REVISED STUDY PLAN

ON

AVIAN DOSING STUDY

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1.0 INTRODUCTION

The U.S. Environmental Protection Agency (EPA), in collaboration with Organization for Economic Cooperation and Development (OECD) is developing a test guideline to assess the impact of chemicals on the reproduction and development of birds over two generations. The guideline will include both conventional and endocrine endpoints. Several methodological issues that could not be resolved from existing literature were discussed during an OECD Endocrine Disruptor Testing and Assessment Task Force consultation with member country experts (OECD Expert Group on Assessment of Endocrine Disrupting Effects in Birds). One of the key issues needing resolution prior to developing a test guideline is the selection of appropriate exposure scenario(s) during a two-generation test. Some experts argue that dietary treatment of the parental (P1) generation should begin after the onset of egg-laying to 1) Allow the option of using pre-treatment measurements as covariates (internal controls), 2) Remove incompatible or infertile pairs before treatment to reduce non-treatment sources of variation and increase the power to statistically evaluate test parameters (i.e., increase the ability to detect treatment effects if they exist). Other experts believe that exposure should begin prior to sexual maturation to detect effects resulting from delayed or inhibited gonadal development and/or changes in the onset of laying of the P1 generation.

Debate over the exposure regimen also extends to the F1 generation, with some member country experts proposing that the F1 generation also receive dietary treatment of the test substance, while others argue that the F1 generation should not be exposed to the test chemical. Arguments in favor of exposing the F1 generation to the test substance during all critical life stages include the ability to account for endocrine–mediated effects that occur during growth and development of the F1 chicks and to represent a worst-case exposure scenario. Not treating the F1 generation focuses the test on the effects of *in ovo* exposure of the developing embryo (e.g., gonadal abnormalities, altered sex ratio) and the subsequent reproductive success of the F1 generation without the potentially confounding influence of direct toxicity of the test substance to the chicks and the sexually maturing juveniles. However, the response of the F2 generation may provide needed *in ovo* effects data if the F1 exposure regimen is used.

This study plan addresses the need for experimental data regarding

1) The relative importance of the timing of onset of treatment of the P1 generation (prior to sexual maturation or after proven egg-laying ability) for detecting reproductive and developmental effects over two generations[1].

2) Whether the F1 generation should receive dietary treatment of the test substance.

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[1] Please note that on page 2 of the Work Assignment Statement of Work the exposure options for the P1 generation are "initiated at sexual maturation or after proven egg-laying ability." Because sexual maturation is often determined by the onset of egg laying, it is assumed that "prior to or during maturation" was intended and conforms to the reference to "pre-breeding" dosing and the exposure scenarios discussed in the OECD documents.

The specific objectives of the study are to

1) Compare dose-response relationships of endocrine and fitness endpoints between the two exposure scenarios to define the most appropriate exposure regimen for detecting and quantifying a range of endocrine-mediated effects. Emphasis will be placed on comparing the relationships on the basis of slope, relative sensitivity and relative variability of the endpoints, and determining endocrine-mediated effects that may not be observed by initiating treatment after the onset of egg laying.

2) Compare dose-response relationships of endocrine and fitness endpoints between the two exposure scenarios for the F1 generation of each P1 exposure scenario and between all F1 exposure groups to define the most appropriate exposure regimen for detecting and quantifying a range of endocrine-mediated effects. Time series data will be used to assess the daily/weekly/etc. within-class variation in response, time lag between exposure and response, and appropriateness of the exposure duration.

1.1 STUDY DESIGN

The exposure options will be evaluated in an experimental design that compares two P1 exposure scenarios (P1A receiving treated diet prior to sexual maturation and P1B receiving treatment after proven egg-laying ability has been established) and F1 exposure options (F1a receiving no dietary treatment and F1b receiving treated diet from hatch through egg laying) using two cohorts of F1 chicks that survived *in ovo* exposure from the P1 parents (Figure 1).

Figure 1. Exposure Design. The F1 Populations are Obtained from the Last Week of Eggs Collected from the P1 Birds.

Both P1 populations will be assigned randomly to a control group or one of a geometrically spaced series of four dietary concentrations. Because the available literature is insufficient to establish appropriate test concentrations, the dietary concentrations will be determined from a range-finding trial. Dietary concentrations for the study will be chosen to define the dose-response relationships for the more sensitive test endpoints. The control group will be fed diet containing any solvents or carriers in amounts equivalent to those used in treated diets. The dietary route of administration was selected because it represents the most likely route

of exposure to avian species in the environment. The diet will be analyzed for caloric content and the presence of natural endocrine-active compounds.

Birds in the P1A population will start on dietary treatment when the gender of the birds can be determined by a visual examination of the plumage (about 3 weeks of age). At this time the birds will be separated by gender and assigned to treatment groups. The P1A birds will continue on treatment through maturation and approximately 8 weeks of egg laying. The date of onset of laying will be recorded. Males and females will be housed separately until the fifth week of egg production at which time they will be paired together. All eggs produced prior to pairing will be collected, counted, candled for cracks and other abnormalities, sub-sampled (1 egg per pen per week) for shell quality measurements (thickness and strength) and discarded. Eggs collected after pairing at the beginning of the fifth week post onset of laying will be counted, candled for cracks or other abnormalities, sub-sampled for eggshell quality measurements and for steroid content (1 egg per pen per week of weeks 5 and 8). All remaining eggs will be incubated and candled on Day 8 of incubation to determine embryo viability. Except for a subset of eggs (4 per pen) from the eggs produced in week 8 of egg laying, all eggs will be discarded after the Day 8 candling. The remaining subset of eggs will be incubated to hatch to produce the F1 breeding population. Hatchlings from this final egg batch will be assigned to one of two cohorts as they are removed from the hatcher. The F1a cohort will receive no dietary treatment. Hatchlings assigned to the F1b cohort will receive dietary treatment from hatch through egg laying. Once chicks have reached the age where gender can be determined, the F1 breeding population will be selected as described in the protocol (Appendix A). Both cohorts will be brought into egg production, and the eggs will be collected for 5 weeks after the onset of laying to measure test endpoints. All intact eggs, except those removed for eggshell quality measurements (1 egg per pen per week) and steroid analysis (1 egg per pen of week 5), will be artificially incubated and hatched on a weekly basis. Embryo viability will be determined on Day 8 of incubation and F2 hatchling survival will be monitored for 14 days. Gross necropsy, body weight, and histological examination of excised tissues will be performed on all adult birds when terminated. Terminal body weight will be obtained for all F2 chicks. Necropsies will be conducted on a subset of the F2 chicks (33% of each pen's chicks) and histological examination of the reproductive system will be performed on a total of one third of the F2 chicks produced from eggs collected during week 5. Genetic sex determination will be performed on about one half of the week 5 F2 chicks. The cloacal gland will be measured in all male birds.

P1B birds will be raised as described for the P1A birds, but without dietary treatment until they are paired. Females laying at least 3 eggs per week by the fifth week post onset of egg production will be considered proven breeders at which time they will be paired with males and the breeding pairs will start on dietary treatment. Treatment will last 4 weeks such that treatment ends at the same time as for the P1A birds. Endpoint measurements will be collected, the F1 breeding populations established and the F1 cohorts treated as described for the P1A birds.

The experimental treatments are shown in Table 1. Figure 2 shows the number of adult birds used, number of eggs collected and produced in the different phases of the study.

Table 1. Proposed Treatment Groups for the Exposure Comparison Study

Onset of Exposure	Pens per P1 or F1 Group (1 cock and 1 hen per pen)	17β-Estradiol Exposure Concentration (ppm)			
		adults	F1a	F1b	F2
P1A (pre-breeding; 2-3 wks old)	8	0	0	-----[2]	0
	8	1x	0	1x	0
	8	0.33x	0	0.33x	0
	8	0.11x	0	0.11x	0
	8	0.033x	0	0.033x	0
P1B (adult; proven layers)	8[1]	0	0	-----	0
	8	1x	0	1x	0
	8	0.33x	0	0.33x	0
	8	0.11x	0	0.11x	0
	8	0.033x	0	0.033x	0

[1] 10 pairs will be established in each group initially to provide for at least 8 pairs of breeding pairs during treatment. (Both birds of a pair will be removed if one of the pair dies or is injured).
[2] No additional control group is used. The F1a control groups serve as controls for both F1a and F1b populations.

1.2 TEST SUBSTANCE(S)

Candidate test substances for avian reproductive tests have not been separately identified by the OECD Expert Group on Assessment of Endocrine Disrupting Effects in Birds for the Endocrine Disruptor Testing and Assessment Task Force, but rather the group proposes using the list of candidate chemicals developed by the Validation Management Group on Mammalian Test Methods of Endocrine Disruption (VMG-Mammal). The VMG-Eco recommended being consistent with this list. Compounds on these lists (Appendix B) were reviewed by Battelle with input from the expert group. The draft OECD list of candidate test substances for test guideline development with fish was also reviewed (Appendix C). A range of pesticides, toxic effects and modes of action are represented. The EPA Office of Pesticide Program's Environmental Fate and Effect Division (EFED) was also contacted for appropriate data from its pesticide toxicity database. A draft list of candidate substances developed previously by Dan Balluff (EFED) for testing in avian reproduction validation studies was provided (Appendix D). The criteria for selecting the listed chemicals from the EFED database were statistically significant reproductive effects in bobwhite quail, with preference to chemicals that showed reproduction toxicity at relatively low levels. Listed compounds, therefore, are reproductive toxicants,

