DRAFT STUDY PLAN

ON

AVIAN DOSING STUDY

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1.0 INTRODUCTION

The US 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.

The specific objectives of the study are to

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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.

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. Within Each Exposure Designation (e.g., P1a, F1b) are Four Dietary Treatment Concentrations and a Control

Both P1 populations will be assigned randomly to a control group or one of a geometrically spaced series of four dietary concentrations. The dietary concentrations will be determined from a range-finding test. 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.

Birds in the P1A population will start on dietary treatment when the gender of the birds can be by a visual examination of the plumage (3 weeks of age). At this time they will be

assigned to treatment pens in pairs. The P1A birds will continue on treatment through approximately eight weeks of egg laying. The date of onset of laying will be recorded. All intact eggs, except those removed for eggshell quality measurements, will be artificially incubated and hatched on a weekly basis. Hatchling survival will be monitored for 14 days. Eggs collected during the fifth and sixth weeks will be pooled 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, banded and weighed. The F1a cohort will receive no dietary treatment. Hatchlings assigned to the F1b cohort will receive dietary treatment as soon as they are moved to assigned brooders 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 six weeks to measure test endpoints. All intact eggs, except those removed for eggshell quality measurements, will be artificially incubated and hatched on a weekly basis. F2 hatchling survival will be monitored for 14 days.

P1B birds will be raised as described for the P1A birds, but without dietary treatment. Breeding pairs will start on dietary treatment after a two-week pre-treatment period. Pairs assigned to treatment groups must have produced at least one egg during the last week prior to the start of pre-treatment. Treatment will last six weeks such that treatment ends at the same time as for the P1A birds. Date of onset of egg laying and other endpoint measurements will be collected, the F1 breeding population established, and the F1 cohorts treated as described for the P1A birds.

The experimental treatments are shown in Table 1.

Table 1. Proposed Treatment Groups for the Exposure Comparison Study

TREATMENT GROUPS

Onset of Exposure	Conc. (ppm a.i.)	Pens per P1 or F1 Group[1] (1 cock and1 hen per pen)	Exposure None (F1a)	All Life Stages (F1b)
P1A (pre-breeding;2-3 wks old)	(Control) 0	16	0	0
	1x	16	0	1x
	0.5x	16	0	0.5x
	0.25x	16	0	0.25x
	0.125x	16	0	0.125x
P1B (adult; proven layers)	0	16	0	0
	1x	16	0	1x
	0.5x	16	0	0.5x
	0.25x	16	0	0.25x
	0.125x	16	0	0.125x

[1] 20 pairs will be established in each group initially to provide for at least 16 pairs of breeding pairs during treatment. (Both birds of a pair will be removed if one of the pair dies or is injured).

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 (VMGmammal). The VMGeco 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 data base. 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, 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 gondatotrphic 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 17β-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 evalutating the F1 exposure regimen in the absence of confounding toxicity. Therefore, 17β-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. A second substance was sought to resolve the question of whether compounds that are directly toxic to the chicks mask or limit the ability of the test to detect endocrine-mediated effects in the F1 generation. To evaluate the suitability of the different F1 exposure scenarios in the presence of non-endocrine related effects, all candidate chemicals that have been shown to be reproductive toxicants but did not act on a hormone system were reviewed, as were those that could potentially cause direct (non-

reproductive) toxicity, particularly in the F1 chicks (e.g., pesticides and metals). Of these compounds, many organphosphorus insecticides (OPs) appear to be chemicals that effectively compromise reproduction in birds without direct involvement of the endocrine system. These pesticides induce anorexia such that food consumption falls below levels required for egg formation, resulting in reduced egg production (Stromborg, 1986a,b; Bennett et al., 1990). Exposure to these anticholinesterase agents can also result in mortality of the chicks. However, some of the OPs induce a greater impact on egg production caused by food consumption alone and may be explained by their effects on hormone secretion (Rattner et al. 1982a,b). Selecting an OP as a test substance that produces both endocrine and non-endocrine-mediated effects and/or one that causes only non-endocrine-related effects in birds will enable an evaluation of the ability of the different exposure regimens to detect endocrine-related effects in the presence of confounding toxicity. Therefore, in addition to 17β-estradiol for evaluating the appropriateness of pre-breeding vs. proven breeder exposure regimens, a second compound, the organophosporus insecticide methyl parathion, is recommended for testing to resolve the issue of F1 toxicity. Methyl parathion causes reproductive deficits in adults (Bennett et al. 1990) at concentrations that likely will induce mortality and toxic effects (e.g. growth reduction) in F1 chicks (Bennett 1989).

