



4/17/2002

WWF submits the following responses to questions posed by EPA during the March 25-27 EDMVS meeting.

Comparative Evaluation of Fathead Minnow Assays for Detecting Endocrine-Disrupting Chemicals

1. *Is the approach proposed appropriate to the objectives?*

Yes.

2. *Does the EDMVS have suggestions to improve the study plan?*

In the discussion following Dr. Ankley's presentation it became apparent that a great deal more well-thought out research took place that was not included in the DRP. For example, the additional exploration he described with methoxychlor and flutamide (hydroxyflutamide) reinforces the feasibility of using this assay as a screen. It is imperative that the possibility of adding a thyroid endpoint to the assay be explored. If the fathead minnow assay proves to be able to detect thyroid disruption, then it may be a possible replacement for *in vivo* mammalian Tier 1 tests (pubertal male and female, Hershberger, uterotrophic, and adult 14-day intact male). This is the first viable option presented to the EDMVS of having a single assay for EAT in one screen and perhaps avoiding using a battery of EAT screens and vast numbers of animals. The husbandry and maintenance of the fathead minnow has been established already. In addition, cost-wise, space-wise, and logistic-wise no mammalian study can compare with the fathead minnow assay. The issue of species extrapolation should not be visited now, but should be discussed after pre-validation studies have been conducted using the same set of core chemicals. Using a species such as the fat-head minnow as an *in vivo* Tier 1 screen should not be a consideration at this point, as the real issue is whether this assay will detect EAT effects.

Another compelling reason to potentially replace mammalian *in vivo* Tier 1 screens with the fathead minnow assay is that the fathead minnow assay appears to be sensitive for detecting low dose effects. This is critical because EPA has stated that it will not require routine low dose testing, but will make this

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determination on a case-by-case basis. This is problematic because it requires that a chemical have been studied thoroughly enough to make this assessment, which is not the case for the majority of environmental chemicals. However, because the fathead minnow assay appears to be extremely sensitive (effects of trenbolone were noted at 50 parts per trillion), this assay could provide the data required to determine whether Tier 2 testing should include low-doses for a particular chemical.

During the pre-validation and validation stages, the fathead minnow assay should contain both apical (survival/growth, behavior, fecundity, fertility, and hatch) and diagnostic (secondary sex characteristics, gonadal condition, sex steroid, and vitellogenin) endpoints. This is important because it will allow EDMVS members to evaluate the relationship between these endpoints and compare their relative sensitivities to chemical exposure. Many EDMVS members have expressed interest in knowing what additional information proposed EAT endpoints will provide compared to more traditional well-established endpoints, which tend to be apical in nature. Only by collecting both diagnostic and apical information will this issue begin to be addressed. The appropriate time to discuss whether apical endpoints should be included in final protocol should come only after EDMVS has evaluated pre-validation and validation study results.

Similarly, EPA should consider assessing the same set of diagnostic and apical endpoints in the offspring of the exposed fat head minnow during the pre-validation and validation phases because developmental sensitivity is such a critical issue for endocrine disruption. Specifically, for screening purposes, the issue is whether any EAT effects are detected in the F1 generation that were not detected in the parental generation. Only by collecting this information now will the EDMVS panel be able to assess whether a Tier 1 screen needs to include a developmental exposure period.

It was suggested at the EDMVS meeting to use trenbolone instead of methyltestosterone. It would be far more relevant and cost-wise to spend EPA resources on environmentally relevant compounds when making choices such as this.



Intra-Uterine Lactation (IUL) Assay Plan Questions

1. *EPA views the IUL assay primarily as a potential substitute for a full Tier 2 study when only endocrine sensitive endpoints need to be examined (i.e., when valid data from a 2-generation study exist). The other potential use includes substitution for all mammalian in vivo assays in Tier 1 (pubertal assay, uterotrophic and Hershberger.) Is the initial study design, breaking the study into three cohorts (uterotrophic with 1 female per litter, pubertal female with 4 females per litter, and pubertal male), consistent with these purposes?*

Yes.

2. *Are the endpoints for each of the cohorts appropriate to the design of the study?*

Yes. However, additional consideration should be given to the adrenals, pituitary and pancreas. Estrogenic, androgenic and thyroid effects have been given much attention to this point, but there is little dispute that the adrenal glands, pituitary, and pancreas are critical endocrine organs. In section 3.6.3 (p 29) of the IUL protocol, the collection of the adrenal and pituitary glands for histology and pathology is considered optional for F1 males and not discussed for F1 females. Adrenal and pituitary histopathology should be examined in the IUL.

