U.S. ENVIRONMENTAL PROTECTION AGENCY

FIFRA SCIENTIFIC ADVISORY PANEL

OPEN MEETING TO CONSIDER AND REVIEW SCIENTIFIC ISSUES ASSOCIATED WITH THE AGENCY'S ENDOCRINE DISRUPTOR SCREENING PROGRAM (EDSP) PROPOSED TIER-1 SCREENING BATTERY

EPA CONFERENCE CENTER

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MR. DOWNING: Good morning, everyone. I'd like to welcome everyone to the second day of our FIFRA SAP, scientific advisory panel meeting discussing the Endocrine Disruptor Screening Program Proposed Tier 1 Screening Battery.

I want to just remind everyone that all the documents that have been presented yesterday as well as what will be presented today will be available in the docket for this meeting which the document information identification information is there at the top of your agenda, and that information should be on the docket within just a day or two, actually, certainly by tomorrow, the end of tomorrow, so you can access all of that information in the EPA docket for this meeting.

As well as I would remind everyone that the final report for this meeting which actually will

serve as the meeting minutes under FACA will be available within 90 days after the conclusion of the meeting. That will also be posted on the FIFRA SAP web site as well as posted in the LPP docket.

And I think, with that, I'd like to turn it over to our chair, Dr. Heeringa, and begin our meeting today.

DR. HEERINGA: Good morning, everyone, and welcome back to the second day of our meeting of the FIFRA SAP on the topic of the Endocrine Disruptor Screening Program, the Proposed Tier 1 Screening Battery. As Jim just indicated and you probably heard yesterday, I'm Steve Heeringa of the University of Michigan. I'm currently the chair of the FIFRA science advisory panel, and I'm here primarily to assist in running this meeting over the next...balance of today and possibly tomorrow.

I'd like to have, again, other members of the panel introduce themselves and their affiliation and a little bit of description of their specialty. Ken?

DR. PORTIER: Good morning. I'm Ken Portier, director of statistics at the American Cancer Society national office in Atlanta. I'm an applied statistician and a member of the permanent panel.

DR. CHAMBERS: I'm Jan Chambers. I'm with the College of Veterinary Medicine at Mississippi State University. My area of expertise is in pesticide toxicology with emphasis on metabolism and neurotoxicity, and I'm a member of the permanent panel.

DR. ISOM: Good morning. I'm Gary Isom, professor of toxicology at Purdue University. My area of interest is chemical induced neural degeneration, and I'm a permanent member of the panel.

DR. BUCHER: I'm John Bucher. I'm an associate director of the National Toxicology Program at NIEHS in Research Triangle Park. I'm a toxicologist by training with interest in chemical carcinogenesis applications in new models in toxicology.

DR. DELCLOS: Barry Delclos from the FDA's National Center for Toxicological Research. My research interests are endocrine disruption and in carcinogenesis.

DR. ELDRIDGE: Charles Eldridge, Wake Forest University, Department of Physiology and Pharmacology. I've been working with steroid hormones receptors, neuroendocrine, and, basically, female reproduction.

DR. DENVER: I'm Bob Denver from the University of Michigan. I'm a professor of molecular,

cellular, and developmental biology and also ecology and evolutionary biology, and my interests are in developmental neuroendocrinology, steroid thyrocrine action on the brain, and amphibian metamorphosis.

DR. VANDENBERGH: I'm John Vandenbergh, professor emeritus of zoology at NC State University, and my area of interest is in hormones and behavior and behavioral endocrinology basically.

DR. LASLEY: I'm Bill Lasley, University of California at Davis. I'm a reproductive toxicologist interested in toxicology and reproduction at the population-based level.

DR. COOKE: I'm Gerard Cooke, Health Canada Food Directorate. I'm a reproductive toxicologist with particular emphasis on male reproduction.

DR. ZOELLER: I'm Tom Zoeller, professor of biology at University of Massachusetts, Amherst, and I work on thyroid hormone action in early brain development and thyroid disruption.