1.3 Endpoints

Selection of the endpoints for this pre-validation study were based on information provided in three 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.

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

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[2] Note that the version of the protocol that was provided listed only the more standard "fitness" endpoints. Consultation with Dr. Ottinger resulted in an expanded list of endpoint measures. These and the endpoints listed in Document 1 above ("Discussion Document of Pre-Validation of an Avian Two-Generation Toxicity Test with the Japanese Quail") were used to select the subset of endpoints in consultation with the expert group.

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 17β-estradiol, the selected endpoints emphasize measures with underlying estrogenic mechanisms and measures for feminization of males (Table 3). 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. Measurement of thyroid-specific endpoints is relatively simple, as T_4/T_3 hormone structures are well-conserved across species and therefore circulating levels are easily measured with commercially available ELISA kits. Thyroid weights also can be measured easily at necropsy, and body weights (especially weight gain in juveniles) are routinely measured in all avian studies.

Table 2. Fitness Endpoints for Exposure Comparison Study

Endocrine Activity

Endpoint	Estrogenic	Thyroidogenic
For F1 and F2		
number of eggs laid per pair	X	
number of fertile eggs per eggs laid	X	
number of cracked eggs (at set and at 2 weeks)		
number eggs hatched per eggs set	X	
number of chicks surviving to 7 and 14 days per eggs set and per eggs hatched		
growth rate of chicks (weight at days 1, 7, 14)	X	X
eggshell strength and thickness	potential	
early & late viability per eggs set	X	
sex ratio of chicks	X	
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	

Table 3. Endocrine or Physiological Endpoints for Exposure Comparison Study

	Endocrine Activity		
Endpoints	**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		
• cloacal gland size, 1st appearance of foam			X
• 1st egg laid	X		
• Sexual behavior	feminization		
Plasma and fecal/urate hormones			
• steroid hormones (estrodiol, testosterone, corticosterone)	X		X
• thyroid hormones and TSH		X	

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, collection of these data was still recommended.

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. Because these studies are conducted using naive males, conducting the tests would require additional birds and housing during the study. Additional birds may be obtained from one of the weeks that the chicks are observed for 14-day survivorship.

1.4 Protocol Issues Needing Resolution and Proposed Courses of Action for Resolving these Outstanding Issues

Several protocol issues have been identified that will impact on the design of the exposure comparison study.

- How should treatment concentrations be determined for F1 hatchlings? As noted above, this study does not address the issue of treatment *concentrations* for the F1 hatchlings. Treating the chicks with their respective parental concentrations (particularly in the high end of the treatment range) may cause toxic effects or mortality, thereby confounding the results (as noted in the OECD pre-validation discussion document). This is a desired result when evaluating the performance of the different exposure scenarios in detecting endocrine-related effects in the presence of other toxic impacts. An OP would be a potential test substance for this comparison with the exposure concentrations selected to include an exposure that will induce some mortality. The appropriate ranges will be determined from the EFED data base. On the other hand, to clearly compare the ability of the different exposure regimens to identify endocrine-mediated effects, confounding toxicity should be minimized. One solution is to not treat the chicks until they reach the age that their parents were placed on treatment (3 weeks of age), but this does not reflect the all-life stages exposure or worst-case scenario that is in question. Therefore, a range-finding study is needed to determine the maximum tolerated dose in the hatchlings, which then can be used so that the hatchlings are treated with the same doses as their parents. Although several studies have dosed birds with estrogen by injection or implants, no direct data are available on exposure levels in diets. A study under way at University of California-Davis is using oral gavage of a synthetic estrogen in finches. To obtain the appropriate dietary test concentrations, a range-finding test will be conducted, drawing as much as possible from these data.