The pancreas should also be examined because it is an endocrine organ critical for proper glucose regulation. Diabetes is now an epidemic in the U.S. and although it is often linked to the growing number of Americans who are overweight, it is important to remember that certain environmental chemicals, such as dioxin and polychlorinated biphenyls, can disrupt glucose and insulin regulation. The association between Agent Orange, an herbicide mixture contaminated with dioxin used during the Vietnam war, and diabetes in Vietnam veterans was recognized by the National Academy of Sciences Institute of Medicine in 2000. Certain pesticides such as amitraz (Cascino, 1989, EPA, 1995, Hugnet, 1996, Ulukaya, 2001), benomyl (Dreisbach, 1983), tebuthiuron (EPA, 1994), and glyphosate (EPA, 1993) have been shown to increase glucose levels and/or cause pancreatic toxicity. Although insulin levels or regulation are not currently included in Tier 2 tests, studies from the open literature suggest this would be appropriate, especially for chemicals found to alter glucose levels or cause pancreatic toxicity. For example, several studies demonstrate that amitraz decreases insulin release following glucose challenge (Abu-Basha, 1999, Chen, 1994, Hsu, 1988, Smith, 1990).

EPA is devoting much effort and money to the proposed screens and assays and should try to assess function in as many endocrine target organs as feasible in order to use test animals as responsibly as possible. EPA should take a forward-

looking approach to endpoint selection and not just limit organ collection to those that were discussed by EDSTAC. For example, during the early and mid-1990s estrogenic and then anti-androgenic chemicals were receiving considerable attention; however, towards the latter 1990s thyroid toxicity was being given more attention. EPA should not try and chase the moving target of attention-getting modes of action, but rather evaluate endocrine function in a more comprehensive manner by assessing all appropriate endocrine organs.

Serious consideration should be given to assessment of auditory function (auditory thresholds/evoked potentials) in neonatal animals in IUL. Auditory thresholds may be This is an endpoint known to be affected by thyroid disruption. There is a significant amount of data in rats, mice and humans that congenital hypothyroidism causes hearing loss or increased auditory thresholds (reduced sensitivity) (Abel, 1999, Bellman, 1996, Crofton, 2000, Crofton, 2000, Goldey, 1998, Goldey, 1995, Herr, 1996, Li, 1999, Ng, 2001, Rovet, 1996, Sher, 1998). Iodide deficiency (DeLong, 1985), resistance to thyroid hormone (Brucker-Davis, 1996) and Pendred syndrome (an autosomal recessive disorder characterized by congenital deafness and thyroid goiter) (Everett, 2001) also cause hearing loss in humans. Chemicals that induce hypothyroidism and affect auditory function include propylthiouracil (Hebert, 1985), methimazole (Knipper, 2000) and Arochlor 1254 (Crofton, 2000, Crofton, 2000, Goldey, 1998, Goldey, 1995). These changes can occur in the absence of thyroid weight changes (Hebert, 1985). Because data suggest that hearing loss is not recovered following developmental hypothyroidism, auditory function could be assessed during developmental time periods or in older animals (Hebert, 1985). In addition, impaired auditory potential in human infants appears to be predictive of later reading ability (Molfese, 2000). The critical period in rats for thyroid sensitive ear development ranges from gestational day 18 to post-natal day 18 (Freeman, 1996). In humans, the critical period ranges from the end of the first trimester to the first month of life (Brucker-Davis, 1996).

It is unclear whether auditory function is a more sensitive measure of thyroid disruption when compared to thyroid hormone levels or thyroid histopathology. Many studies that evaluate both thyroid hormone status and auditory function have only used one chemical dose and do not typically evaluate thyroid histopathology. EPA should consider inclusion of auditory function at this stage in order to generate dose-response data appropriate for determining endpoint sensitivity to thyroid disruption. By collecting information on auditory function, thyroid hormone levels, and thyroid histopathology, EDMVS members will be in a better position to evaluate potential Tier 2 guideline changes later. Although assay sensitivity may be more of a Tier 2 issue, if EPA does not collect this information now, there will be no possibility of making critical endpoint sensitivity evaluations later.