DR. BROWN: Terry Brown, Johns Hopkins University Department of Biochemistry and Molecular Biology, and my areas of interest are in male reproduction, particularly androgens and androgen receptors.

DR. BELCHER: Scott Belcher, University of Cincinnati Department of Pharmacology. My major interests are mechanisms mediated through primarily estrogen receptor beta and signaling and the role of endocrine disruptors in brain development.

DR. KULLMAN: I'm Seth Kullman at North Carolina State University Department of Environmental and Molecular Toxicology, and I'm a molecular toxicologist interested in endocrine receptors and comparative genomics.

DR. FURLOW: David Furlow, Department of Neurobiology, Physiology and Behavior, University of California at Davis, and I'm a developmental endocrinologist interest in thyroid hormones and control of amphibian metamorphosis but steroid hormone control of muscle mass as well in...in mammals.

MR. DOWNING: Jim Downing, Designated Federal Official for the FIFRA SAP.

DR. HEERINGA: Thank you very much, panel members. Just a few administrative notes at this point. I want to mention that, as chair, I will be here through the morning. This afternoon, I have a teaching commitment, a regular teaching commitment at the University of Maryland. Ken Portier will be assuming chair duties at that point in time.

So, at this point in the process, we have concluded the presentations from the EPA scientists with regard to the endocrine disruptor screening battery, the tier 1 battery, and we have heard public comment from a number of parties. We have written materials.

The period of public comment is closed at this point. However, there is...if you have additional materials you would like to provide to the panel, you may do so in writing before the close of the meetings, and I would assume that would carry over into tomorrow as well, to be fair, so that if, again, additional public comments or clarifications, things you would like to bring before the panel, you may do so in writing after this point.

At this point, I would like to turn to Gary Timm for some additional comments before we turn to the charge questions.

DR. TIMM: Thank you, Dr. Heeringa. In listening to the...not only the questions posed by the panel but...but some of the public comments, it seemed to us that it might be useful to clarify a few points before we actually got into the charge questions, and these...these are not point/counterpoint to anything we heard yesterday but just general clarifications.

There are about four topic areas, I guess. We would like to take probably 15 or 20 minutes to...to go over these, first of all, updating the battery, quality assurance for...for assays, the status of the OECD fish test guideline, and some illustration of weight of the evidence. That seemed to...to come up a number of different times yesterday, and I think we can give some additional examples to show you how the...the assays in the battery work and how...how some of the...some other cases would be...would be assessed.

So, let me start off with a couple of things, and I'll turn to my colleagues for...for help on the others.

With respect to updating the battery, there's a lot of research going on in EPA, outside of EPA, but within our own laboratory down at RTP, we have developed and a number of laboratories have already used, probably tested hundreds of chemicals already, some transcriptional activation assays for estrogen binding the...using the MDA KB2 cell line and for androgens using the 247DK blood cell line, and there is going to be probably an effort...we're going to, next week, tell the OECD that we believe that a project should...should be initiated to develop a generic test guideline for transcriptional activation assays for

using the estrogen receptor.

This will, I think, be very useful rather than going assay by assay. Right now, the Japanese have validated an assay, and there is a test guideline being developed for that particular assay, but I think that this would open up the field by having a generic test guideline.

We're also working on an alternative to the existing AR binding assay which, of course, uses the rat prostate cytosol. It would be using the...the chimp AR binding, and that will become an OECD project as well, and we mentioned the status of the H295R assay which is a replacement for the testes assay that the...the EDSTAC recommended, and that assay has completed validation. The report will be submitted next...next month for peer review.

So, that's kind of an update on...on the battery. We...we clearly intend to be flexible and to introduce new technology as...as it is validated.

And with respect to quality assurance, I know one of the presentations yesterday talked about that. I think it's well to recognize that all of the assays have quality assurance built into them. There are performance standards in them. There are controls, and laboratories must demonstrate, in fact, that they

can run the assays correctly before we will accept the...the data.