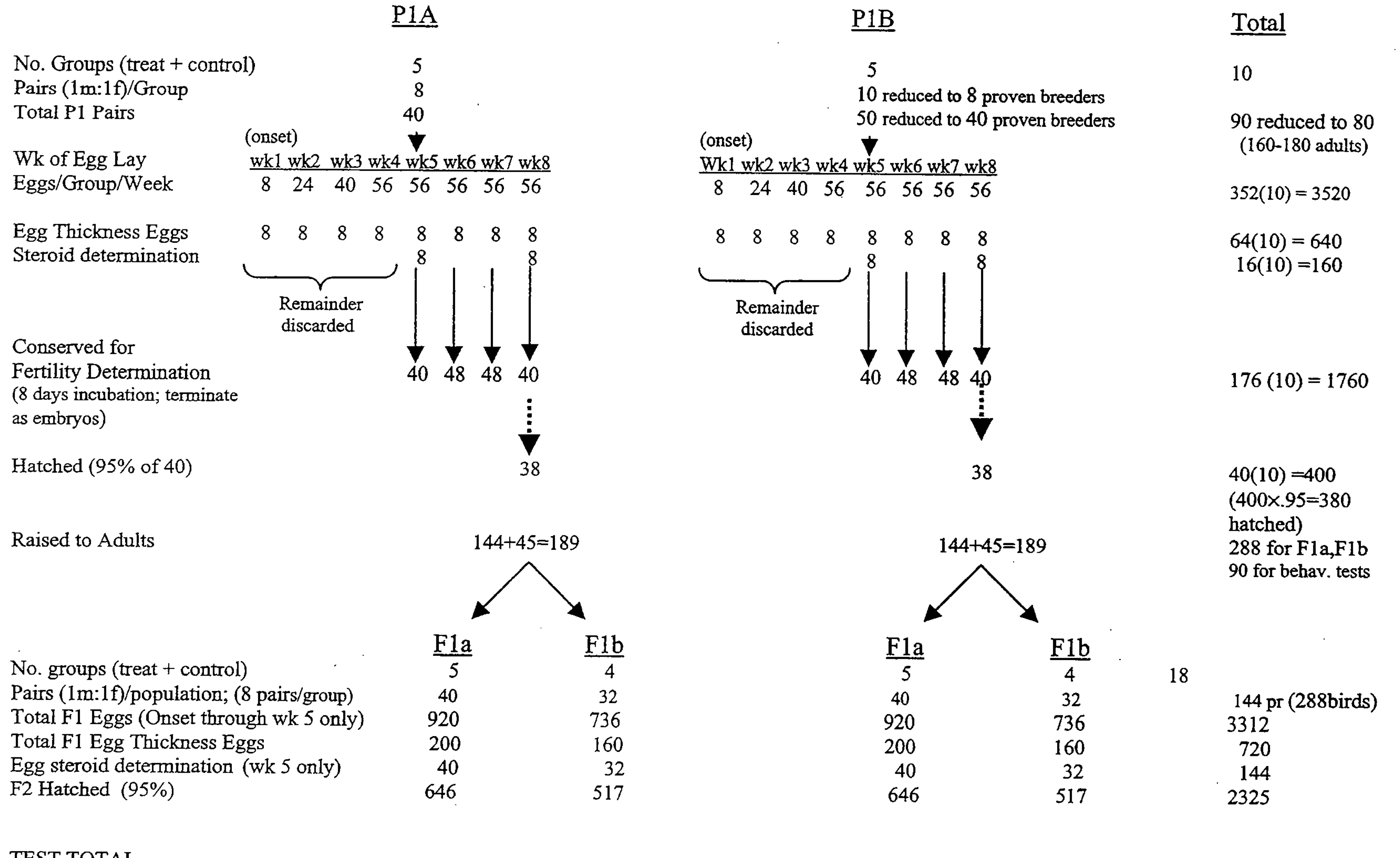

Figure 2. Number of Adults, Eggs, and Hatchlings Used During Different Phases of the Dosing Study

but not all of them are endocrine disruptors. A similar list ("Substances Classified in the EU as Toxic to Reproduction") was obtained from the OECD Series on Testing and Assessment; document Number 15, Detailed Review Document on Classification Systems for Reproductive Toxicity in OECD member countries (Appendix E). Also, the scientific literature on avian and mammalian endocrine physiology and endocrine disruptors was searched for additional candidate substances. Compounds with toxicological importance for reproduction and development compiled from recent reviews (Soto et al., 1995; GEA, 1996; U.S. EPA, 1997) and other papers are listed in Appendix F.

The following criteria for selecting a suitable test substance for comparing exposure regimens were applied to the candidate compounds:

1) The test substance should have the potential to affect the maturation of parents in such a way to determine what endocrine-mediated effects may not be observed by starting treatment during the egg laying period (P1) or by not treating the F1 chicks.

2) The test substance should give rise to inter-generational effects so that the impact on reproductive/endocrine endpoints in the F1 generations of the two P1 exposure regimes can be compared. This also provides for a comparison of the reproductive performance of untreated F1 and treated F1 birds and the survivability of their offspring.

3) The test compound must clearly act on a hormone system (not simply alter a process that is under normal endocrine control).

4) There should be sufficient knowledge of the effects and/or mode of action of the test substance that appropriate, sensitive endpoints can be selected.

In comparing the exposure scenarios of the F1 generation, two issues need resolution that have conflicting requirements related to test substance selection. First, determining whether endocrine-mediated effects occur during maturation that are overlooked when only *in ovo* exposure is considered necessitates the use of a compound that does not exert confounding non-endocrine-mediated effects on the growing chicks. However, many of the compounds that will be subject to endocrine disruption testing will probably be highly toxic. Therefore, resolving the question of whether compounds that are directly toxic to the chicks at exposure levels that are appropriate for measuring reproductive effects mask or limit the ability of the test to detect endocrine-mediated effects in the F1 generation requires using a compound that does exert non-endocrine-mediated effects (juvenile mortality, non-endocrine-related changes in behavior). Also, the ability of the exposure regimens to detect disrupted endocrine function amid effects such as food avoidance and chronic toxicity in adults should be assessed.

Accordingly, candidate compounds reviewed for this study were evaluated relative to the above criteria and separated according to their potential to exert confounding effects in the F1 generation. In general, those compounds that have no or minimal maternal transfer to the egg

were considered to be less useful for the study. This eliminated compounds such as bisphenol A (Berg et al., 2001) and those with low Log K_{ow} (e.g., amitrol, fenoxycarb). Other compounds also were considered less useful if they appeared to affect pathways that could result in confusing results. For example, TCDD is both anti-androgenic and anti-estrogenic in northern bobwhite quail (*Colinus virginianus*), depending upon dose and tissue (McMurry and Dickerson, 2001). Organochlorine compounds were not rated highly for selection because relatively great concentrations of these compounds are required to illicit effects (Feyk and Giesy, 1998).

No data were found in the literature on the effect of avian exposure to anti-estrogenic chemicals. Pharmaceuticals such as tamoxofen and relaxofene are selective estrogen antagonists, blocking the estrogen receptors on the uterus and breast tissue of mammals, but are agonists in other tissues such as those in bone. It is not known if any of these drugs would have similar effects in birds; therefore, they were eliminated from consideration as potential test substances for the dosing comparison study.

Likewise, review of the literature indicated that there are few studies on the effects of anti-androgens on the developing embryo or hatchlings. Birds appear to be affected by androgenic or anti-androgenic substances to a much lesser extent than they are affected by estrogens. Because males are the homozygous sex in birds, sexual dimorphism results from a demasculinization of female embryos under the influence of estrogen (i.e., lack of estrogen results in production of phenotypic males). On the other hand, excess or insufficient testosterone is unlikely to affect female birds unless levels are so low that there is no substrate for the action of aromatase and, therefore, no production of estrogens. It has been shown that administration of excess testosterone or its active metabolites has little effect in the adult birds as well (Balthazart et al., 1983). However, vinclozolin ((R,S)-3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione) is a compound with apparent anti-androgenic properties in Japanese quail (McGary et al., 2001). Exposure of embryos on day 4 of incubation significantly altered gonadotrophic releasing hormone (GnRH) levels in male hatchlings, and the onset of male reproductive behavior was delayed and significantly reduced during the beginning of the reproduction period, but the effects appeared to diminish with time. However, fertility measures were inconclusive (EFED pesticide database). On the other hand, data from the EFED pesticide data base (Appendix D in the protocol) indicates that the compound inhibits egg production and affects shell thickness. Although artificial dosing of embryos has been studied, maternal transfer of vinclozolin to the egg has not yet been demonstrated. A study in progress at the University of Maryland may provide additional information on other measurable effects that would increase the compound's usefulness in a dosing comparison study.

Too little work with thyroidogenic compounds in birds has been conducted to identify a useful test substance from this class of endocrine disruptors. Generally, in animals, there is an excess of circulating T_4 sufficient to produce an adequate amount of T_3. Therefore, unless a chemical acts directly on conversion of T_4 to T_3, or binds to T_3 receptors, sufficiently high doses would be required that systemic toxicity is likely to result prior to the onset of signs of thyroid dysfunction. To date, only the pharmaceutical PTU is known to inhibit the conversion of T_4 to T_3. PTU also inhibits the action of thyroid peroxidase, thereby reducing the amount of T_4 produced. It is likely, however, that thyroid-related fitness endpoints will be much less sensitive

than other hormone-related effects and may occur at exposure concentrations that also result in generalized systemic toxicity.