- How should treatment concentrations be determined for the P1 generation? Determining the appropriate concentration range of the estrogen compound for the P1 generation exposures poses the problem of which effects to measure: those that manifest themselves in the P1 generation and have the potential for affecting reproduction (e.g., delayed maturation, abnormal mating behaviors) or the transfer of the compound to the egg that thereby exposes the developing embyro. The protocol for the proposed study plan does briefly describe a range-finding test that uses for its endpoints some of the more sensitive responses to estrogenic exposure (delayed gonadal development and sexual maturation in males). From these data it also will be apparent which concentrations will be tolerated by maturing chicks. However, it does not provide information on female response or maternal transfer of the test compound to the egg. Another range-finding test wherein 8-week-old females are given a range of dietary concentrations of the compound for 3 weeks would allow collection of eggs and analysis of their contents for the estrogen to determine which concentrations result in *in ovo* exposure. Incubation of the remaining eggs would provide data on

concentrations likely to result in impacts on chicks (e.g, altered sex ratio).

- Should mating behavior endpoints be measured in the F1 breeding pairs or a separate cohort from the F1 generation? As noted above, sexual behavior changes appear to be among the most sensitive measures for detecting endocrine-mediated effects, particularly for estrogenic compounds. However, 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). The probable source of a separate population of birds would be one of the 14-day survivor groups that would otherwise be terminated. A separate cohort is the most non-confounding option, wherein males are not paired with females for the 3 to 4 days of egg laying (fertility measurements, etc.). Females not on test would have to be used for the behavioral tests to avoid receptivity issues caused by treatment.

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APPENDIX A

STUDY PROTOCOL

Battelle
. . . Putting Technology To Work

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

Proposed Study In-Life Dates:

Amendments:

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

Approvals:		
AUTHOR: Crystal Driver		
	Signature	Date
TECHNICAL REVIEWER: Michael L. Blanton		
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PROGRAM MANAGER: David P. Houchens		
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PROGRAM QUALITY ASSURANCE: Charles Lawrie		
	Signature	Date
EPA WORK ASSIGNMENT MANAGER: Gary Timm		
	Signature	Date
EPA PROJECT OFFICER L. Greg Schweer		
	Signature	Date

APPENDIX A
Study Protocol

EXPERIMENTAL DESIGN

Test Substance

At least two test substances will be used for the study, one with low hatching toxicity, but established *in ovo* transfer and endocrine-mediated effects during both maturation of the juveniles and egg formation (e.g., 17β-estradiol) and one that induces non-endocrine-related reproductive toxicity or direct toxicity in the F1 generation (e.g., OP). Test concentrations will be determined by a range finding test. For each compound, four treatment groups and one control group will be used for each of two P1 test populations and for each of two F1 test populations. Two additional F1 test populations will not receive dietary treatment. The test concentrations will be geometrically spaced between the highest and lowest doses. The highest concentration will be below levels shown to cause mortality or severe signs of parental toxicity in the range finding test, but will be of a level that is expected to reveal significant effects on reproductive and endocrine endpoints. Concurrent controls will be run with each exposure regimen. The 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 diet 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.

Test Groups and Numbers of Birds

Two tests will be run in parallel for each compound, one parental population (P1A) receiving dietary treatment from about three weeks of age through egg laying and the other parental population (P1B) receiving dietary treatment after two weeks of egg laying (proven breeders) to test termination. Each of the P1 populations will be divided into four treatment groups and a control group. Each group will initially consist of 20 replicate pens to assure that there will be at least 16 replicate pens in each group by termination of the test.

Eggs from each of the treatment groups and the control group from the P1 test populations will be collected from the last two weeks of the test period and incubated. 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 two male and two female F1 chicks will be randomly selected from each P1 pair. From each P1 group, two F1 groups will be formed with equitable representation and assigned to one of two exposure regimens. F1a chicks will receive no dietary treatment and F1b will receive treated diet from hatch through egg laying (Figure 1). The F1a populations will be divided into groups of 20

replicate pens according to their parental test diet, but will receive no treatment. F1b clients also will be divided into groups of 20 replicate pens according to their parental test diet and will receive the same diet as their parents from hatch through egg laying. (Table 1).

