1, estrous cyclicity is monitored for three weeks in female rats. Following this, F1 non littermate pairs of males and females from the same dose group are established for a two week period in order to produce the F2 generation. Upon delivery, F2 pups are counted by sex and weighed, being terminated at weaning after individual body weights are taken. The F2 litters are not standardized to eight pups per litter.

After mating the F1 males are necropsied and reproductive organs (SV, VP, LA, testis, epididymides, pituitary, etc.) are weighed and preserved for histological examinations. In addition, one testis and caput/corpus and cauda epididymis is used for enumeration of sperm numbers. The thyroid gland also is weighed and saved for histological examination and serum is taken for determination of serum T4, T3 and TSH (especially if testing was triggered by thyroid endpoints in screening). Liver, kidney, and brain weights also should be determined.

F1 females should be necropsied immediately after weaning of the F2 pups and reproductive organs (ovary, uterus, pituitary) weighed and saved for histopathological examination. Liver, kidney and brain weights also should be taken. The thyroid gland also should be weighed and saved.

III. Study Design for the One-Generation Test

A. Rats of both sexes seven-nine weeks of age, quarantined for one week, distributed into treatment groups (four is usual, can use any number depending on previous information) ten/sex/group, then exposed separately (housed individually) to chemical substance or mixture by whatever route (we have used gavage, IV injection, inhalation, dosed feed, dosed water) for a prebreed exposure period (suggest two weeks but would depend on any prior information on chemical substance or mixture in terms of bioaccumulation, cumulative toxicity, etc.; could delete to shorten duration)

B. Animals mated one: one within groups for two weeks (can use one week to shorten duration); vaginal sperm/copulation plug indicative of insemination with date designated gestational day (gd) 0. On gd 0, females ("dams") separated from males and housed individually.

C. Dams deliver on gd 22 ± one (for rats), date of delivery designated postnatal day (pnd) 0. Anogenital distance measured in newborn pups (pnd 0 or one). Litters (designated F1) culled to standard size on pnd four (usually eight-ten/litter, with as even a sex ratio as possible). Culled pups could be examined, or discarded. F0 males necropsied after all dams deliver, with organ weights, blood samples (?), andrological assessments (?).