The majority of the environmental chemicals identified as endocrine-active compounds are estrogenic. Existing data show that administration of endogenous estrogen in young birds, in adults, and *in ovo* causes clear changes in reproduction, sexual behavior, and sexual differentiation (Yoshimura et al., 2000). Furthermore, maternal transfer of estradiol to egg yolks in hens injected or implanted with the hormone has been demonstrated and resulted in changes in sexual differentiation of the offspring (Adkins-Regan et al., 1995). Exposure to elevated levels of estrogen may also cause eggshell thinning and changes in reproductive behavior (Enstrom et al., 1997; Brewer et al., in prep). Because of the potential for environmental chemicals to mimic the effects of estrogen in birds and potential for such interactions to result in feminization of males, a large amount of work has been done in the past decade to develop sensitive assays for detecting the estrogenic effects of xenobiotics, mostly focusing on mammalian systems, but more recently assessing applications with birds (Ankley et al., 1998; DiGuilio and Tillett, 1999). A compound such as 17ß-estradiol would satisfy the general selection criteria (obvious action on a hormonal system, ability to affect maturation, documented transfer from hen to egg, induction of intergenerational effects) for the dosing study and would be applicable to evaluating the F1 exposure regimen in the absence of confounding toxicity. Therefore, 17ß-estradiol is recommended for use in the dosing study specifically to evaluate the relative importance of the timing of onset of treatment of the P1 generation (prior to sexual maturation or after proven egg-laying ability) for detecting reproductive and developmental effects. An additional advantage of using 17β-estradiol for this dosing study, is the availability of data on the effects of dietary estradiol on the reproductive performance of the northern bobwhite (*Colinus virginianus*). Studies by Lien et al. (1985 and 1987) provide information on potential dose levels, endpoint sensitivity and range testing. In addition, using 17β-estradiol in this study with Japanese quail will allow a comparison of the relative sensitivity of this more domesticated species with the bobwhite, a species that has experienced less inbreeding pressure and presumably is more representative of wildlife responses. Although estradiol is not volatile (1.26E-008 mm Hg vapor pressure), care must be taken to avoid exposure of staff and cross-contamination of controls to this potent estrogen. Staff handling the preparation and distribution of the treated diets and accessing the animal rooms will be trained relative to the hazards and safe handling of this potent estrogen and will wear appropriate personal protective equipment as determined by an Environmental Safety and Health subject area expert at Battelle Northwest and formalized in a Chemical Process Permit (CPP). Access to those rooms in which the estrogen will be in use will be restricted by a proximity access card system. Access criteria include completion of all required safety and specific CPP training and approval of the Cognizant Space Manager of the space within the Integrated Operations System of the specific building. To minimize cross-contamination of the test diets, the dietary treatments will be mixed in increasing concentration from control to high dose. All mixing equipment will be cleaned with ethanol prior to the control feed and between each dietary concentration. An initial "control" diet will be mixed and discarded, and a second batch made following cleaning of the equipment to assure no transfer of estrogen to the control diet. Where possible dedicated mixing vessels will be used per dietary concentration. Cages will be positioned to minimize cross-contamination of feed.

The scope of this study plan encompasses the evaluation of the effectiveness of pre-breeding vs. proven breeder exposure regimens for detecting endocrine-mediated effects in birds exposed to dietary test substances. Resolution of the question of whether compounds that are directly toxic to the chicks limit the ability of the test to detect endocrine-mediated effects in the F1 generation could be accomplished in future studies using one of the OP compounds identified during the test substance review for this study.

1.3 ENDPOINTS

Selection of the endpoints for this pre-validation study were based on information provided in four documents:

1) "Discussion Document of Pre-Validation of an Avian Two-Generation Toxicity Test with the Japanese Quail," R. Bennett, K. Brugger, A. Fairbrother, A. Leopold, N. Mastrota, and M.A. Ottinger, OECD Draft Document, March 2001.

2) A draft protocol developed by Dr. Mary Ann Ottinger (University of Maryland) entitled "(Test Substance): A Two-Generation Reproduction Study with the Japanese quail (Coturnix coturnix japonica)"[2].

3) Proposal for a New Test Guideline, "Avian Two-generation Toxicity Test in the Japanese quail (Coturnix coturnix japonica)," OECD Guideline for Testing of Chemicals, First Draft, December 1999.

4) Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) Final Report. Office of Prevention, Pesticides, and Toxic Substances, U.S. EPA 1998.

Battelle also consulted with individuals that have served on the OECD Expert Group on Assessment of Endocrine Disrupting Effects in Birds for input on selecting of endpoint measures. The expert group members contacted were Drs. Rick Bennet (EPA-ORD), Nick Mastrota (EPA-OPP), Anne Fairbrother (formerly of Parametrix, Inc. and currently with EPA-ORD), and Mary Ann Ottinger. Dr. Ottinger also provided information on refinements to the above-mentioned endpoints and protocols based on preliminary results from two-generation endocrine studies conducted at the University of Maryland.

The general consensus of the expert group was to include in the pre-validation most of the "fitness" endpoints (Table 2) described in the above documents and to apply a subset of "physiological or "endocrine" endpoints that identify endocrine-mediated effects during sexual maturation and egg production. Because the proposed test substance is 17ß-estradiol, the selected endpoints emphasize measures with underlying estrogenic mechanisms and measures for feminization of males (Table 3). As recommended by the EDSTAC 1998 report, feminization of the P1 and F1 offspring will be measured by determining genetic sex in hatchlings (Table 2) and internal sexual characteristics of a subset of genetic male and female chicks at 14-days of age (Table 3). Hormone status will be measured non-invasively in the P1 adults at the end of the

treatment period. Steroid levels in fecal-urate samples have been shown to reflect the plasma levels and steroid status in a variety of species, including birds (Wasser et al. 2000). The samples will be collected from the drop pans under each of the breeder cages and prepared for analysis by commercially available Enzyme Immunoassay (EIA) kits for testosterone and estradiol according to methods modified for avian species by Brewer et al. (in prep.) and Wasser (2000). Steroid status will also be monitored in egg contents. Composites of a subset (1 egg per pen per week) of eggs collected during the 5th and 8th weeks of egg laying of the P1 birds and one egg per pen during week 5 of the F1 parents will assayed for testosterone and estradiol content by EIA. Yolks will be separated from albumin by differential thawing and the free steroids extracted and cleaned by a method described Schwabl (1993) and modified by Lipar et al. (1999).

Because of the apparent sensitivity of the oviduct to endocrine changes (Lien et al, 1985), the oviduct will be weighed and the degree of differentiation in the organ will also be evaluated. Some measures also are relevant for general toxicity assessment, especially in embryonic stages. Also, because there are a number of interactions among various endocrine axes and estrogen is known to depress production of thyroid hormone, Dr. Fairbrother suggests the addition of thyroidogenic endpoint measures to monitor this interaction and begin to develop baseline information for the Japanese quail including measurement of T_4/T_3 because T_4/T_3 hormone structures are well-conserved across species and therefore circulating levels are easily measured with commercially available ELISA kits. However, plasma concentrations of thyroid hormones are influenced by handling, bleeding, and other stressors in birds (Williamson and Davidson 1985). In addition, plasma T3 and T4 concentrations fluctuate significantly because of their short half-life in avian blood, making it difficult to document T4/T3 increases or decreases from a single sample. Therefore a means of non-invasively sampling thyroid hormones over time is needed to provide a more informative evaluation of dose regime impacts on the detectability of thyroid response to endocrine active chemicals. (T3 and T4 are hormones that are excreted in bile, which suggests that an RIA or ELISA assay for these hormones could be readily developed for non-invasive monitoring of thyroid status using fecal/urate samples.) Because of the cost and interpretational problems with T3 and T4, these hormones will not be evaluated during this dosing study. Those measures of thyroid function that will be included in this study are body weight, major organ weights, wing and bone length, thyroid weight and histopathology. EDSTAC (1998) also recommends measurement of several additional growth parameters that are indicative of adequate thyroid function in the subset of 14-day-old chicks described above. These measures should provide adequate and sensitive assessment of thyroid function. The use of skeletal x-ray to detect changes in thyroid function has also been proposed by EDSTAC (1998). However, morphometric measurements of bone structure at necropsy provide more quantitative information on bone growth than can be derived from conventional radiography (Cruickshank and Sim 1986). In addition, availability of expensive portable x-ray equipment and processing are limited among testing laboratories. Therefore, this measure will not be included as an endpoint. Instead, wing bone morphology will be examined as indicated in Table 3.

Table 2. Fitness Endpoints for Exposure Comparison Study

Endpoint	Endocrine Activity	
	Estrogenic	Thyroidogenic
For Breeding birds (P1 and F1)		
Body weight at start and end of treatment		X
Food consumption weekly during treatment		X
Survival	toxicity	
Signs of toxicity	toxicity	
Number of eggs laid per pair	X	
Number of fertile eggs per eggs laid	X	
Number of cracked eggs (at set)	potential	potential
Number of eggs hatched per eggs set[1]	X	
Eggshell strength and thickness	potential	
Early and late viability per eggs set[2]		
F1 and F2 Chicks		
Sex ratio of chicks	X	
Number of chicks surviving to 7 and 14 days per eggs set and per eggs hatched[3]		
Growth rate of chicks (weight at days 1, 14)[3]	X	X

[1] Only F1 eggs from the last week of egg-laying (week 8) will be hatched. F2 eggs from all collection periods will be hatched.
[2] Late viability will be determined on all F2 eggs and F1 eggs from last week of egg-laying.
[3] F2 chicks and those F1 chicks hatched from the last week of eggs.

Table 3. Endocrine or Physiological Endpoints for Exposure Comparison Study

Endpoints	Endocrine Activity: Estrogenic	Thyroidogenic	Androgenic
For Breeding Birds (P1 andF1)			
Gross morphology & histology			
weight of testes, ovaries, thyroid, adrenals, oviduct, cloacal gland, liver	X	X	X
histology of thyroid, adrenals, gonads, brain	X	X	X
testicular spermatid counts and morphology	feminization	X	
gross anomalies of the genital tract	X		X
Developmental Landmarks			
feather dimorphism	X	X	
cloacal gland size, 1st appearance of foam			X
1st egg laid	X		
Sexual behavior[1]	feminization		
Fecal/urate hormones			
steroid hormones (estradiol, testosterone)[2]	X		X
For F2 Chicks			
steroid hormones (estradiol, testosterone)	X		X
Gross morphology & histology			
size and dimorphism of gonads	X		X
histology of gonads (relative amount of cortex and oocytes), thyroid, oviduct	X		X
presence, weight and differentiation of oviduct	X		X
thyroid, cloacal gland, liver, brain, pancreas	X	X	X
wing and bone length		X	

[1] F1 males only
[2] P1 only

Three functional endpoint tests of ecological importance were also suggested in the final EDSTAC Report (1998) and include a nest attentiveness/incubation behavior test, a visual cliff test, and a cold stress test. The nest attentiveness/incubation behavior test is not applicable to the non-nesting cage-system used for Japanese quail to provide the large number of eggs required for the two-generation tests. Although it may give an indication of fitness/survivability of chicks, the sensitivity of the visual cliff test in detecting endocrine-mediated effects is not known nor is a standardized method for performing the behavioral test available. Also, effects measured in the visual cliff test can be a result of altered food consumption and body weight loss (Fleming et al. 1985) from food avoidance when only contaminated feed is available to chicks. Therefore, without a paired feeding design where concomitant groups of untreated birds are fed diets restricted to the amounts consumed by treated birds, endocrine-induced effects cannot be separated from effects induced by reduced food intake. In cold stress tests conducted with organophosphorus pesticides, food avoidance contributes significantly to weight loss and mortality under hypothermic conditions (e.g. Stromborg 1986a and b, Maguire and Williams 1987, Martin and Solomon 1991). Conditions related to cholinesterase inhibition such as vaso-

dialation on the skin (Meeter 1973) may also contribute to hypothermia and confound any endocrine-mediated effects. Under the conditions of the dosing study, reduced food consumption and body weight loss would appear to be as effective as the functional tests in indicating a potential endocrine response.