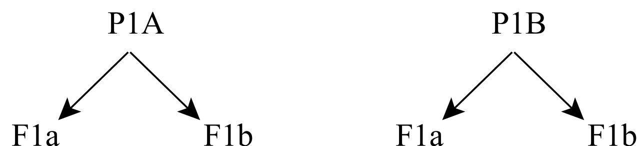

Figure 1. Exposure Design. Within Each Exposure Designation (E.g, P1a, F1b) are Four Dietary Treatment Concentrations and a Control

Table 1. Proposed Treatment Groups for the Exposure Comparison Study

TREATMENT GROUPS

Onset of Exposure	Conc. (ppm a.i.)	Pens per P1 or F1 Group[1] (1 cock and1 hen per pen)	Exposure None (F1a)	All Life Stages (F1b)
P1A (pre-breeding;2-3 wks old)	(Control) 0	16	0	0
	1x	16	0	1x
	0.5x	16	0	0.5x
	0.25x	16	0	0.25x
	0.125x	16	0	0.125x
P1B (adult; proven layers)	0	16	0	0
	1x	16	0	1x
	0.5x	16	0	0.5x
	0.25x	16	0	0.25x
	0.125x	16	0	0.125x

[1] 20 pairs will be established in each group initially to provide for at least 16 pairs of breeding pairs during treatment. (Both birds of a pair will be removed if one of the pair dies or is injured).

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, from initiation to termination, will be approximately 24 to 29 weeks. The primary phases of the study and their approximate durations are:

P1A
Acclimation of P1A birds: from hatch until approximately 3 weeks of age.
Pre-laying exposure of P1A: about 4 weeks
Egg-laying P1A: about 4 weeks

Incubation and hatching of F1: hatches set weekly; 17 days of incubation per hatch or about 9 weeks total.
Brooding of F1 to 14 days of age: 2 weeks per hatch or about 6 weeks total.

F1a and F1b from P1A population (F1a is not treated, F1b receives treatment from hatch)
Selection, rearing, and pairing of F1a and F1b: about 3 weeks
Pre-laying period of F1a and F1b: about 4-5 weeks
Egg-laying F1a and F1b: about 6 weeks
Incubation and hatching of F2: hatches set weekly; 17 days of incubation per hatch or about 9 weeks total.
Brooding of F_2 to 14 days of age: about 2 weeks per hatch or about 6 weeks total.

P1B (concurrent with P1A)
Acclimation of P1B birds: from hatch until approximately 3 weeks of age.
Pre-laying acclimation of P1B birds: about 4-5 weeks
Egg-laying P1B: about 2 weeks to establish baseline production (no treatment)
Exposure during egg laying P1B: 4 weeks
Incubation and hatching of F1: hatches set weekly; 17 days of incubation per hatch or about 9 weeks total.
Brooding of F1 to 14 days of age: 2 weeks per hatch or about 6 weeks total.

F1a and F1b from P1B population (concurrent with F1a and F1b from P1A population; F1a is not treated, F1b receives treatment from hatch)
Selection, rearing, and pairing of F1a and F1b: about 3 weeks
Pre-laying period of F1a and F1b: about 4 weeks
Egg-laying F1a and F1b: about 6 weeks
Incubation and hatching of F2: hatches set weekly; 17 days of incubation per hatch or about 9 weeks total.
Brooding of F_2 to 14 days of age: about 2 weeks per hatch or about 6 weeks total.

Photoperiod is maintained at 9 h of light per day until birds are paired at about 3 weeks. At this time the photoperiod is increased to 16 h of light.

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. 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.

Eggs will be set weekly for incubation.

Early and late embryo viability: Eggs will be candled on Day 8 and at Day 15 of incubation to determine early and late embryonation (embryo viability), respectively.

Hatching success: 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. 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: Offspring 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.