2. *Is the rationale clear as to why the pubertal cohorts are further subdivided into dosed and undosed groups?*

Yes. This exercise, along with evaluation of methoxychlor in the pubertal and uterotrophic assays, is important because comparisons among animals exposed in



utero-lactation, in utero-lactation-young adult, and prepubertal only (such as in the female uterotrophic study) should allow the EDMVS panel to make several “age at exposure sensitivity comparisons”.

Age at exposure differences in response has major implications for implementation of the Food Quality Protection Act (FQPA), specifically with respect to the magnitude of the FQPA Safety Factor that is applied to a pesticide or group of pesticides which have been determined to share a common mechanism of action. Although it is clearly beyond the charge of EDMVS to advise on the policy implications of the endocrine disruptor screening and testing program for FQPA implementation, it is necessary for the EDMVS panel to understand the data required to make scientifically sound regulatory decisions under FQPA. This will be especially important when the time comes to evaluate the adequacy of Tier 2 studies for addressing potential increased developmental susceptibility.

The FQPA 10X safety factor represents an additional safety and/or uncertainty than can be applied to a No Observed Effect Levels (NOEL) in addition to 10X interspecies and 10X intraspecies factors, to account primarily for potential increased susceptibility in fetuses, infants and children. This safety/uncertainty factor primarily exists to ensure that there is a “reasonable certainty of no harm” to embryos, fetuses, infants and children resulting from pesticide exposure. In order to evaluate potential increased susceptibility in developmentally exposed animals, EPA typically compares the dose levels corresponding to the parental and developmental NOELs obtained in the developmental rat, developmental rabbit, and reproductive rat (multigenerational) toxicity studies. If the developmental or fetal NOEL occurs at the same or higher dose than the maternal or parental NOEL, EPA concludes there is no evidence of increased susceptibility. Conversely, if the developmental NOEL is lower than the parental or maternal NOEL, EPA concludes there is potential increased developmental sensitivity.

There are two major problems with this approach. First, the developmental rat and rabbit studies do not evaluate post-natal development and tend to be less sensitive than studies that do assess postnatal function. For example, a 1998 EPA draft report titled “A Retrospective Analysis of Twelve Developmental Neurotoxicity Studies Submitted to the USEPA Office of Prevention, Pesticides and Toxic Substances (OPPTS)”¹ found that the developmental neurotoxicity study resulted in a lower NOEL for 10 of the 12 chemicals surveyed when compared to the developmental rat study (comparison to rabbit developmental study was not discussed).

¹ Makris et al., A Retrospective Analysis of Twelve Developmental Neurotoxicity Studies Submitted to USEPA Office of Prevention, Pesticides and Toxic Substances (OPPTS), 11/12/98

Second, although the rat reproduction study does assess postnatal function in developmentally exposed animals, it is very difficult to separate developmental effects from dosing duration effects. On one hand, effects that occur in the adult F1 animals at lower doses than P animals could be interpreted as evidence of increased susceptibility. However, this interpretation is complicated because the parental F1 animals have also been exposed to the chemical for a longer period of time than the parental P animals (F1: in utero, lactation, dietary for 10 wks prior to breeding through lactation compared to the P animals which are exposed 10 wks prior to breeding through lactation). In general, EPA does not appear to consider the adult F1 effects as being developmental unless the effects observed in the F1 animals are related to malformations that could only occur during development (i.e. nipple retention or epididymal malformation). However, this logic does not translate to the evaluation of continuous response measures such as organ weight or hormone level.

Comparison of NOELs obtained from the developmental neurotoxicity and rat reproduction study suggest that the two-generation design does not adequately assess developmental susceptibility. The “Retrospective Analysis of Twelve Developmental Neurotoxicity Studies” document described above found that the NOEL obtained from the developmental neurotoxicity study was lower than that obtained from the rat reproduction study for 6 of the 10 chemicals compared. In summary, the data obtained from the IUL and pubertal studies will be useful for the EDMVS panel to have when considering changes to the multigenerational study.

Will this design result in sufficient power in the test to detect the endpoints of interest?