A number of endocrine endpoints were eliminated by the expert panel because they were not considered to be integrative for the purpose of the study (brain catecholamine measures), in need of refinement (aromatase, histological evaluations), or have proved to be of relatively low sensitivity (GnH system effects). Although there is a question as to whether thyroid weight is a valuable or pragmatic parameter to measure because of inter-laboratory variation in surgical technique and the difficulty of excising the embedded tissue, collection of these data was still recommended. Therefore, the thyroid will be excised within the surrounding connective tissue and fixed in preservative prior to trimming. Trimming and weighing the preserved gland will minimize damage to the gland during excision preserving its integrity for subsequent histopathological examination and allow for more consistent trimming and weight determination.

A specific point was made by several of the experts regarding egg quality measurement. Because comparative studies indicate that shell strength is considerably more sensitive than shell thickness (Bennett et al. 1988, Henny and Bennett 1990), it was recommended by the majority of the group that shell strength be measured in addition to eggshell thickness. Also, mating behavior of male Japanese quail was suggested by one member of the expert group. It appears to be among the most sensitive endpoints measured in birds exposed to estrogen or estrogen-agonists (Halldin et al. 1999). Males are housed singly and then observed following introduction of a receptive female into their cage. Such tests usually involve the use of sexually mature, naive males and mature, receptive females. This would entail either keeping the males separate from the females, conducting the behavioral tests, and then pairing the males and females for the subsequent reproductive tests, or raising a separate cohort of males (and a few females). Females not on test would have to be used for the behavioral tests to avoid receptivity issues caused by treatment. Because these studies are conducted using naive males, conducting the tests requires additional birds and housing during the study. Birds used for the male behavioral trials will be obtained from a random selection of excess F1 birds from the P1A and P1B week 8 egg cohorts (see Figure 2) that are not used to form the F1 pairs.

1.4 RANGE FINDING TEST

The range finding test will consist of three treatment levels with no controls. Each treatment level will expose three reproducing pairs of Japanese quail to dosed feed (six birds * three treatment levels or 18 birds total). The treatment levels will be 1 ppm, 10 ppm and 100 ppm 17ß-estradiol in feed.

The test duration will evaluate adult birds through 14 days of egg-laying, including adult survival, egg production and embryo viability at Day 8 of incubation. Eggs from the second week of egg laying will be incubated until hatching. Hatchlings will be evaluated at 3 days for normality.

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APPENDIX A

STUDY PROTOCOL

EFFECTIVE DATE:

PROTOCOL **Battelle #-**

Battelle
Pacific Northwest Laboratories
Richland, Washington

EPA Contract No. 68-W-01-023

Title: Avian Dosing Study

Sponsor: Environmental Protection Agency
Endocrine Disruptor Screening Program
Washington, D.C.

Testing Facility: Battelle Pacific Northwest Laboratories
Natural Resources Department
908 Battelle Boulevard
Richland, Washington 99352

Amendments:

Number	Date	Section(s)	Page(s)
1			
2			
3			
4			

Approvals:		
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APPENDIX A
STUDY PROTOCOL: EXPERIMENTAL DESIGN

Test Substance

One compound with low hatching toxicity, but established *in ovo* transfer and endocrine-mediated effects during both maturation of juveniles and egg formation (17β-estradiol) will be used to evaluate the appropriateness of pre-breeding vs. proven breeder exposure regimens. Because the available literature is insufficient to establish appropriate test concentrations, the dietary concentrations will be determined from a range finding test. In the definitive test, four treatment groups will be used for all P1 and F1 populations. A concurrent control group will be used for each of the two P1 test populations and each of the two F1A populations. F2 chicks will not receive dietary treatment. The test concentrations will be geometrically spaced between the highest and lowest doses. The highest concentrations will be below levels shown to cause mortality or severe signs of parental toxicity as determined in the range-finding test, but will be of a level that is expected to reveal significant effects on reproductive and endocrine endpoints. Control birds will be from the same hatch as the test groups and will be kept under the same experimental conditions as the test birds. Control diets will consist of the same basal diets that the test birds receive with no test substance added. The carrier will be added to the control diet in the highest concentration used for the test diets.

The test substance will be analytically pure, and the purity will be reported, along with the percentage of each impurity.

Range Finding Test

The range finding trial will consist of three treatment levels with no controls. Each treatment level will expose three reproducing pairs of Japanese quail to dosed feed (six birds * three treatment levels or 18 birds total). The treatment levels will be 1 ppm, 10 ppm and 100 ppm 17ß-estradiol in feed.

The test duration will evaluate adult birds through 14 days of egg-laying, including adult survival, egg production and embryo viability at Day 8 of incubation. Eggs from the second week of egg laying will be incubated until hatching. Hatchlings will be evaluated at 3 days for normality.

If the 100 ppm test concentration manifests no adverse effects, then the dosing study will use 100 ppm as the highest dose. If the 100 ppm test concentration reveals effects, but the 10 ppm test concentration shows no effects, then the maximum dose to be used in the dosing study will be between 10 and 100 ppm. Depending on the severity of effects seen, the dose selected may be closer to 10 ppm or to 100 ppm, Similarly, if the 10 ppm test concentration manifests effects and the 1 ppm test concentration does not, the maximum dose for the dosing study should be in between the two concentrations.

If the 1 ppm test concentration manifests effects (other than total adult mortality), then 200 ppb (20% of the range-finder effect level) will be the maximum dose for the dosing study. If the 1 ppm test concentration results in mortality to all adults in pens, then a second range finding test will be needed to assess the test concentrations below the 1 ppm level until no adult mortality effects are observed.

Test Groups and Number of Birds

One parental population (P1A) will receive dietary treatment form about three weeks of age through egg laying and the other parental population (P1B) will receive dietary treatment at the beginning of the fifth week post onset of egg laying (proven breeders). Proven breeders are females that are laying at least 3 eggs per week by the fifth week post onset of egg production. Males and females of both populations will be housed separately at three weeks of age (when gender can be determined by plumage) until they are paired at the beginning of the fifth week of egg production. Each of the P1 populations will be divided into four treatment groups and a control group. Each P1B group will initially consist of 10 replicate pens to assure that there will be at least 8 replicated pens in each group by termination of the test.

A subset of eggs (4 eggs per pen) from each of the treatment groups and the control group from the P1 test populations will be collected from eggs in week 8 of egg laying. The eggs and hatchlings will be marked to identify parental origin. Once the sex of the F1 chicks can be determined (about three weeks of age), chicks from each group will be paired so that F1 breeding pairs are formed from non-siblings of their associated P1 parents. From each P1 group, two F1 groups will be formed with equitable representation and assigned to one of two exposure regimens. A control group will be formed only for the F1a populations; no control groups will be formed for the F1b populations. The F1a population will be divided into groups of 8 replicate pens according to their parental test diet, but will receive no treatment (Figure 1). F1b chicks will also be divided into groups of 8 replicate pens according to their parental test diet and will receive the same diet as their parents from hatch through egg laying (Table 1). The number of adults and the estimated number of eggs collected and hatched in the P1 and F1 generations are shown in Figure 2.

Figure 1. Exposure Design. The F1 Populations are Obtained from the hatch of the Last Week of Eggs Collected from the P1 Birds

Table 1. Proposed Treatment Groups for the Exposure Comparison Study

Onset of Exposure	Pens per P1 or F1 Group (1 cock and 1 hen per pen)	17β-Estradiol Exposure Concentration (ppm)			
		adults	F1a	F1b	F2
P1A (pre-breeding; 2-3 wks old)	8	0	0	-----[2]	0
	8	1x	0	1x	0
	8	0.33x	0	0.33x	0
	8	0.11x	0	0.11x	0
	8	0.033x	0	0.033x	0
P1B (adult; proven layers)	8[1]	0	0	-----	0
	8	1x	0	1x	0
	8	0.33x	0	0.33x	0
	8	0.11x	0	0.11x	0
	8	0.033x	0	0.033x	0

[1] 10 pairs will be established in each group initially to provide for at least 8 pairs of breeding pairs during treatment. (Both birds of a pair will be removed if one of the pair dies or is injured).

[2] No additional control group is used. The F1a control groups serve as controls for both F1a and F1b populations.