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 test initiation; on Weeks 2, 4, 6, 8; and at adult termination. Body weights will not measured during egg laying because of the possible adverse effects that handling may have on egg production. Group 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 weekly and at test termination. 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:

- Eggs laid
- Eggs cracked
- Eggs broken
- Description of all egg abnormalities
- Egg shell thickness
- Egg shell strength
- Body weight of 14-Day-old Survivors

- Eggs incubated (Set)
- Infertile or clear eggs
- Dead embryos (Days 8, Day 15)
- Viable embryos (Day 8, Day 15)
- Unhatched eggs
- Hatchlings
- Body weight of hatchlings
- 14-day old survivors

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 2-kg 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: 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, spleen, and pancreas and cloacal gland will be excised and their weight recorded. Blood, brain, and gonad samples will be frozen for further analysis. All lesions will be recorded.

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.

Fecal/Urate and Plasma Hormones: Fecal/urate matter (1-5 g) will be collected from the drop pans under each of the brooder cages and breeder cages two times per week until birds reach sexual maturity, at which time samples will be collected once per week. Samples will be collected to avoid contamination by feed and adjoining cage occupants as described by Brewer et al. (in prep).

Blood samples will be collected from the wing vein of the adults at termination of the test. All blood samples will be collected in the morning to avoid sampling close to the reported nocturnal surge of testosterone secretion (Halldin et al. 1999). Blood samples will be immediately placed on ice, centrifuged for 20 min at 2,500 x g, and frozen at -20ºC until analyzed for hormone content.

The left ovary and both testes will be excised at the termination of the test, homogenized in phosphate buffer (pH 7.4), extracted twice in ethyl ether, and dried. The tissues will then be reconstitued with diluent and analyzed for hormone content. This procedure will also be used to determine the hormone content of the eggs used in the eggshell quality tests.

Fecal/rate samples will be prepared for analysis as described by Tell and Lasley (1991) and modified by Brewer et al. (in prep).

The hormone content of the blood and fecal/urate samples will be determined using a solid-phase radioimmunoassay kit (Coat-A-Count, Diagnostic Products C., Los Angeles, CA).

Thyroxin Hormone: The blood collected at termination of the test will also be analyzed for T_4/T_3 . Measurement of T_4/T_3 will be conducted with commercially available ELISA kits.

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 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 by a combination of the fifth and sixth week eggs laid 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% of either sex becomes debilitated in the 7-day period immediately prior to test initiation. Test birds for the P1 generation should be approximately 14 days old and will be $\leq$ 18 days of age at test initiation.

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.

<u>Identification</u>: Adult and juvenile birds will be identified by individual leg bands. All birds will be identified so that they can be traced to their parental pen or origin. 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.

<u>Feed and Water</u>: 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, pulverized limestone will be added to the diets of adults at 5% (w/w) to provide the necessary calcium for proper eggshell formation. Offspring will receive diets prepared without the addition of limestone.

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: All treatment and control diets will be prepared at the Chemical Repository at Sequim Marine Sciences Laboratory (MSL) and sent to Battelle Pacific Northwest Laboratories. 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 weekly diet preparation. 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).

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 four food hopper 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. Samples will be analyzed by the Chemical Repository at MSL. 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., 17β-estradiol) will be analyzed by 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. For an OP or other test substance, analysis will be by a similarly appropriate method.

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 (Allentown). 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 (over 3 weeks of age) will be 16 hours of light per day throughout the test. Birds will receive about 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 wet bulb temperature of approximately 30.0˚C (or an average relative humidity of approximately 56%). The incubator (Humidaire, Inc, New Madison, OH) 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 two hours.

Eggs will be candled on Day 8 and at Day 15 of incubation 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˚C and a wet bulb temperature of 33˚C (or an average relative humidity of approximately 76%). 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 (at two weeks post-hatch or at eight weeks after proven laying ability is established) is more biologically sensitive to chemically induced reproductive/endocrine disrupting impacts to species fitness. The study design, as already discussed, 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 analysis of variance (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 EC50 using the slope of the response. The difference in the slope and EC50s between generations will also allow the evaluation of a potential carry-over effect between generations.