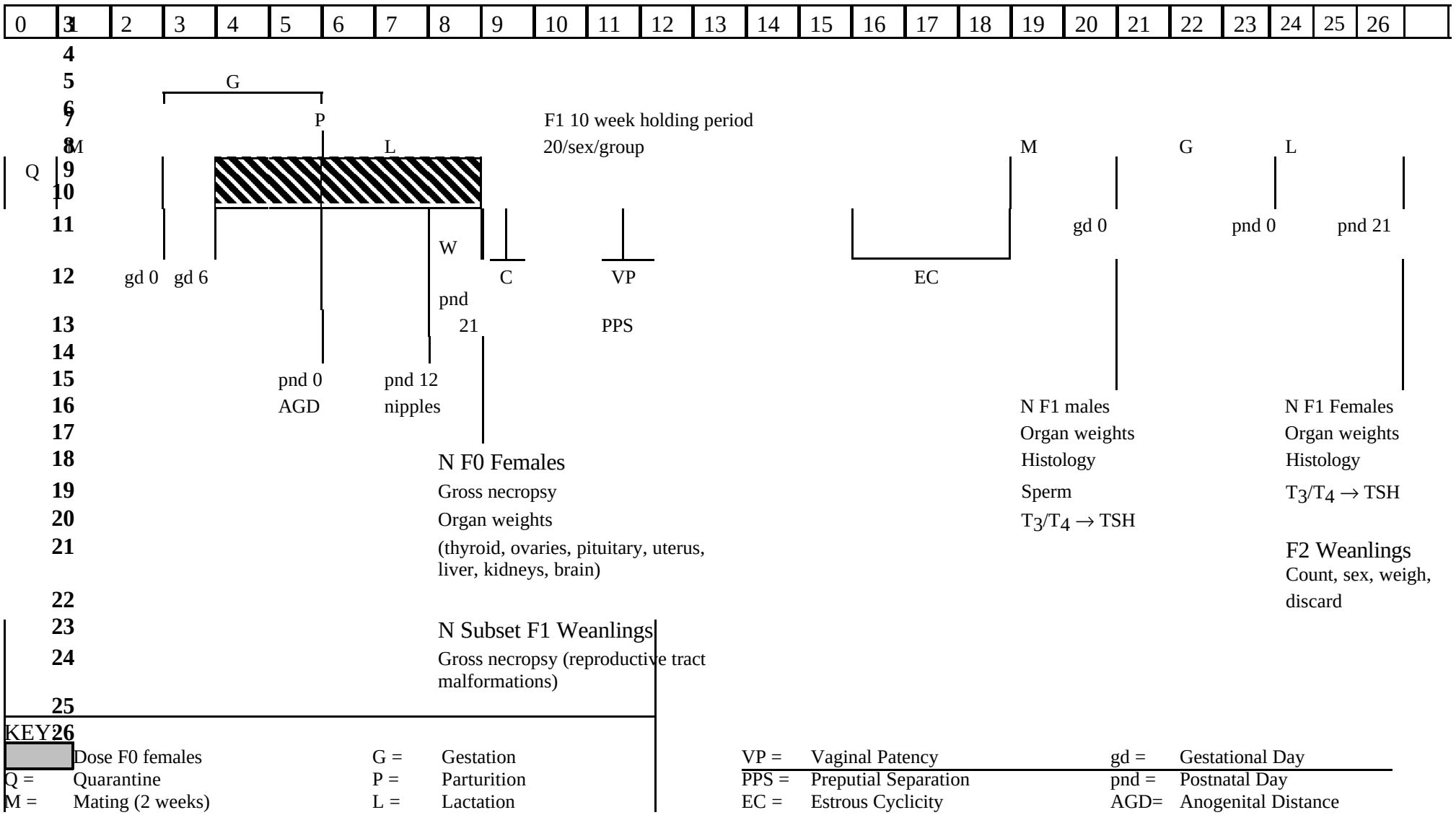
D. At weaning on pnd 21, F0 dams necropsied, with organ weights, blood samples (?), etc., F1 one-three pups/sex/litter necropsied with organ weights, blood sampling (?).

E. Also at weaning, pups selected from all litters, ten/sex/group, to undergo postwean exposure.

F. During ten-week post-wean exposure period, F1 females assessed for vaginal patency (VP; starting on pnd 22), F1 males assessed for preputial separation (preputial separation; starting on pnd 35). For the last three weeks, F1 females assessed for vaginal cyclicity by daily vaginal smears (can terminate after VP and preputial separation).

G. On pnd 91, F1 males and females necropsied with organ weights, blood sampling, andrological assessments, etc.

Figure Q.2:Alternative Mammalian Reproductive Test (AMRT)



N = Necropsy
C = Cull F1 pups on pnd 4

H. Information on:

- General toxicity (body weights, feed and/or water consumption, chemical substance or mixture intake, clinical observations, etc.)
- Mating
- Fecundity
- Fertility
- Parturition (number total, dead, live, sex, weight, AGD, gross exam)
- Prenatal survival and growth
- Postnatal survival and growth
- Reproductive development (VP, preputial separation)
- Vaginal cyclicity in F1 females
- Male epididymal sperm number, motility, morphology; testicular homogenization-resistant spermatid head counts (SHC), daily sperm production (DSP), efficiency of DSP
- Blood samples for E, A, T (T_4, TSH)
- Organ weights: reproductive organs, thyroid, liver (EROD?)
- Histology of reproductive organs, thyroid, brain, etc., optional