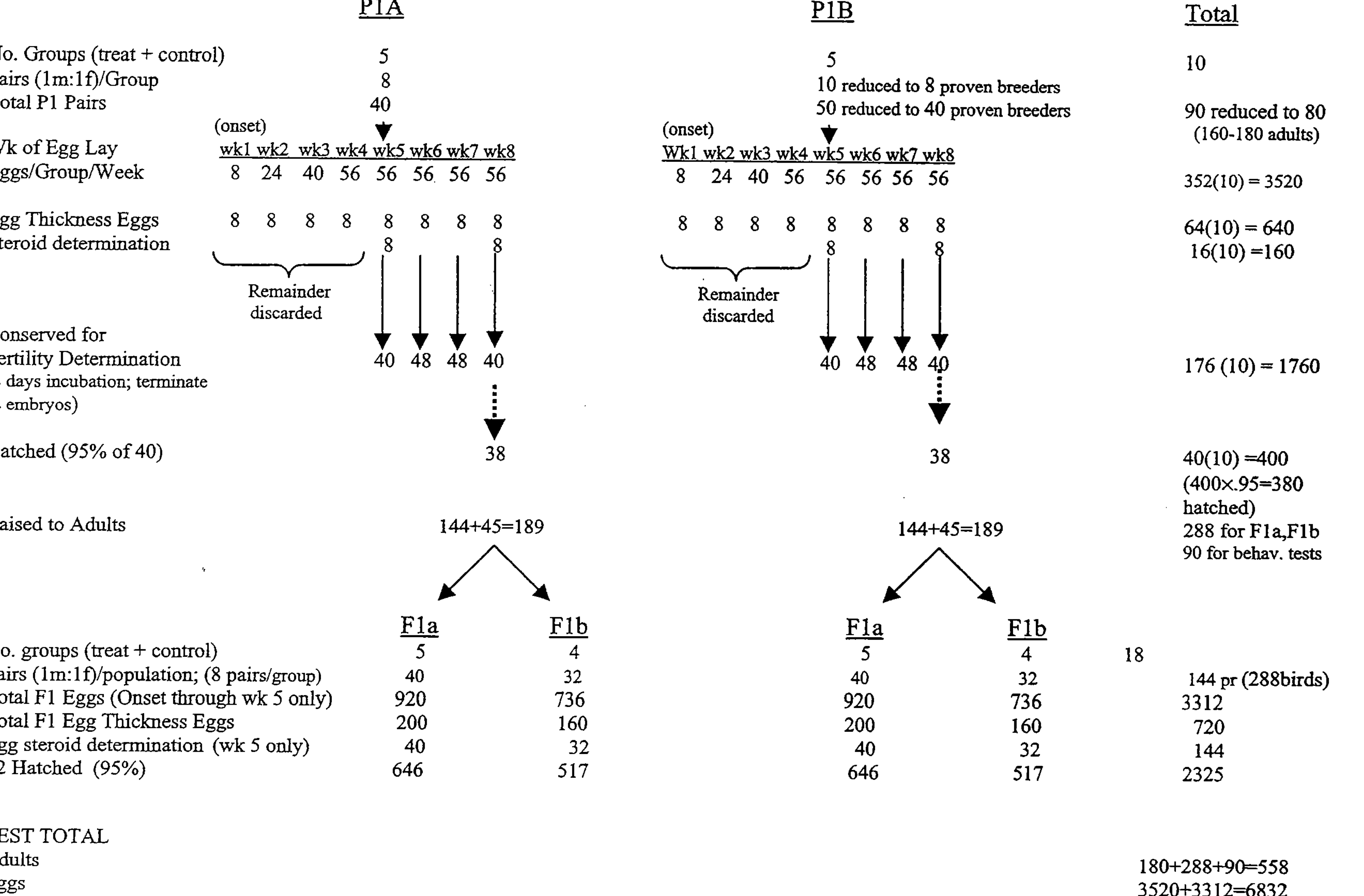

Figure 2. Number of Adults, Eggs, and Hatchlings Used During Different Phases of the Dosing Study

Duration of Test

The duration of the test may be impacted by the strain of birds and their variability in maturation. The expected duration of the in-life portion of the test will be approximately 40 to 44 weeks. The primary phases of the study and their approximate durations are:

<u>P1A</u>
Incubation and hatch of eggs: about 18 days
Growth of P1A birds: from hatch until approximately 3 weeks of age.
Pre-laying exposure P1A: about 4 to 5 weeks
Egg-laying exposure of P1A: about 8 to 9 weeks (4 weeks of egg laying of paired birds)
Incubation of F1; set eggs weekly and incubate for 8 days and terminate: about 4 weeks
Incubation and hatching eggs for F1a and F1b population and mating behavior tests: set the fourth week of eggs and incubate 18 days.

<u>F1a and F1b from P1A population (F1a is not treated, F1b receives treatment from hatch)</u>
Selection and rearing, of F1a and F1b: about 3 weeks
Pre-laying period of F1a and F1b: about 4-5 weeks
Egg-laying period of F1a and F1b: about 5 weeks
Incubation and hatching of F2: eggs set weekly; about 18 days of incubation per hatch or about 13 weeks total.
Brooding of F2 to 14 days of age: about 2 weeks per hatch or about 8 weeks total.

<u>P1B (concurrent with P1A)</u>
Incubation and hatch of eggs: about 18 days
Growth of P1B birds: from hatch to pairing: about 12 weeks of age
Pre-laying acclimation of P1B birds: 4 to 5 weeks
Egg-laying of P1B: about 4 weeks to establish baseline production (no treatment)
Exposure during peak egg laying of P1B: 4 weeks
Incubation of F1; set eggs weekly and incubate for 8 days and terminate: about 4 weeks
Incubation and hatching eggs for F1a and F1b population and mating behavior tests: set fourth week of eggs and incubate18 days.

<u>F1a and F1b from P1B population (concurrent with F1a and F1b from P1A population; F1a is not treated, F1b receives treatment from hatch)</u>
Selection, rearing, and pairing of F1a and F1b: about 3 weeks
Pre-laying period of F1a and F1b: about 4-5 weeks
Egg-laying period of F1a and F1b: about 5 weeks
Incubation and hatching of F2: eggs set weekly; about 18 days of incubation per hatch or about 13 weeks total.
Brooding of F2 to 14 days of age: about 2 weeks per hatch or about 8 weeks total.

Observation of Record

Eggs: Eggs will be collected daily and marked with a soft lead pencil or permanent ink according to the pen from which they were collected. At the end of the weekly interval, all eggs will be removed from the cold room, counted, and selected eggs taken for eggshell quality measurements (1 egg per pen per week). The remaining eggs will be candled with an egg-candling lamp to detect eggshell cracks or abnormal eggs. Cracked or abnormal eggs will be recorded and discarded.

F2 eggs will be set weekly for incubation. F1 eggs collected from the beginning of the 5th week after the onset of laying will be set weekly for incubation.

Early embryo viability: Eggs will be candled on Day 8 of incubation to determine early embryonation (embryo viability). Eggs set for F1a and F1b breeding populations and male mating behavior trials will also be candled on Day 15.

Hatching success: On Day 15, viable eggs will be placed in pedigree baskets and transferred to another incubator where they will be allowed to hatch. Those chicks that have not hatched within about 24 hours of the majority of chicks, will be considered unhatched. The number hatched and unhatched will be recorded.

Survivability of hatchlings: F1 offspring (F2) will be observed over a 14-day period beginning when birds are first removed from the incubator. The number surviving to 14 days will be recorded. The observation period will be extended if late mortality occurs that appears to be treatment related. Survivability of chicks hatched for F1a and F1b populations and male mating behavior trials will be also recorded.

Clinical Observations: All adults and offspring will be observed daily throughout the test for overt signs of toxicity or abnormal clinical observations. A record will be maintained of all mortalities and observations.

Body Weight: Individual body weights of the adults will be measured at start and end of treatment. Body weights will not be measured during egg laying because of the possible adverse effects that handling may have on egg production. Individual body weights of offspring by parental pen will be recorded at hatching and on Day 14 post hatch.

Feed consumption: Feed consumption for each pen will be measured at least weekly and at test termination. More frequent measurements will be made if feed must be changed more often than weekly to maintain 80% of the original concentration under the test conditions. Feed consumption is determined by weighing the freshly filled feeder on Day 0, recording the amount of any additional diet added during the week and weighing the feeder and remaining feed at the end of the seven-day feeding period. The accuracy of feed consumption values may be affected by unavoidable wastage of feed by birds. Since wasted feed normally is scattered and mixed with water and excreta, no attempt will be made to quantify the amount of feed wasted by the birds. Therefore, feed consumption will be presented as an estimate.

Reproductive Parameters: The following reproductive parameters will be measured and recorded by pen:

- Body weight of F2 14-Day-old Survivors and chicks raised for F1a and F1b populations and male mating behavior trials
- Dead embryos (Day 8 for F1; Day 8 and Day 15 for F2 and for eggs set for F1a and F1b populations and mating behavior trials)
- Viable embryos (Day 8 for F1; Day 8 and Day 15 for F2 and for eggs set for F1a and F1b populations and mating behavior trials)
- Unhatched eggs (F2 and for eggs set for F1a and F1b populations and mating behavior trials)
- Hatchlings (F2 and for eggs set for F1a and F1b populations and mating behavior trials)
- 14-day-old survivors (F2 and 14-day-old chicks raised for F1a and F1b populations and mating behavior trials

Eggshell Strength Measurements: Each week's eggs will be selected from those eggs laid during that week for eggshell thickness measurement. One egg will be collected from each of the odd-numbered pens during odd-numbered weeks (1, 3, 5, etc.), and one egg will be collected from each of the even-numbered pens during even-numbered weeks (2, 4, 6, etc.). Shell strength will be measured with a universal testing instrument. The egg will be placed on its side on the test stand so that the compression head will contact the egg at the equator between two parallel stainless steel surfaces advancing at a constant rate of 4 mm/min with a 50 Newton (N) maximum load range. The load (±1%) will be recorded in Newtons.

Eggshell Thickness Measurements: Following the shell strength test, the same eggs will be prepared for shell thickness measurements. Each egg will be cut open at the waist, the contents removed, and the empty shell rinsed with tap water. (Yolks will be frozen for analysis of the test compound.) The shells then will be allowed to air dry with the membrane intact for at least 48 hours at room temperature. The mean thickness of the dried shell, including membranes, will be determined by measuring five points around the waist of the egg with a micrometer. Measurements will be made to the nearest 0.002 mm.

Offspring Body Weights: The mean weight of all surviving offspring will be determined both at hatch and at 14 days of age. Mean weights may be determined from either individual or group body weight measurements and will be determined from all offspring originating from a given parental pen during a specific week of egg laying.

Necropsy, Organ Weights, Histology: All adult test birds that die during the course of the test and all adults remaining at the termination of the adult portion of the test will be subjected to a gross necropsy. The necropsy will include an examination of the overall condition of the birds,

as well as any external or internal observations. The examination will include, but not be limited to, gross observations of the liver, gonads, and general condition of the organs. Gonads, oviduct, thyroid, adrenal glands, liver, brain and cloacal gland will be excised and their weight recorded. Thyroid and adrenal glands will be excised with adjoining tissue and injected with fixative prior to trimming and organ weight determination to diminish handling damage to the tissues and decrease time required for excisement. All lesions will be recorded.