This is unclear because there has not been a discussion of what magnitude of effect the screen should assess. For example, a 1.95 day change in vaginal opening might not be considered a large effect by some. However, a change of this magnitude would be a change equal to a 1.5 standard deviation² shift. A change equal to 0.8 standard deviation would be considered a large effect using normal power analysis conventions³. Using this example, a sample size of 8 in each of four treatment groups (as presented in the IUL EDMVS presentation) would detect a 1.95 change approximately 66 % of the time (based on ANOVA, not ANCOVA). The power would decrease if females did not become pregnant. For example, the power would decrease to ~56 percent based on a sample size of 7 and ~48% based on a sample size of 6. Although the exact power calculations will vary depending upon the analysis used (i.e ANCOVA vs. ANOVA, non-parametric, etc.), the IUL as presented likely would miss biologically significant changes in endpoints. For this reason it is imperative that the study results include not only statistical significance, but also the magnitude of effect, because at some point the EDMVS panel should have a discussion of desired magnitude of effect to detect when evaluating group sizes.

² based on TherImmune historical data presented in December 2001

³ refer to WWF comments on December 2001 EDMVS meeting



Do you agree with this design as being appropriate for a test and/or a screen?

This study was not supposed to be used for one or the other. It is a feasibility study. It was designed to be heuristic, to ask questions, to incorporate as many endpoints as possible, and to look at all the outcomes. After it was done and the data analyzed, we were to decide whether it could be used as a screen; or some part of it be used as a screen; or some of the endpoints could be incorporated into other screens; or whether it might even become a potential substitute for a full Tier 2 study when only endocrine sensitive endpoints need to be examined (i.e., when valid data from a 2-generation study exist).

3. *Does the EDMVS agree with the conduct of a demonstration study using one chemical? Do you agree with methoxychlor as the choice for that chemical?*

Yes. Methoxychlor is a good choice because of its known tri-hormonal activity not only in one tissue but also across tissues and at various life stages.

4. *Are the choices of doses (0, 25, 50, 100 mg/kg/day MXC in corn oil to the dams, and pubertal cohorts and the same administered sc to the uterotrophic cohort) appropriate?*

No. This assay should be designed to detect a NOEL because it has been discussed as a potential substitute for a full Tier 2 study when only endocrine sensitive endpoints need to be examined (i.e., when valid data from a 2-generation study exist). The lowest dose should be less than any existing methoxychlor mammalian LOEL. The most recent minimal risk level (MRL) cited by ATSDR is 0.005 mg/kg/d based on a LOEL of 5 mg/kg/d detected in a study that assessed neurological, immunological and reproductive outcomes following in utero and lactation exposure (Chapin, 1997). In addition, a dose of approximately 0.02 mg/kg has been shown to increase prostate weight following in utero exposure (Welshons, 1999). Thus, EPA should consider either adjusting the dose range downward to encompass these doses or expand the number of treatment groups used in the study to cover the low dose range.

5. *Do you agree with the data collection and analysis procedures recommended in the protocol?*

In general, yes. However, as noted above, there has not been adequate discussion of desired magnitude of effect to detect and power analysis, although this is true for all of the *in vivo* assays presented thus far. An additional issue of concern is the decision to throw out statistical outliers. At this point, given the exploratory nature of the assay, it is probably more appropriate to include all data points unless it is known for sure that a technical error led to the outlier. This is also

important because inclusion of all outliers (except those arising from a technical issues) will present a more realistic estimate of variability and be important when assessing results from multiple laboratories.



Aromatase DRP

1. *Is the DRP complete and accurate?*

The DRP is extremely informative. However, it does not devote much discussion to the H295R human adrenocortical carcinomas cell line, which appears to be the cell system most appropriate for detecting aromatase induction. This may leave the reader with the impression that there are no viable cell-based methods to detect aromatase induction.

Does it form an adequate basis for making decisions about what pre-validation studies to initiate?

The combination of the DRP and oral presentation by Susan Laws are adequate for making initial pre-validation recommendations, although it appears that additional preliminary studies need to be conducted in order for the EDMVS panel to have a better idea of aromatase pre-validation specifics. For example, there are outstanding issues surrounding the recombinant assay and two cell-line cell system approaches.

2. *Do you agree with the recommendation to proceed with pre-validation studies on placental aromatase?*

Only in conjunction with further exploration of the two-cell based system (such as the JEG-3 and H295R). In addition, further consideration should be given to determining whether aromatase activity could be assessed in the steroidogenesis assay.

Do you agree with the recommended protocol?

Yes. It would also be useful if information regarding the placental material (such as smoking status, pregnancy complications, basal aromatase levels) could be reported in the pre-validation and validation results.