I. Advantages include:

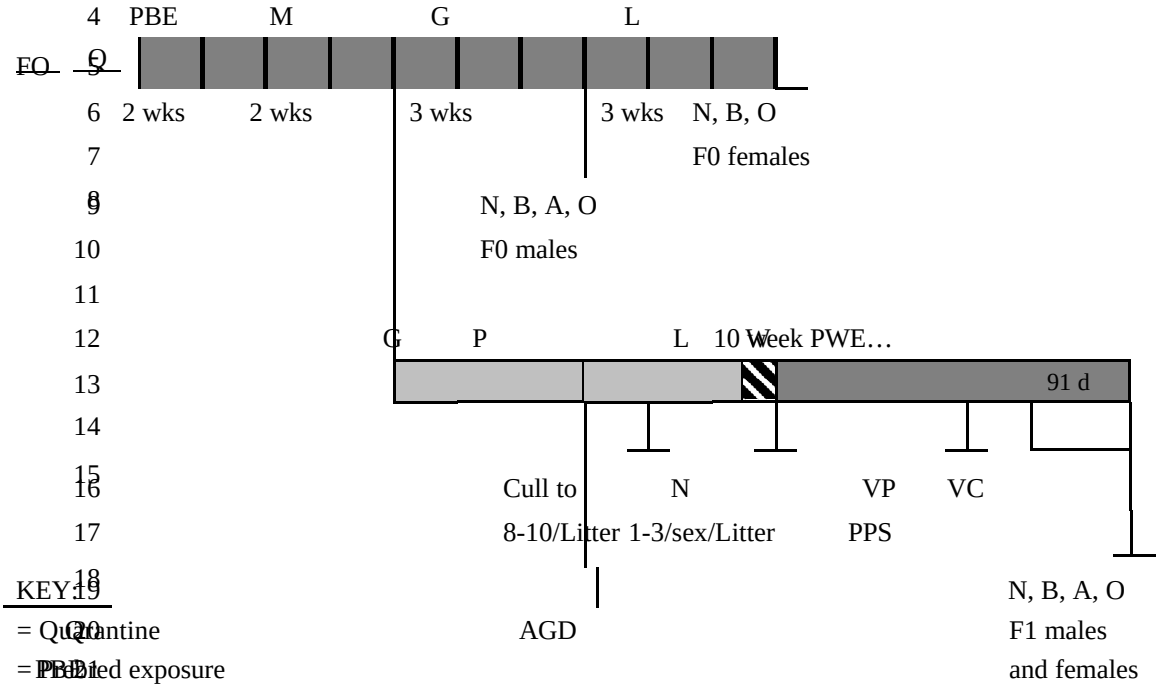
- Involves *in utero* exposure
- Assesses reproductive development, structures and functions.
- Can be modified as needed, e.g., shorten/lengthen/delete prebred exposure, shorten mating, shorten postwean exposure (to only through reproductive development or to ~pnd 50), after postwean exposure, mate F1 to produce F2 generation (terminate F2 on pnd four, etc.), add other assessments (e.g., more blood samples, histopathology, etc.), add F1 developmental landmarks such as surface righting reflex, pinna detachment, eye opening, acquisition of startle reflex, midair righting reflex, motor activity, learning and memory, etc.
- Covers the mammalian "waterfront" for E, A, and T.
- Any contract lab currently performing multi-generation studies has the necessary capabilities (staff, facilities, SOPs, experience). This is relatively "low tech" except for the blood assays and andrological assessments.

J. Sensitivity:

The sensitivity is dependent on the number of animals/group (for statistical power), the type of assessments included, the number of treatment groups and the range of doses. It is a powerful, apical assay.

Figure Q.3

One-Generation Mammalian Reproductive Toxicity Test



KEY:

= Quarantine
= Pre-mating exposure
= Mating
= Gestation
= Lactation
= Necropsy
= Blood samples
= Anatomical assessments
= Organ weights
= Parturition (delivery, pnd 0)
= Anogenital distance
= Vaginal patency
= Preputial separation
= Vaginal cyclicity
= Post-weaning exposure

Direct exposure

Possible indirect transplacental and/or translactational exposure

Direct and possible indirect exposure if route is dosed feed or water

1

IV.Recommended Extensions to the Avian Reproduction Guideline

A.The following modifications of OPPTS 850.2300 are recommended to assess development in the offspring of exposed adults.