Necropsies will be conducted on a subset of the F2 chicks. One-third of the chicks from each pen will be necropsied. In one-third of the chicks from the Week 5 eggs, the gonads, oviduct, and thyroid will be examined histologically. The wing or leg length will be measured in F2 chicks and the bone removed and measured.

Organ weights will be normalized by body weight (100 X organ weight/body weight) and the testis weight asymmetry (left testis weight/right testis weight) calculated.

Sexual Maturation: Sexual maturation of males will be determined by the protrusion and secretion of foam from the cloacal gland. Cloacal gland measurements will be taken weekly until maturation. Semen samples will be taken at maturation and at termination of the study and sperm quality/viability evaluated. Female maturation will be recorded as the day the first egg is laid. The number of follicles in rapid development (>4mm in diameter and yellow in color) will be determined at termination of the study.

Feather Color and Pattern: Appearance of feather dimorphism will be recorded. Gender will be confirmed at necropsy and recorded.

Steroid Content of Fecal-Urate Samples: Fecal-urate matter (0.2-2 g) will be collected from the drop pans under each of the breeder cages at termination of the P1 birds. Samples will be collected to avoid contamination by feed and adjoining cage occupants as described by Brewer et al. (in prep). The samples will be prepared for analysis as described by Tell and Lasley (1991) and as modified by Brewer et al. (in prep) and Wasser et al. (2000). The hormone content of the fecal-urate sample preparations will be determined using commercially available Enzyme Immunological Assay kits for testosterone and estradiol.

Steroid Content of Eggs: The steroid content of a subset (1 egg per pen per week) of eggs collected during the 5th and 8th weeks of egg laying of the P1 birds and one egg per pen during week 5 of the F1 parents will be determined by Enzyme Immunoloical Assay. Four eggs from each group will be composited for the analyses (2 composites of 4 eggs each per group). Yolks will be separated from albumin by differential thawing and the yolks homogenized and mixed. Free steroids will be extracted with petroleum and diethyl ethers, the proteins and excess lipids precipitated, the steroids extracted in ethanol and the extracts cleaned using chromatography columns as described by Schwabl (1993) and modified by Lipar et al. (1999). The eluents will be analyzed for steroid content using Enzyme Immunological Assay kits for testosterone and estradiol.

Male Sexual Behavior: A receptive egg-laying female will be placed in a 50 x 40 x 30 cm-high test arena. The male will be introduced and sexual interaction observed for 2 minutes.

Mount attempts will be recorded when a male, while grabbing neck of the female, places one leg over the female's back. One test will be performed each day for 4 consecutive days. Mounts and cloacal contact movements will also be noted. However, because they are more dependent on the receptivity of the female than the mount attempt (Halldin et al. 1999), only the mount attempt will used in statistical analysis of the behavioral data.

Test Animals

The species to be tested will be the Japanese quail (*Coturnix japonica*). Birds used in this test will be obtained from a commercial source as eggs and reared at Battelle Northwest Laboratory. All treatment and control birds used in a test will be from the same hatch for both P1 populations. F1 breeders will be obtained from the eggs produced during the eighth week of egg laying by the P1 birds. Control and test birds will be kept under the same experimental conditions. All birds will be in good health and free of abnormalities or injuries that may affect test results at test initiation. Daily observations and health records will be maintained from hatch until test termination.

A population of birds will not be used if more than 3-5% of either sex becomes debilitated in the 7-day period immediately prior to test initiation. If greater than 5% debilitation is observed, then the EPA Project Officer and Work Assignment Manager will be consulted.

The quail will be acclimated to the test facilities and an untreated diet until test initiation. Acclimation typically will occur in brooding pens. Birds will be weighed and randomly assigned to treatment and control pens. To avoid pairing siblings, within control and treatment group F1 birds will be randomly assigned to pens by pairs with males from odd-numbered pen parents being paired with females from even-numbered pen parents and males from even-numbered pen parents being paired with females from odd-numbered pen parents. The sex of the birds will be determined by a visual examination of the plumage. However, if birds in a pen are incompatible, they may be replaced or rearranged within a control or treatment group at any time prior to egg laying.

Identification: All birds will be identified by individual leg or wing bands. Each pen will be identified with a unique number. Groups of pens will be identified by exposure type (e.g., established breeder, P1B, or during maturation, P1A) and concentration. All eggs laid during the study will be marked with a soft lead pencil or permanent ink marker for identification.

Feed and Water: All birds and their offspring will be given feed and water ad libitum during acclimation and testing. Basal diet used to prepare the treated and control diets of both adults and offspring will be obtained from Purina Mills, in Spokane, WA. The basal ration will contain at least 27% protein and 2.5% fat and will contain no more than 6.5% fiber. During the test, adults will receive a basal diet supplemented with calcium (Layeena) for proper eggshell formation. Offspring will receive diets prepared without the addition of limestone (Starteena).

All birds will receive filtered tap water. Water is supplied by the City of Richland municipal water system. All offspring will receive a water-soluble vitamin and electrolyte mix (Durvet, Incorporated, Blue Springs, MO 64015) in their water. Neither the adults nor offspring

will receive any form of medication in their feed or water during the test. Birds will not be medicated beginning seven days prior to the start of the treatment until the test is terminated.

Diet Preparation and Chemical Handling: Test diets will be prepared by dissolving or suspending the test substance in a solvent or vehicle prior to mixing with the feed. If a diluent such as corn oil is used it will not comprise more than 2% by weight of the treated diet.

A premix may be used to facilitate diet preparation. All treatment and control premixes will be prepared at the Chemical Repository. Diet premixes will be prepared in a Hobart mixer or equivalent. If not used immediately, the premix will be frozen until used. Data will be generated to indicate whether or not the test substance degrades or volatilizes. Premixes will be prepared as frequently as necessary to assure stability of the test substance (less than 20% loss of test substance). The assay used to determine test substance stability will be reported. Once each week or more frequently, aliquots of the premix will be blended into bulk quantities of the basal ration to achieve the desired dietary concentrations of the test substance. Bulk diet mixing will be done in a Hobart mixer or equivalent. Homogeneity of the test substance in the diet will be evaluated prior to the test. Samples of diets fed to the birds will be collected every time new diet is mixed during the treatment period to allow measurement of the actual concentration of the test substance. All test substance calculations will be based on the purity of the test substance as received or will be corrected for purity of the active ingredient in the test substance. Dietary concentrations will be adjusted for purity of the test substance expressed as ppm (active ingredient).

Although the estradiol is minimally volatile (1.26 E-008 mm Hg), there may be potential for dermal and respiratory exposure of staff through handling the diet and/or from airborne fines from the feed. Therefore staff handling the preparation and distribution of the treated diets and accessing the animal rooms will be trained relative to the hazards and safe handling of this potent estrogen and will wear appropriate personal protective equipment as determined by an Environmental Safety and Health subject area expert. Area access is restricted by a proximity access card system. Access criteria requires completion of all required training and approval of the Cognizant Space Manager of the space within the Integrated Operations System. To minimize cross-contamination of the test diets, the dietary treatments will be mixed in increasing concentration from control to high dose. All mixing equipment will be cleaned with ethanol prior to the control feed and between each dose. An initial "control" diet will mixed and discarded, and a second batch made following cleaning of the equipment to assure no transfer of estrogen to the control diet. Where possible dedicated mixing vessels will be used per dose. Cages in the animal rooms will be positioned to minimize contamination of feed.

Diet Sampling and Analysis: Samples of the treated and control diets will be collected and analyzed to evaluate the homogeneity of the test substance in avian diet and to confirm test concentrations in the prepared diets. Samples from all test substance concentrations will be collected from food troughs within each treatment concentration at the end of the first feeding period (before the diet in the hoppers is renewed) and again at the end of the last feeding period of the study. The Chemical Repository will analyze samples. The nutrient analysis supplied by the manufacturer will be reported. The composition of the vitamin supplement will also be recorded.

The test substance, (e.g., 17ß-estradiol) will be analyzed by gas chromatography-mass spectroscopy (GC-MS). The food will be solvent extracted; the extract will be derivitized to form compounds of estradiol and degradation products of estradiol that can be quantified by GC-MS. The caloric content of the feed and the amount of natural endocrine-active compounds in untreated diet will also be determined.

Housing and Environmental Conditions: Adult birds will be housed indoors in stainless steel or galvanized cages designed to house one pair of quail. The pens have sloping floors, individual cage feeders, and automatic water. Only birds associated with this study will be maintained in the study room. The study room has controlled light, temperature, and humidity.

Hatchlings will be housed in stainless steel or galvanized brooding pens by treatment group. Thermostats in the brooding compartment of each pen will be set to maintain a temperature of approximately 38°C for one week, and then temperature will be lowered 3 to 5°C per week over the following three weeks. Hatchlings will be housed in brooding pens until 14 days of age.

The photoperiod in the rooms housing both the adults and hatchlings will be maintained by time clocks. The photoperiod for both adults and hatchlings will be 16 hours of light per day throughout the test. Birds will receive a minimum of 6 foot candles of illumination at the level of the bird. Light will be provided by fluorescent lights that emit a spectrum simulating that of daylight.

Egg Collection and Storage: Eggs will be collected daily and marked with a soft lead pencil or permanent ink according to the pen from which they were collected. Eggs then will be stored at an average temperature of 10 to 16°C and an overall relative humidity of 40 to 95%. All eggs laid in weekly intervals will be considered as one lot. Lots will be identified by a lot code.

Incubation and Hatching: Eggs will be set weekly for incubation. The eggs will be incubated at 37.5°C, with an average relative humidity of approximately 60%). The incubator is equipped with a fan and blades that produce a mild breathing air movement that is designed to eliminate intracabinet temperature and humidity variation during incubation. To prevent adhesion of the embryo to the shell membrane, the incubator also is equipped with an automatic egg rotation device, designed to rotate the eggs from 50° off of vertical in one direction to 50° off of vertical in the opposite direction (a total arc of rotation of 100°) every 2 to 4 hours.