3. *Do you agree with the DRP's conclusion that the disadvantages of cell based systems outweigh the advantages?*

Not necessarily. While the placental aromatase assay may not require specialized lab set-ups (such as cell culture) and may be somewhat cheaper, it is critical to see how the placental aromatase assay compares with other suggested assays, such as the two-cell based system which could detect aromatase inhibition and induction.

4. *Do you agree with the recommendation to proceed with pre-validation studies on*

placental aromatase? Do you agree with the recommended protocol?

See answer to question 2.

5. *Do you agree that pre-validation studies should include optimization of concentrations of substrate, microsomal protein and co-factors? Is there any guidance you might provide regarding the design of such a study?*

While optimization of these factors would be desirable if they increased assay sensitivity and/or specificity, it would be useful to know how much more time and money would be required for assay optimization.

6. *The DRP recommends an initial protocol demonstration of 1-3 chemicals. It recommends a short list of strong steroidal (4-hydroxyandrostenedione, exemestane, 7 alpha-substituted androstenedione) and non-steroidal inhibitors (aminogluthimide, anastrole, letrozole). What chemicals should be selected for the initial demonstration?*

It would be preferable to include chemicals that are environmentally relevant if at all possible. For example, atrazine would be appropriate to include because it is an aromatase inducer and may also be selected as a core chemical.

Chemicals Used in Prevalidation of EDSP-Related Assays

1. *Are the modes of action (columns) broken out at the right level of detail? For example, should we break out "HPG axis" into more detailed levels?*

The table is a good first attempt at an “at a glance” breakdown of pre-validation chemicals. Clearly the main modes of EAT activity could be expanded and this may be especially worthwhile for the chemicals ultimately used in the pre-validation phase. For example, the thyroid columns appear to only address hypothyroidism; a more complete breakdown would include hyperthyroidism as well (such as Table 1).

Table 1: Suggestions for additional mechanisms of thyroid disruption

Thyroid					
hypothyroidism			hyperthyroidism		
decreased iodide (includes ↓ iodide uptake into thyroid and ↓ iodide incorporation)	inhibits T4 to T3 conversion (such as ↓ deiodinase activity)	accelerates T4 breakdown (includes ↑ glucuronidation, sulfatase activity, and biliary excretion)	increase TSH	inhibition of T4 breakdown	increase T3/T4

Similarly, increased aromatase activity should be included.

2. *Are all modes of action that we're interested in listed here?*



No (see previous comment). However, it is recognized that it would be extremely difficult, if not impossible, to cover all relevant modes of action at this point.

3. *Are there other candidates for the core set that should be included here?*

Thyroid appears to be underrepresented compared to chemicals likely to affect reproductive function. The pre-validation phase should consider including a chemical likely to cause hyperthyroidism in addition to a chemical(s) that can cause hypothyroidism such as phenobarbital or PTU. The pesticide imazalil (a conazole fungicide) has been shown to cause hypothyroidism (decrease T4, increase TSH) in the short-term (1 week), but cause hyperthyroidism (increase T4, decrease TSH) after a longer exposure period (4 weeks) (EPA, 2002). Imazalil may not be the best candidate for inclusion in the pre-validation studies because it displays biphasic thyroid hormone effects over time, but EPA should consider adding it or thyroxin to the pre-validation list of core chemicals to cover the hyperthyroid mode of action.

The core set of chemicals should be used in all pre-validation assays if possible. EPA has determined that exceptions to testing core chemicals include “special studies” (one-generation, avian dosing, etc) and “for cause” (for example, no need to test thyroid chemicals in aromatase assay). It is inappropriate to exclude chemicals for cause at this point since we do not really know *a priori* the extent to which many chemicals may “cross talk” across modes of action. For example, imazalil affects thyroid hormone levels (see preceding paragraph) and was also discussed in the aromatase DRP as having been shown to inhibit aromatase *in vivo*.

This information would be especially desirable to have when interpreting the relationship between *in vitro* and *in vivo* results as well as to establish the ability of apical assays to respond to different modes of action. In addition, this type of information would be important to have when interpreting dose specific effects.