•modify *(d) Test procedures (2) Definitive test, (viii) Chick ...,* to require that all offspring be maintained separately by parental (dose) group; and,

•modify *(d) Test procedures (2) Definitive test, (ix) Observations of record on chicks...* to include:

1.the genetic sex ratio at hatching; genetic sex can be determined routinely by the identification of restriction fragments from sex chromosomes derived from red blood cells in a small blood sample (~0.3ml) (Halvorsen 1990; Dvorak et al. 1992); identification of the genetic sex of offspring allows closer control of subsequent operations, as well as providing an endpoint of interest.

2.at 14 days of age, for 12 genetic males at each dose level:

•external characteristics, the size of cloacal protuberance in ducks and cloacal gland size in quail (Ottinger, M.A. and H.J. Brinkley., other refs); and,

•internal characteristics, the size and dimorphism of gonads, the presence of oviducts, especially on the left side, and the histology of the gonad, looking especially for the relative amount of cortex and the presence of primary germ cells located there, (Fry et al., 1987; Ottinger and Bakst, 1981); also, serum sex steroid (estrogens, androgens) should be determined (more refs needed)

3.at 14 days of age, for 12 genetic females at each dose level:

•internal measurements only, looking especially for presence of structures on the right side, and histologically the relative amount of cortex and medulla and the development of oocytes; also, serum sex steroid (estrogens, androgens) should be determined.

4.at 14 days of age, both sexes at each dose level will be assessed for general growth parameters indicative of adequate thyroid function major organ weights, including brain, body weights, wing and bone length, thyroid weight, and skeletal x-ray. If there are significant differences among exposure groups in these responses, thyroid histopathology will be performed on all groups. Otherwise, only the high dose and control groups will be so assessed.

5.at 14 days of age all surviving chicks will be subjected to the visual cliff test (Baxter et al., 1969; Dahlgren and Linder, 1971; Emlen, 1963; Fleming et al., 1985; Fox, 1976). The F1 chicks will be challenged with a cold stress test (Fleming et al., 1985a; Fleming et al., 1985b; Maguire and Williams, 1987; Martin and Solomon, 1991; Rattner et al., 1982; Rattner et al., 1987; Tori and Mayer, 1981) and the nest attentiveness test (Fox et al., 1978; McArthur et al., 1983; Kubiak et al., 1989).

·Breeding the Second Generation:

Complete assessment of the potential endocrine disruption of chemical substances or mixtures requires a test to the reproductive capability of birds exposed as embryos. This is distinct from data gathered in part A that evaluates the progress of sexual differentiation and reproductive development in birds exposed as embryos. There are no codified guidelines for a two-generation avian reproduction test, however, an OECD work group is currently designing protocols for such a test. These guidelines should be used if they will be available soon enough to implement in Tier 2 Testing. If not, development of a protocol by the EPA should be a very high priority.

A two-generation avian reproduction test should have the following features:

- uses subjects that mature and breed in a short time; Japanese quail are an obvious choice, and in addition there is substantial information on the husbandry, development, endocrinology and reproduction of this species,
- exposure of the parental generation should be exposed through the feed before and continue through egg-laying; chicks (F1 generation) should be fed from hatching diets at the same dose levels as P1,
- breeding of F1 should begin as soon as possible, consistent with the biology of the species used; Japanese quail mature in about eight weeks, and should be kept on long day photoperiod from hatching,
- responses measured should follow the guidelines for the Avian Reproduction test (OPPTS 850.2300) with the addition of measurement of the age at reproduction in both sexes.
- From the 12 hens per treatment group, 60 eggs will be needed with 30 eggs or survivors thereof sacrificed at 14 days and 30 eggs/chicks maintained for F1 testing.