So, I think that that...that certainly has been addressed by...by the existing work.

Let me turn now to the...to Les for the update on the...on the fish, a clarification on the fish.

DR. TOUART: Thanks, Gary. I'm Les Touart, and what I want to do is just...there might have been some confusions with regard to the status of the fish test guideline, at least from an international standpoint, within the Organization of Economic Cooperation and Development. In this light, it may be difficult for folks in the back of the room, you know, to see it, and they may want to get the full paragraph from the summary record of the meeting of the OECD validation management group for ecotoxicity testing, and, basically, first in this, these are recommendations to the EDTA task force. EDTA is the Endocrine Disruptor Testing and Assessment Task Force, and then the working group of the National Coordinators of the Test Guidelines which oversee the EDTA and...and the B and G activities.

If you come down to the...to the last bullet, it basically saying subject to the provision of

data generated according to the OECD foundation principles, you know, that is the guidance document 34, to integrate fecundity and histopathology into a revised OECD test guideline for fish screening for endocrine active substances.

The context here is the...the existing test guideline needs to accommodate new endpoints and those endpoints, in particular, and once these have been demonstrated to be valid, these would be incorporated into the OECD version. The U.S. has completed a...a peer review of the...of the assay and these endpoints. We provided responses to those, and I think the panel has copies of the peer review report as well as the comments to that.

These have also been provided to the OECD and will be an item on the agency for the EDTA and the National Coordinators which is scheduled for next week with the proposal that these endpoints now become part of the...the fish screen.

DR. TIMM: Thanks, Les. Earl, you and...and was Gary going to join you at the table?

DR. GRAY: I certainly hope so.

DR. TIMM: Okay, excellent. Why don't you introduce yourselves, Earl Gray and Gary Ankley., at this time.

DR. GRAY: I'm Earl Gray, and that's Gary Ankley. What we'd like to do is talk about the ability of the tier 1 screening battery to detect lower potency environmentally relevant endocrine disruptor chemicals. We've talked about a few, and many of those focused on potent pharmaceuticals that have a single mode of action that produce rather diagnostic profiles.

What will be the importance of this, I think, is to highlight how the mammalian and non-mammalian assays compliment one another in the battery...they're not necessarily redundant...and how, on occasion, an endpoint that you might think that's not very useful or unnecessary provides useful information, because the purpose of the battery is not only to detect chemicals with EAT; it's to help design the tier 2 testing. So, the information that you gain from this can help design.

So, these, as...and we've stolen some slides from Les. These are...this is the tier 1 screening battery and the assays that are recommended by EDSP, and this is Les' summary slide of how your...how your potent chemicals would be detected in the screening battery.

What...what happens, though, when you go to chemicals with multiple modes of action or when you

go to weaker chemicals, in particular, a weaker estrogen, for example? Some of these bright green boxes kind of get a little light or disappear, and you don't have these multiple hits, and it's at this point where a single endpoint can be very useful in the assays. The fish and frog and rodent assays, compliment one another.

And...and as...as we all are aware, all estrogens don't act through all of the same...on all of the same tissues, and it's the same with androgens, anti-androgens, and et cetera. You know, the chemicals that interfere with steroid hormone synthesis do so often by inhibiting an array of p450 enzymes that are throughout the body.

So, what I wanted to just...we made up these lists on how environmentally relevant chemicals would behave, and...and it sort of gives you a...an idea of the emergence of the weight of evidence approach. If you look down a column, you begin to see with different chemicals, you know, what...what's positives.

So, what I have on here, we started off with your potent estrogen. These are, for the most case, environmentally relevant chemicals.

And so, ethylestradiol is a

pharmaceutical in the environment. It's a classic estrogen. It produces all of the typical responses in the assay, including the hormonal changes in the hypothalamic-pituitary-gonadal axis.