4. *Is the table filled out correctly? "X" is essentially a check-mark, "S" means "strong", and "W" means "weak".*

The main modes of action portion of the table is a good start although the literature review should be expanded for the chemicals that will ultimately be used during pre-validation. For example, fenarimol is listed as an aromatase inhibitor on the table, but has also been shown to interact with the estrogen receptor *in vitro* (Vinggaard, 1999). A more thorough assessment of the pre-validation chemicals list is necessary in order to develop a useful table of “predicted effects” and to better understand the relationship of Tier 1 and 2 *in vitro* and *in vivo* results. This literature review should include effects that may

only be relevant at high concentrations or doses because this information will be useful to have when comparing Tier 1 and Tier 2 study results. It might also be useful to include agonist or antagonist information in the binding column when possible. For example, estradiol could be described as S+ for estrogen receptor (ER) binding because it is a potent ER agonist. Similarly ICI 182,780 could be described as a S- because it is a strong ER antagonist. In addition, the errors noted by certain EDMVS panel members should be corrected.

5. *Should all the core chemicals be used in validation as well as pre-validation?*

No.

6. *Which chemicals should be in the core set?*

EPA has proposed a “full set” of chemicals (n = 16) and a “limited set” of chemicals (n = 7). Although the limited set of chemicals would certainly be less expensive, 7 may be too small a set. EPA should consider having one chemical for each major mode of action (Table 2). The core chemical list could include 10 chemicals (atrazine fulfills both aromatase inducer and CNS/pituitary toxicant) and a negative control condition.

Table 2: Suggested core chemicals

mode of action	potential chemicals
E+	methoxychlor bisphenol A
E-	ICI 182,780
A+	trenbolone
A-	p,p'-DDE
T+	thyroxin imazalil
T-	phenobarbital
aromatase inhibitor	fenarimol
aromatase inducer	atrazine
steroidogenesis inhibitor	ketoconazole
CNS/pituitary	atrazine
“negative toxic”	feed-restriction

EPA should discuss more clearly why a feed-restriction group would not be an appropriate negative ED condition. If the goal of the negative toxic is to decrease body weight and ensure that resulting toxicity is not attributable to chemically induced ED toxicity, then feed restriction would be the easiest way to assess this. Selection of a “negative toxic” chemical would be extremely difficult, especially since the screens and assays designed to detect ED are far from finalized. The ideal negative toxic would be one that has been well studied and shown not to affect endocrine target organs or reproductive parameters. It is extremely important that the chemical be well studied, including a recent multigeneration study, so that the determination of endocrine or reproductive toxicity is based on



negative findings rather than the absence of data. Many of the suggested negative toxics are not appropriate for consideration because they have endocrine or reproductive activity or because they do not appear to have an adequate database to support no evidence of endocrine disruption (Table 3). EPA could have an extremely difficult time selecting a chemical appropriate as a negative ED control especially since the battery of screens and assays required to determine this is still being formulated.

EPA may want to consider choosing a pesticide as a negative chemical if feed restriction is not determined to be appropriate. The biggest advantage of this approach is that the chemical would have a “complete” toxicity database, especially if the rat reproduction study was conducted after 1996 guideline changes that included more complete reproduction assessments. In justifying the choice of a negative chemical EPA should provide a summary of the toxicity database (including the open literature) for that chemical and list any endocrine or reproductive effects noted.

Table 3. “Negative toxics” and evidence of endocrine disruption and/or inadequate database

chemical	endocrine-related toxicology
methoxyacetic acid	↓ testis weight; ↓ epididymis weight; ↓ epididymal sperm quantity and quality; (Chapin, 1997) testicular germ cell apoptosis damage and sperm quantity and quality (Chapin, 1997); testicular histopathology; decreased sperm fertilizing ability (Peiris, 2001)
cadmium chloride	alter plasma cortisol levels and decrease thyroid hormone levels in rainbow trout (Ricard, 1998) increased plasma levels of GH, TSH, LH, and FSH; decreased prolactin levels (some effects opposite after acute (6-hours) exposure: decreased GH, TSH, and LH (Lafuente, 1997); alters hypothalamic 5-hydroxytryptamine (5-HT), noradrenaline (NA) and dopamine (DA) levels (Shrivastava, 1988); increased corticosterone secretion (Hidalgo, 1987); <u>cadmium</u> decreased thyroid/parathyroid calcitonin (CT), calcitonin-gene related peptide (GGRP), parathormone (PTH), somatostatin (ST), synaptophysin (SPh) and neuron-specific enolase (NSE) (Pilat-Marcinkiewicz, 2001); inhibits estrogen receptor activity (Guevel, 2000); testicular degeneration; Leydig cell tumors; decreased testosterone (Waalkes, 1997);
alpha-2u-globulin inhibitor	????
bromobenzene	???? existing NTP data sets: 4-day acute; 13-week inhalation; 13-week gavage no ATSDR review; no WHO EHC review; no IRIS review;
phenacetin	urinary tract tumors (NTP); estrogens and androgens can affect urinary tract
acetaminophen	antagonize E2 induced uterotrophic responses (Patel, 2001); inhibit vitellogenin production (Miller, 1999); stimulate human breast cancer cell proliferation (Gadd, 2002, Harnagea-Theophilus, 1998, Harnagea-Theophilus, 1999), which appears to require estrogen receptor (Harnagea-Theophilus, 1999)