An efficient method for conducting a two-generation test would be to use the Japanese quail in a modified Avian Reproduction test as described in part A, and produce extra offspring that would the F1 breeders in a two-generation test.

B.In the expanded two generation testing protocol described in section A above, exposure of F1 embryos occurs only through maternal depositions of chemical substance or mixtures to eggs. Often avian egg exposure occurs directly, such as during spraying of pesticides and pesticide-fertilizer mixtures or crude oils and derivatives during spills, or with certain air pollutants. Direct exposure differs from maternal deposition because the parent compound is not being introduced to the egg as adults metabolize the parent compounds. These facts argue for an alternative exposure to embryos via the direct pathway. For chemicals that have pathways directly to eggs, (certain pesticides, fertilizers, oils, and some air pollutants), a direct exposure protocol needs to further amend existing EPA method (OPPTS 850.2300). Experience with this assessment protocol is well documented (Hoffman and Eastin, 1981; Hoffman and Albers, 1984; Hoffman, 1990; Somers et al., 1974). It is recommended that eggs be dipped in appropriate solutions, including registered carrier solvents for pesticides for ten seconds at 84 hrs of incubation for bobwhite quail and mallards and 63 hours for Japanese quail. This assures embryonic development is proceeding uniformly prior to exposure (eggs can be candled) and during the period of organogenesis. Eggs will be air dried for five minutes prior to return to the incubator. All eggs will be clean eggs with no previous adult exposures. Surplus control group eggs or purchased eggs may be used. Testing of these eggs and offspring will be identical to those prescribed in Section A, 1, 5 above.

•Functional Endpoint Tests for the Two-Generation Avian Reproduction Test:

i.Nest Attentiveness/Incubation Behavior: Measuring nest attentiveness assessment of adult birds during incubation would complement other proposed toxicity endpoints. It is a functional test of adult reproduction behavior following embryonic and subsequent dietary exposure and can provide a functional endpoint of high ecological relevance.

ii.Visual Cliff Test: A short-term behavioral assessment of hatchling chicks to a visual challenge. It evaluates depth perception and motivation needed for escape responses and predatory performance to capture live prey. A functional deficit in these behavior patterns early in life can result in reduced growth and survival, both of which are ecologically important.

iii.Cold Stress Test: The ability of chicks and adult birds to regulate body temperature is a vital physiological response and largely controlled by thyroid hormones. Delay or impairment of thermoregulatory ability might significantly affect survival during inclement weather. Likewise, adult birds are exposed to winter cold stress and inclement weather during migration periods. This test provides a functional evaluation of the effect of cold stress on body temperature and the ability to regulate body temperature within normal ranges.

V.Fish Life Cycle Test

A.Introduction

1.Purpose

The fish life cycle test is designed to evaluate growth, maturation, and reproduction of fish exposed to a chemical, substance, or mixture from a continuous exposure through a complete reproductive life cycle.

2.Test Material

The test material must be soluble or dispersable to allow distribution to test aquaria.

3.Acceptable Protocols

Test protocols which provide guidance for performing a fish life cycle test can be found in the following references.

Benoit, D.A., "User's Guide for Conducting Life-Cycle Chronic Toxicity Tests with Fathead Minnows *(Pimephales promelas)*," Environ. Res. Lab. - Duluth, MN. EPA 600/8-81-011, 1981

Hansen, D.J., P.R. Parrish, S.C. Schimmel, and L. R. Goodman, "Toxicity Test Using Sheepshead Minnows (*Cyprinodon variegatus*)," *Bioassay Procedures for the Ocean Disposal Permit Program*, EPA-600/9:78-010, 1978.

American Public Health Association, American Water Works Association and Water Pollution Control Federation, "Standard Methods for the Examination of Water and Wastewater," 16:854, 1985.

B. Materials, Methods, and Reporting Requirements

[Note: numbers in parentheses refer to the numbered references at the end of this section.]