Eggs will be candled on Day 8 and again at Day 15 of incubation (for those that are to be incubated to hatch) to determine early and late embryonation (embryo viability), respectively. On Day 15, the eggs will be placed in pedigree baskets constructed of galvanized steel wire mesh. The eggs then will be transferred to another incubator where they will be allowed to hatch. Eggs will not be rotated during hatching, and the pedigree baskets will be designed to keep hatchlings separated by their parental pen or origin. The incubator will be set to maintain a temperature of 37.5°C and an average relative humidity of approximately 60%. Wet and dry bulb temperatures in the incubator will be recorded twice daily during incubation and hatching.

Hatchlings will be removed from the incubator over an approximately 24-hour period beginning on approximately Day 18. All unhatched eggs and eggshells will be removed from the hatcher by the end of Day 20.

Disposition of Test Birds: At test termination, all surviving adults will be euthanized using carbon dioxide gas, cervical dislocation, or any other appropriate methods. Following measurement of body weight at 14 days of age, all chicks also will be euthanized. All euthanasia methods will be documented in the raw data. All birds will be disposed of by incineration or other appropriate methods.

Statistical Analyses

The overall objective of the statistical analysis is to determine which dosing scenario for the P1 generation birds (during maturation or after proven breeding ability is established) and F1 birds (exposure from hatch or no additional exposure above in ovo exposure) are more biologically sensitive to chemically induced reproductive/endocrine disrupting impacts to species fitness. The study design will produce a time series of reproductive parameters for P1 adults under both dosing scenarios for each concentration, a dose-response curve for each generation, plus the pen mean responses for each concentration, dosing scenario, and generation. Thus, three statistical approaches will be used: a regression against time for a given concentration, dosing scenario, and generation; a regression against chemical concentration for a given dosing scenario and generation; and an ANOVA approach based on the mean pen responses for a given concentration, dosing scenario, and generation.

The time series produced by the P1 and F1 generation birds for a given concentration and dosing scenario will allow the evaluation of a possible delay in response time, the form of the time series response (i.e., linear, curvilinear, spline), and the potential carry-over effect of the reproductive response to the F1 generation.

The dose-response for each generation and dosing scenario will allow the estimation of the EC_{50} and slope of the response. The difference in the slope and EC_{50}s between generations will also allow the evaluation of a potential carry-over effect between generations. Curve fitting procedures will also be used as an alternative to the traditional NOAEL approach to better assess the appropriateness of the dosing regimens. Specific effect levels for quantal and continuous endpoints will be identified in the range of biologically observable data and confidence limits generated around this concentration. The Benchmark Dose (the lower confidence limit producing a specified percentage of change in a response) determined under each exposure regimen will be compared.

ANOVA analysis of the pen mean responses will be conducted to compare specific contrast between treatment groups. Specific parameters will be tested only against the control using Dunnett's Analysis. Appropriate data transformations will be applied to maintain homogeneity of the within class variances (i.e., data expressed as a percentage will be arcsine-square root transformed, counts will be square root transformed, and continuous data will be transformed to the natural logarithm). Nonparametric statistic will be used when the data

transformation is not successful in controlling heterogeneity. Any pen in which an adult mortality occurs will not be used in statistical comparisons of the reproductive data.

Analyses will be performed on each of the following parameters:

1. Adult Body Weight- Individual body weight will be measured at the test initiation, at test initiation and at adult termination. Statistical comparisons will be made by sex between the control group and each treatment group at each weighing interval using dose- response and Dunnett's Analysis.

2. Adult Feed Consumption- Feed consumption expressed as grams of feed per bird per day will be examined by pen at weekly intervals during the test. Statistical comparisons will be made between the control group and each treatment group using time series analysis, dose- response, and Dunnett's Analysis.

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. This transformation is used to convert the number of eggs laid to a percentile value less than or equal to 100. The value is correlated with eggs laid per pen per day. Statistical analysis of egg production will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses.

4. Eggs Cracked of Eggs Laid (%)- The number of cracked eggs (determining by candling) divided by the number of eggs laid per pen. Statistical analysis of the percentage of eggs cracked will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses.

5. Viable Embryos of Eggs Incubated (%)- The number of viable embryos as determined by candling on Day 8 and 15 divided by the number of eggs set per pen. Statistical analysis of the percentage of viable embryos will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses.

6. Hatchlings of Viable Embryos (%)- The number of hatchlings removed from the hatcher divided by the number of viable embryos per pen. Statistical analysis of the percentage hatching will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses.

7. Hatchlings of Fertile Eggs (%)- The number of live hatchlings divided by the number of fertile eggs per pen. Statistical analysis of the percentage hatching will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses.

8. 14-Day Old Survivors of Normal Hatchlings (%)- The number of hatchlings divided by the number of eggs set per week by pen. Statistical analysis of the percentage normal will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses.

9. Normal Hatchlings as a Percentage of the Maximum Number of Eggs Incubated- The number of hatchlings per hen divided by the largest number of eggs set from any one hen. This transformation is used to convert the number of hatchlings to a percentile value equal to or less than 100. Statistical analysis of the percentage normal will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses.

10. 14- Day Old Survivors of Eggs Set (%)- The number of 14-day old survivors divided by the number of eggs set per week by pen. Statistical analysis of the percentage surviving will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses

11. 14- Day Old Survivors of Maximum Set (%)- The number of 14-day old survivors per pen divided by the largest number of eggs set. Statistical analysis of the percentage surviving will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses.

12. Hatchling Body Weight- The group body weights of surviving hatchlings and 14-day old survivors will be measured by parental pen group will be analyzed by dose-response and ANOVA.

13. 14- Day Survivor Body Weight- The group body weights of surviving hatchlings and 14-day old survivors measured by parental pen group will be analyzed by dose-response and ANOVA.

14. Eggshell Thickness and Eggshell Strength- The average eggshell thickness of indiscriminately selected eggs per pen will be measured and analyzed by dose-response and ANOVA.

15. Male Sexual Behavior- The average number of mountings per pen will be analyses by dose-response and ANOVA.

16. Hormone level in egg contents and fecal/urate matter- Concentrations of hormones averaged per groups and pen respectively will be analyzed by dose-response and ANOVA.

17. Sexual Maturation- The time to sexual maturation averaged per pen will be analyzed by dose-response and ANOVA. If significant photoperiod drift occurs in the strain of Japanese quail used, onset of egg laying may be analyzed by comparing the percentage of the group laying eggs over time. Onset of lay will be recorded as the number of days to first egg laid in the group and days until 33% hen-day egg production is reached in each group (Lien, et al., 1987).

18. Genetic Sex Ratio- The ratio of the number of males to females by blood analysis will be analyzed by the time series analysis, dose-response, and ANOVA.

19. Incidence of Abnormal Reproductive Structures – The number of abnormal reproductive structures found in the 14-day old chicks will be analyzed by regression analysis.

20. Organ Weights – The absolute value, the somatic index of organ weight to body weight, and the organ weight to brain weight of 14-day old chicks and adults will be analyzed by the time series analysis, dose-response, and ANOVA.

21. Oocyte Development – The number of oocytes in rapid development per adult female will be analyzed by dose response and ANOVA.

22. Cloacal gland size – Coacal gland size will be calculated as the volume of the cloacal gland using the following formula:

$$4/3pab^2$$

where a is half of the length of the long axis, and b is half of the length of the short axis. The cloacal volume will be analyzed by dose-response, and ANOVA.

23. Gonad Lesions- Histological scores of testicular and ovarian abnormalities will be will be analyzed by dose-response, and ANOVA.

24. Spermatid Counts- Histological counts of spermatids will be analyzed by dose-response, and ANOVA.

RECORDS TO BE MAINTAINED

Records to be maintained include:

1. Copy of the signed protocol.
2. Identification and characterization of the test substance, if available.
3. Date of initiation, critical phases, and termination of the test.
4. Animal history.
5. Husbandry and environmental conditions.
6. Dietary concentration calculations and diet preparation.
7. Dietary Concentration of phyto-estrogens and the Caloric content of the basal diet.
8. Individual body weight measurements of adults and group body weight measurements of offspring.
9. Feed consumption measurements of adults.
10. Daily observations.
11. Necropsy findings.
12. Records of all reproductive parameters detailed in this protocol.
13. Analytical chemistry methods, results, and chromatograms, if applicable.
14. Statistical calculations, if applicable.
15. A copy of the final report.

FINAL REPORT

A final report of the results will include, but not be limited to, the following:

1. Name and address of the facility performing the study.
2. Experimental start and experimental termination dates and study completion date.
3. Objectives and procedures stated in the approved protocol, including any changes in the original protocol.
4. Statistical methods employed for analyzing the data, when applicable.
5. The test and control substances identified by name, chemical abstracts number or code number, strength, purity, and composition or other appropriate characteristics, if available.
6. Stability and, when relevant to the conduct of the study, the solubility of the test and control substances under the conditions of administration.
7. A description of the methods used.
8. A description of the test system used. Where applicable, the final report shall include the number of animals used, sex, body weight range, source of supply, species, age, and procedure used for identification.
9. A description of the dosage, dosage regimen, route of administration, and duration.
10. A description of all circumstances that may have affected the quality or integrity of the data.
11. The name of the PI, the names of other scientists or professionals, and the names of all supervisory personnel involved in the study.
12. A description of the transformations, calculations, or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis.
13. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
14. The location where all specimens, raw data, and the final report are to be stored.