	many relevant reproductive measures not assessed in Reproductive Assessment by Continuous Breeding (RACB) (Chapin, 1997)
eugenol	<u>methyleugenol</u> has been found to cause adrenal gland cortical hypertrophy; cytoplasmic alteration in the submandibular salivary glands, adrenal glands, testis and uterus of rats (Abdo, 2001)
chloroform	↓ thyroid follicular size and colloid density, ↑ epithelial cell height and occasional collapse of follicles (WHO, 1994) many relevant reproductive measures not assessed in Reproductive Assessment by Continuous Breeding (RACB) (Chapin, 1997)
acetylaldehyde	increased glycogenolysis; decreased serum T4 and iodine uptake; pancreatic histopathology; (WHO, 1995) reproductive studies not identified (WHO, 1995)
pentachlorophenol	hypothyroidism [Beard, 1999 #88; Beard, 1999 #89; Rawlings, 1998 #94; Jekat, 1994 #96; (van Raaij, 1993); transthyretin (TTR) binding (van den Berg, 1990); increased insulin (Rawlings, 1998); oviductal intraepithelial cysts (Rawlings, 1998)
2,4-dinitrophenol	prevents the development of goiter and the histological signs of thyroid activation in propylthiouracil (PTU) treated rats with or without intact adrenal glands. DNP alone leads to thyroid atrophy (Goldberg, 1957)
cyanide	increased TSH (human), increased T3 (human), thyroid enlargement (human) (ATSDR, 1997) reproductive studies not identified , endocrine studies not identified in animals (ATSDR, 1997)

Avian Dosing Study

1. *Is the approach proposed appropriate to the objectives?*

Yes. However, EPA should consider comments made by various EDMVS panel members that the DRP as described may be overly ambitious and EPA should proceed by conducting appropriate pilot studies.

2. *Does the EDMVS have suggestions to improve the study plan?*

Yes. The study plan should include additional thyroid responsive endpoints. Specifically, EDSTAC proposed two functional test to assess thyroid function, the cold-stress test and the visual cliff test. The visual cliff tests addresses depth perception and chicks that jump off the cliff (versus choosing the ramp) may have neuro-optic damage. In this way, the visual cliff test may assess thyroid function as thyroid hormones affect optic nerve development. In addition, jumping of the cliff may be indicative of a fright response and these chicks may be more susceptible to predation.

3. *Is 17 β -estradiol acceptable as a test compound in this study?*

Yes. Although inclusion of other mode of action chemicals (such as thyroid or androgen disruptor chemicals) taken from a finalized core set of chemicals would



be appropriate at some point when the experimental design is further refined during the multi-chemical phase.

4. *Is methyl parathion appropriate as a test compound to evaluate “chick mortality” influences?*

Not necessarily. Methyl parathion was considered as a candidate chemical that would help resolve the issue of whether compounds that are directly toxic to chicks mask or limit the ability of the test to detect endocrine-mediated effects in the F1 generation. Methyl parathion was chosen because it was believed to be a reproductive toxicant without acting on the hormone system. However, the latest EPA revised risk assessment for methyl parathion indicates that it affects the testes and ovarian weight in rodents (EPA, 1999). In addition, slight increases of thyroid adenomas, pituitary adenomas, Leydig cell tumors and uterine adenocarcinomas were also observed in rodents, although determined not to be biologically significant (EPA, 1999).

5. *Is the approach to determine treatment concentrations for F1 hatchlings and P1 generation appropriate?*

No comment.

6. *Is the approach for considering mating behavior endpoints appropriate?*

Yes.

Literature cited:

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