1.Biological System

a)Preferred Species

The preferred test species are fathead minnow (*Pimephales promelas*) and sheepshead minnow (*Cyprinodon variegatus*).

b)Source and Acclimation of Fish

Adult fish are obtained from either wild populations or suitable culture laboratories. Fathead minnows should be maintained at 25^{o} C. Sheepshead minnows are to be held in flowing 30^{o} C seawater of > 15 $^{o}/_{oo}$ salinity. Source fish should be held for at least two weeks prior to breeding and should not exhibit mortalities > 5 %.

c)Eggs from Adult Fish

Artificial inducement and natural spawning are the two methods for obtaining a sufficient number of eggs for a chronic exposure. Artificial inducement entails the stimulation of egg production by injection of human gonadotrophic hormone. Sheepshead minnow females can be injected intraperitoneally with five IU HCG on two consecutive days. Two days following the second injection, ova from females are stripped and mixed with sperm derived from excised macerated testes. Usually ten females and five males should be used.(l)

Natural spawning is possible with a few considerations for each fish species and is preferred. Fathead minnows require paired spawning in order to eliminate fighting and competition. Culture units for this fish can consist of one tank measuring 30.5 x 30.5 x 61 cm with a water depth of 18 cm and four individual spawning chambers (15.2 x 30.5 cm) formed by stainless steel screen dividers (5 mesh, 0.89 mm wire).(2) Sheepshead minnow embryos are obtained by combining five or more females and three males in spawning chambers measuring 20 x 35 x 22 cm. Mature adults should attain a minimum standard length of 2 > mm and display courtship characteristics (sexual dimorphism, territoriality, and aggressive behavior by the male). Fish from each spawning group are left in chambers for a minimum of 14 days.(2)

Adult deaths during spawning should be noted; dead animals are removed, but not replaced. At termination of each spawning group, lengths and weights of individual fish are measured.

d)Feeding

Fry of both fish species should be fed equal portions of live brine shrimp nauplii at least two times daily about six hours apart for three weeks (frozen nauplii are not to be used). juveniles (four weeks posthatch) and adults can be fed twice daily on equal portions of dry food (e.g., Tetramin or Biorell) supplemented with frozen adult brine shrimp. Each batch of food should be checked for pesticides and metals.

e)Embryo Removal

A record of numbers and egg fertility must be maintained daily. All embryos are examined daily with a dissecting scope or magnifying viewer to remove empty shells and opaque, or abnormal appearing embryos. If less than 50 percent of the embryos from a spawn appear to be healthy and fertile, all embryos from that spawn should be discarded. (2) Embryos should be removed at a fixed time each day so spawning activity is not disturbed unnecessarily.

f)Embryo Exposure (Four-Five Days)

The life-cycle chronic toxicity test must begin with embryos from at least three separate spawnings that are < 24 hours old and have soaked in dilution water for at least t-waugh hours.

(2) Testing begins by randomly distributing 50 embryos to each of the four replicate larval growth chambers. (2) Ten embryos are transferred with a large bore eye dropper to successive incubation cups which are standing in dilution water. This is repeated until 50 embryos are in each cup. The incubation cups are then distributed to each replicate larval chamber.

Survival of embryos, time required to hatch, hatching success, and survival of fry for four weeks are determined and recorded. Dead embryos usually turn opaque and must be counted and removed each day until hatching is complete. Live fungused embryos must be removed daily and counted as dead.(2)

g)Larval-Juvenile Exposure (Eight Weeks)

After hatching, each group of larvae is randomly reduced to 25, and released in replicate larval growth chambers. (2) This random selection must include any fish that are lethargic or deformed. Survival should be determined in each replicate growth chamber at least once a week. Survival during this period is determined by counting the number of live fish, since dead larvae deteriorate rapidly.

At four and eight weeks after hatching, total lengths (mm) of all fish must be recorded. (2) Techniques suggested for measuring fish include direct measurement and a photographic method outlined by McKim and Benoit 1971. (3) In order to treat growth as a valid endpoint, the amount of food given to the control and treated fish must be kept constant between exposures.

h)Juvenile-Adult Exposure (32-40 Weeks)

All fish are transferred to the adult spawning tank (same concentration) eight weeks after hatching. (2) Each tank should have 25 randomly selected fish (deformed individuals included).