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APPENDIX B

ENDOCRINE DISRUPTION CANDIDATE TEST SUBSTANCES FOR THE RAT UTEROTROPHIC ASSAY AND THE RAT SUBCHRONIC STUDY (OECD 407) OECD VALIDATION MANAGEMENT GROUP ON MAMMALIAN TEST METHODS

VMG-Mammal

Rat Uterotrophic Assay:

estrogenic
- a high and low positive control group of ethinyl estradiol

estrogen agonist
- methoxycholr
- bisphenol A
- genistein
- o, p' DDT
- nonylphenol

no estrogenic effect
- butylbenxyl phthalate

RAT Hershberger Assay:

androgenic
- testosterone proprionate

antiandrogenic
- flutamide

Rat Subchronic (OECD 407):

Main Study
- ethinyl estradiol
- tamoxifen
- methyl testosterone
- flutamide
- thyroxin
- propyl thiouracil
- genistein
- nonylphenol
- DDE
- fenarimol

APPENDIX C

DRAFT OECD LIST OF CANDIDATE TEST SUBSTANCES FOR TEST GUIDELINE DEVELOPMENT WITH FISH

B-estradiol
Vinclozolin
Methoxychlor
Methy testosterone

APPENDIX D

DRAFT PROPOSED LIST FOR AVIAN 1- GENERATION REPRODUCTION TESTING WITH JAPANESE QUAIL OFFICE OF PESTICIDE PROGRAM'S ENVIRONMENTAL FATE AND EFFECT DIVISION, U.S. EPA

Chemical name, PC Code	Chronic toxicity	Parameters affected	Laboratory and date	Comments
Cyproconazole (triazole fungicide) 128993	NOEC = 50 ppm LOEC = 250 ppm	significant effects in eggs laid, set, viable embryos, live 21-day embryos normal hatch, 14-day survivors, etc.	Wildlife International 1993	Appears to be a pretty good study
Triphenyltin hydroxide (Fentin hydroxide) (organotin) 083601	NOEC = 3 ppm LOEC = 30 ppm	Significant differences in 14-day-old survivors/set, normal hatch/ live embryos 14-day survivors/normal hatch and food consumption	1994	Did not find DER but did find RED
Dicrotophos (OP) 035201	NOEC = 0.5 ppm LOEC = 1.5 ppm	egg production, food consumption viable embryos, survival	1996	Did not find DER. Mallards also affected at low levels
Acetaldehyde (aldehyde) 112403	NOEC = 10 ppm LOEC = 50 ppm	reduced adult survival, chronic lesions, reduced reproduction due to parental and embryonic toxicity	1983	25-week exposure supplemental study
Chlorfenapir (Pirate) 129093	NOEC = 0.5 ppm LOEC = 1.5 ppm	hatchling weight at 1.5 ppm, 14-day-old survivors at 4.5 ppm	1994	Supplemental
Dimethoate (OP) 035001	NOEC = 6 ppm LOEC = 30 ppm	eggs laid, eggs cracked, viable embryos, eggs hatched growth	1986	
Vinclozolin 113201	NOEC = 50 ppm LOEC 125 ppm	significantly reduced eggs laid, egg shell thickness and14-day-old survivors/hatched chicks	428689-01 1993	another study shows reduction infertility at 5 and 50 ppm but not significantly (p>.05)
Amitraz 106201	NOEC = 25 ppm LOEC = 100 ppm another study NOEC = 24.6 ppm LOEC = 50.5 ppm	hatchlings, egg set 14-day survivors/egg set 14-day survivors/eggs laid food consumption 14-day-old body weight viable embryos/egg set	Huntington 1992 Wildlife international 1992	24 pens per concentration supplemental study 3 northern bobwhite studies in total

Chemical name, PC Code	Chronic toxicity	Parameters affected	Laboratory and date	Comments
Triclorfon 057901	NOEC = 9 ppm LOEC = 30 ppm	14 day old hatchlings	Biolife 1994	Laura Dye (core study)
Clofentezine 125501	NOEC = 30 ppm LOEC = 90 ppm	embryo viability at 90 and 270 ppm and hatchling rate and body weight at 270 ppm	Huntington 1994	20 pens paired
Linuron 035506	NOEC = 100 ppm LOEC = 300 ppm	egg production, hatch ability, offspring survival	Wildlife international 1992	core
Cyanazine 100101	NOEC = 100 ppm LOEC = 250 ppm	14-day-old survivors of egg set, hatchlings, hatchling weight and 14-day-old survivors	Wildlife international 1996	core
Bensulide 009801 (OP herbicide)	NOEC = 2.5 ppm LOEC = 25 ppm	egg shell thickness	Huntington Life Science, 1998	Core

APPENDIX E

SUBSTANCES CLASSIFIED IN THE EU AS TOXIC TO REPRODUCTION OECD SERIES ON TESTING AND ASSESSMENT DOCUMENT NUMBER 15 DETAILED REVIEW DOCUMENT ON CLASSIFICATION SYSTEMS FOR REPRODUCTIVE TOXICITY IN OECD MEMBER COUNTRIES

SUBSTANCE	CAS NUMBER	INDEX	CLASSIFICATION
Benzo(a)pyrene	000050-32-8	601-032-00-3	Cat 2; R60/61
Binapacryl (iso)	000485-31-4	609-024-00-1	Cat 2; R61
Bromoxynil (iso)	001689-84-5	608-006-00-0	Cat 3; R63
Bromoxynil octanate	001689-99-2	608-017-00-0	Cat 3; R63
Carbon disulphide	000075-15-0	006-003-00-3	Cat 3; R62/63
Carbon monoxide	000630-08-0	006-001-00-2	Cat 1; R61
C I Pigment Red 104	012656-85-8	082-010-00-5	Cat 1; R61 Cat 3; R62
C I Pigment yellow 34	001344-37-2	082-009-00-X	Cat 1; R62 Cat 3; R62
Dimethyl formamide	000068-12-2	616-001-00-X	Cat 2; R61
Dinoseb	000088-85-7	609-025-00-7	Cat 2; R61 Cat 3; R62
Dinoseb salts and esters of	000000-00-0	609-028-00-2	Cat 2; R61 Cat 3; R62
Dinoseb	001420-07-1	609-030-00-4	Cat 2; R61
Dinotseb salts and esters of	000000-00-0	609-031-00-X	Cat 2; R61
2-Ethoxyethanol	000110-80-5	603-012-00-X	Cat 2; R60/61
2-Ethoxyethyl acetate	000111-15-9	607-037-00-7	Cat 2; R60/61
Lead acetate	001335-32-6	082-007-00-9	Cat 1; R61 Cat 3; R62
Lead alkyls	000000-00-0	082-002-00-1	Cat 1; R61 Cat 3; R62
Lead azide	013424-46-9	082-003-00-7	Cat 1; R61 Cat 3; R62
Lead chromate	007758-97-6	082-004-00-2	Cat 1; R61 Cat 3; R62

Lead compounds (except those listed)	000000-00-0	082-001-00-6	Cat 1; R61 Cat 3; R62
Lead di-acetate	000301-04-2	082-005-00-8	Cat 1; R61 Cat 3; R62
Lead hexafluorosilicate	025808-74-6	009-014-00-1	Cat 1; R61 Cat 3; R62
Lead hydrogen arsenate	007784-40-9	082-011-00-0	Cat 1; R61 Cat 3; R62
Lead methanesulphonate	017570-76-2	082-008-00-4	Cat 1; R61 Cat 3; R62
Lead 2,4,6-trinitroresorcinoxide	015245-44-0	609-019-00-4	Cat 1; R61 Cat 3; R62
2-Methoxyethanol	000109-86-4	603-011-99-4	Cat 2; R60/61
2-Methoxyethyl acetate	000110-49-6	607-036-00-1	Cat 2; R60/61
Methyl-onn-azoxymethyl acetate	000592-62-1	611-004-00-2	Cat 2; R61
Mirex	002385-85-5	601-077-00-1	Cat 3; R62/63-64
Nickel tetracarbonyl	013643-39-3	028-001-00-1	Cat 2; R61
Nitrofen (iso)	001836-75-5	609-040-00-9	Cat 2; R61
Tri-lead bis (orthophosphate)	007446-27-7	082-006-00-3	Cat 1; R61 Cat 3; R62
Warfarin	000081-81-2	607-056-00-0	Cat 1; R61

APPENDIX F

CHEMICALS WITH REPORTED REPRODUCTIVE AND DEVELOPMENTAL EFFECTS ADAPTED FROM KLEINOW ET AL. 1999

Compound	Known Effects	Log K_{ow}
Trifluralin	mitosis inhibitor	3.97
Atrazine		2.5
Amitrol	thyroid tumors	<1
Benomyl	teratogenicity	1.1
Iprodione	developmental toxicity	3
Mancozeb	thyroid tumors	
Ethylene thiourea	thyroid tumors	- 0.66
Metiram	thyroid tumors	2
Tributyl tin oxide	imposex	3.8
Vinclozolin	anti-androgenic	3
Diflubenzuron chitin	growth inhibitor	3.89
Azdirachtin	echysone blocker	1.09
Fenoxycarb	molt inhibitor	4.07
Carbaryl	AchE inhibitor	2.36
Parathion	AchE inhibitor	0.2
Dicofol	weak estrogen	4.7
Dieldrin/aldrin	weak estrogen	5.4
Endosulfan	weak estrogen	3.8
Toxaphene	weak estrogen	6.4
Methoxychlor	weak estrogen	3.9
p,p'-DDE	anti-androgen	5.7

o,p'-DDT	weak estrogen	6
Chlordecone	weak estrogen	4.5
Tetrachlorobiphenyls	adrenocorticoid, thyroid and estrogenic effects	5.6
Trichlorobiphenyls	adrenocorticoid, thyroid and estrogenic effects	5.5
Hydroxytrichloro-biphenyls	weak estrogen, thyroid mimic	4.9
2.3.7.8-TCDD	weak estrogen, thyroid mimic	6.8
p-terbutyl hydroxy anisole	weak estrogen	3.8
p-terbutylphenol	weak estrogen	3.3
p-nonylphenol	weak estrogen	4.5
p-octylphenol	weak estrogen	5.4
Nonylphenyl ethoxylate	weak estrogen	- 5.9
Nonylphenol carboxylate	weak estrogen	4.4
Bisphenol A	weak estrogen	3.3
4-hydroxbiphenyl	weak estrogen	3.2
Phthalate ester	weak estrogen	4.7
Butylbenzyl phthalate	weak estrogen	4.9
Ethynylestradiol	syn. estrogen	3.7
Beta-sitosterol	phyoestrogen	> 5

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Kleinow, K., J. Baker, J. Nichols, F. Gobas, T. Parerton, D. Muir, G. Monteverdi, and P. Mastrodone. 1999. Exposure, uptake, and disposition of chemicals in reproductive and developmental stages of selected oviparous vertebrates. pp. 9-111, in: R. DiGiulio and D. Tillitt (eds) Reproductive and Developmental Effects of Contaminants in Oviparous Vertebrates. SETAC Press, Pensacola, FL.