ANOVA analysis of the pen mean responses will be conducted to compare the 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 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). Non-parametric statistics 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; Weeks 2, 4, 6, 8; and at adult termination. Statistical comparisons will be made by sex between the control group and each treatment group at each weighing interval using 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 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 (determined by candling) divided by the number of eggs laid per pen. Statistical analysis of the percentage of egg's 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 10 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 and analyzed by ANOVA.

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

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

15. Male Sexual Behavior. The average number of mountings per pen will be analysed by ANOVA.

16. Hormone level in plasma, egg contents, organs, and fecal/urate matter. Concentrations of hormones averaged per pen will be analyzed by ANOVA.

17. Sexual Maturation. The time to sexual maturation averaged per pen will be analyzed by ANOVA.

18. Thyroxin Concentration. Concentrations of thyroxin averaged per pen will be analyzed by ANOVA.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated at University of Maryland at College Park 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. Individual body weight measurements of adults and group body weight measurements of offspring.
8. Feed consumption measurements of adults.
9. Daily observations.
10. Necropsy findings.
11. Records of all reproductive parameters detailed in this protocol.
12. Analytical chemistry methods, results, and chromatograms, if applicable.
13. Statistical calculations, if applicable.
14. 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, control and reference 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, control and reference 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 study director, 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.

Range Finding Tests

17β-estradiol:

At the age of two weeks, 16 pairs (1 male and 1 female) of birds will be randomly assigned to each of four test groups or a control group and will be housed in breeder cages. The birds will be fed food treated with the test substance (e.g.,17β-estradiol) for 30 days. The dietary concentrations of estradiol will be spaced logarithmically between the low and high dose. Half of the birds in each group will be sacrificed after two weeks of treatment and the testes, cloacal gland, and ovary and oviduct weighed. The remainder of the birds will be observed for sexual maturation. Sexual maturation will be recorded when the cloacal gland of the male protrudes and secretes foam (not liquid) or the first egg is laid by the female. Birds will also be observed for signs of toxicity. The highest oncentration not resulting in mortality or severe toxicity in the quail, but causing significant delay in maturation and/or decreased organ size at 28 days, will be used as the high concentration in the exposure comparison study.

Hormone content of the eggs will be determined to verify transfer of the compound from the hen to the egg and to aid in exposure concentration selection.

OP or other test substance:

Existing data will be used to determine exposure concentrations if available. For example, data from Bennet (1989) and Bennet et al.(1990) on mortality and reproductive toxicity of adult bobwhite and mortality and growth deficits in juvenile bobwhite exposed to methyl parathion will be used to define an exposure range. The potential dose range will then be confirmed in a range finding test using Japanese quail. If data is lacking, a maximum tolerated dose will be determined based on chick toxicity.

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Bennet. R. S. 1989. Role of dietary choices in the ability of bobwhite to discriminate betweeen insecticide-treated and untreated food. *Enivron. Toxicol. Chem*. 8(8):731-738.

Bennett, R. S., R. Bentley, T. Shiroyama, and J. K. Bennett. 1990. Effects of the duration and timing of dietary methyl parathion exposure on bobwhite reproduction. *Environ. Toxciol. Chem.* 9(12): 1473-1480.

Brewer, L., H. McQuillen, and A. Fairbrother. (in prep). Effects of 17β-estradiol on reproductive behaviors of the house finch (*Carpodacus mexicanus*).

Tell, L. A. and B. L. Lasley. 1991. An automated assay for fecal estrogen conjugates in the determination of sex in avian species. *Zoo. Biology* 10:361-367.

APPENDIX B

Endocrine Disruption Candidate Test Substances For The Rat Uterotrophic Assay and The Rat Subchronic Study (OECD 407OECD 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, US 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	

Chemical name, PC Code	Chronic toxicity	Parameters affected	Laboratory and date	Comments
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
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 Kow
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	weak estrogen	4.9

phthalate		
Ethynylestradiol	syn. estrogen	3.7
Beta-sitosterol	phyoestrogen	> 5

REFERENCE

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.