When secondary sexual characteristics are well-developed, fathead minnow (20-24 week post hatch) males will exhibit tubercles, pads and body color, while females will exhibit extended transparent and canals (urogenital papilla). At this time, mature fish should be placed in spawning tank, separate from undeveloped fish. (2) The spawning tank will be divided into four

individual spawning chambers with appropriate spawning substrates. Four males and four females are randomly chosen and assigned to spawning chambers. Substrates are examined daily and embryos removed, counted, and recorded separately for each pair.

The adult exposure (fathead minnow) should be terminated when, during the decreasing day-length photoperiod, a one-week period passes in which no spawning occurs. (2) Testing using sheepshead minnows should terminate after spawning is observed for two weeks because this fish spawns readily and almost daily unless immature or affected by a pollutant. (l)

i)Second Generation Embryo Exposure (Four-Five Days)

Fifty embryos from each concentration level are randomly selected and transferred to incubation cups for hatch. Those embryos not selected are discarded. Test procedures used during embryo removal and embryo exposure (sections A, 5 and A, 6, respectively) are repeated with second generation embryo reexposure. (l, 2)

j)Second Generation Larval-juvenile Exposure (Four-Eight Weeks)

Eight week exposure begins with the release of two groups of 25 larvae in replicate growth chambers. These larvae should have been produced from different breeding pairs in each spawning tank. Selection of each group should be from early spawnings. (1, 2) Testing procedures are the same as those described in section A, 7.

Each group of second generation fish is terminated eight weeks after hatching. Fish are blotted, weighed, and measured before being discarded. (l, 2)

2. <u>Physical System</u>

a)Test Water

•Fathead Minnow

i.Test water can be supplied from a well or spring provided that the source is not polluted;

ii.Water should be sterilized with ultra violet irradiation and tested for pesticides, heavy metals, and other possible contaminants;

iii.Hardness of 40 to 48 mg/L as CaCO3 and pH of 7.2 to 7.6 is recommended;

iv.Reconstituted water can be used. Detailed descriptions of acceptable procedures for preparing diluent are found in the protocols by the American Society of Testing Materials 1980. (4)

•Sheepshead Minnow

i.Test water may be natural (sterilized and filtered to remove particles 15 microns and larger) or a commercial mixture (provided that there are no adverse affects to test organisms or alterations in test material toxicity);

ii.Natural seawater is considered to be of constant quality if the weekly range of salinity is less than six percent, and if monthly pH range is less than 0.8 of a pH unit;

iii.Salinity should be $\geq$ 15 parts per thousand;

iv.Water must be sterilized and free of Pollutants. (l) Irradiation with ultraviolet light is recommended to Sterilize test water.

b) Temperature

A continuous record of temperature of test water must be kept.

•Fathead Minnow

Temperature should be maintained at 25°C and should not remain outside the range of 24 to 26°C for more than 48 hours. (2)

•Sheepshead Minnow

Temperature should be maintained at 30°C. (l)

c)Photoperiod

Lighting above each replicate must be balanced and must simulate the wavelength spectra of sunlight. Light intensities at the water surface should range from 10 to 100 lumens. One lumen per square meter is equal to one lux.

•Fathead Minnow

A graduated photoperiod as described in Benoit (1981) is used.

•Sheepshead Minnow

A 16-hour light/8-hour dark cycle is maintained throughout the test. (l)

d)Dosing Apparatus

Intermittent-flow proportional diluters as described by Mount and Brungs (5) or continuous-flow serial diluters, as described by Garton (6) should be employed. A minimum of five toxicant concentrations with a dilution factor not greater than 0.50 and one control should be used.

e)Toxicant Mixing

A mixing chamber is recommended to assure adequate mixing of test material. Aeration should not be used for mixing. Separate flow splitter delivery tubes should run from this container to each replicate larval and adult tank. (2) Depending upon the apparatus used a mixing chamber may not be required. T-t must, however, be demonstrated that the test solution is completely mixed before introduction into the test system. Flow splitting accuracy must be within 10 percent and should be checked Periodically for accurate distribution of test water to each tank. (2)

f)Test Tanks

All test tanks should be of either all glass or glass with a plastic or stainless steel frame.

•Fathead Minnow

Adult spawning tanks should measure 30.5 x 30.5 x 91.4 cm or 30.5 x 30.5 x 61 cm long with a screened-off or separate larval tank. (2) Each larval section is divided in half allowing for two larval growth chambers for each adult spawning tank. Larval chambers should be designed with glass bottoms and drains that allow water to be drawn down to 3 cm. (2) Test water must be delivered separately to each adult tank and larval section, with one-third of the water volume going to the latter. Larval tanks can also be conveniently located directly above spawning tanks containing test solutions of the same concentrations so they can be drained directly into the spawning tank. Test water depth in adult tanks and larval chambers should be a minimum of 15 cm. (2)

•Sheepshead Minnow

Tanks 45 x 90 x 26 cm with a water depth of 19 cm have been successful. Larval chamber design and test water divided are the same as described for fathead minnow. (l)

g)Embryo and Fry Chambers

Embryo incubation chambers should be made from 120 ml glass jars with the bottoms replaced with 40 mesh stainless steel or nylon screen. Chambers can be oscillated vertically (2.5 to 4.0 cm) in the test water (rocker arm apparatus, 2 rpm motor) or placed in separate chambers with self-starting siphons. Both methods should insure adequate exchange of water and test material. (1, 2)

h)Flow Rate

Flow rates to adult tanks or larval chambers should provide 90 percent replacement in 8 to 12 hours. (2) Flow rate must be capable of maintaining dissolved oxygen at above 75 percent of saturation and maintain the toxicant level (concentration cannot drop below 20% with fish in the tank).

i)Aeration

Dilution water should be aerated vigorously insuring that dissolved oxygen concentration will be at or near 90 to 100 percent saturation. Test tanks and embryo chambers should not be aerated. (l, 2)

3. Chemical System

a)Concentrations

A minimum of five concentrations of toxicant and a control (all duplicated) are used in this chronic test. A solvent control is added if a solvent is utilized. As a minimum, the concentration of toxicant must be measured in one tank at each toxicant level every week. Water samples should be taken about midway between top and bottom and the sides of the tank.

One concentration selected must adversely affect a life-stage and one concentration must not affect any life-stage. (1)

b)Measurement of other Variables

Dissolved oxygen must be measured at each concentration at least once a week. Freshwater parameters in a control and one concentration must be analyzed once a week. These parameters should include pH, alkalinity, hardness, and conductance. Natural seawater must maintain a constant salinity and not fluctuate more than six percent weekly or a monthly pH range of less than 0.8 of a pH unit. (2)

c)Solvents

If solvents other than water are necessary, they should be used sparingly and not to exceed 0.1 mL/L in a flow-through system. The following solvents are acceptable: (4)

- dimethylformamide
- triethylene glycol
- methanol
- acetone
- ethanol

The development of chemical saturators for use with hydrophobic chemicals may be used with most test chemicals. (7, 8, 9)

d)Calculations

Data from these toxicity studies are of two types, continuous (i.e., length, weight) and discrete (i.e., number of fish hatching or surviving). In general, continuous data should be analyzed with the appropriate analysis of variance (ANOVA) technique followed by an appropriate multiple comparison test. Dichotomous data should be analyzed using some form of a 2 x 2 contingency table.

As a part of the ANOVA, it is desirable to plot the residuals versus concentration and determine whether there have been any obvious violations of homoscedasticity on the assumption of normality. All test results must be accompanied by the original (raw) data for the reviewer's evaluation.

C.Literature Cited

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