But if you look at the...the weakly estrogenic and weakly anti-androgenic pesticide, methoxychlor, the profile is...is a little...is a little different. And...and here, this chemical is really more effective orally which is why I highlighted the...the pubertal female assay in red, because it really provides a really rapid response to this particular estrogen and not necessarily the bisphenol A on the next column which is also an environmental estrogen.

And the effects of methoxychlor are complimented nicely by the talogen in the conduction and other changes in fish assays. The...the big no on the pubertal male is there have been issues raised about there haven't been anything run through these pubertal assays that were negative and, in fact, methoxychlor is...is pretty much a negative in the male and in...in this assay. And bisphenol A to the right is negative in the pubertal male and the pubertal female assays with oral exposure.

And it's here that the in vitro assays

and the uterotrophic assay for bisphenol A and the fish screening show that it's under certain...at least certain routes of exposure, it's clearly estrogenic. And so, you can see that it's profile is quite different than methoxychlor, and the value of different endpoints and different assays is not the same.

Tamoxifen was...is an interesting...it's also a pharmaceutical found in the environment. It produced an interesting and expected profile based on we knew that it's a selective estrogen receptor modulator. It accelerated vaginal opening as much as estradiol, but it reduced uterine weight by 60 percent. So, if you say uterine weight's too variable, the animals are cycling, yes, uterine weight is variable in the cycling animal, but these females are not cycling, and there is very little variability in that data. This is a highly significant effect.

And it inhibits..tamoxifen inhibited the telogen and...is that correct, Gary? So, I think it provided some really interes...an interesting profile for tamoxifen with dramatic, paradoxical responses in vaginal opening and uterine weight. And...and there are a number of other chemicals where things like uterine weight, ovarian histopathology were useful.

This is sort of our androgen page, and

we started off with your potent classic pharmaceutical in the environment, trenbolone, and it produces a response almost testosterone or methyltestosterone, the difference being that you're not going to get any estrogenic responses in the...with this with oral administration or subcutaneous administration, because it can't be aromatized, so it's a...a little different, but it...but it's still more of a classic profile.

And then you look at the androgen receptor antagonists. The one to the far right, vinclozolin, is...is probably more like flutamide, and it's one of the...maybe one of the more potent pesticides with anti-androgenic activity, and...and in that particular chemical, AR binding is...is positive in some assays and...and not in others, because it requires some degradation or metabolic activation.

It's really the Hershberger and pubertal assays that nail that one, and there are positive effects of vinclozolin and other androgen receptor antagonists in the fish assay, but they're not nearly as diagnostic as what you get with the Hershberger and the pu...pubertal assays on multiple endpoints at relatively low doses.

And I would add that if you looked at the endocrine profile of the vinclozolin in males, it

looks like you would expect from flutamide in that testosterone and LH both go up.

Which is not the case for the linuron and DDE, and many of the other xenoestrogens don't affect hypothalamic-pituitary-gonadal hormones the way you'd expect. And the profile of linuron and DDE is much less dramatic than vinclozolin, and...and they have mixed modes of action. Linuron is also a little bit hypothyroid, and DDE really turns on liver enzymes and other things and affects the adrenal.

So, you know, and...and if you were to do a toxicology study, you're never sure how important the AR antagonism is, but it will get us a nice vocation.

And we call this story genesis and et cetera, and...and it's really...you know, ketoconazole is a nice chemical. It's a potent drug, and...and it shows the characteristic profile that you'd expect would stimulate hormones and inhibits aromatase. In the fish, it was just...it was, what, just testis histopathology?

DR. ANKLEY: Yeah, what we saw was a Leydig cell proliferation. Essentially, it was a compensatory response to the reduction in testosterone synthesis. It's a rather unique example in the fish of

an endpoint that you would pick up only with this knowledge. I'll come back to that in a minute, though.

DR. GRAY: In the...in the pubertal female what was interesting was that was the chemical where vaginal opening some feel is the keystone endpoint in that assay, and it's really not. Vaginal opening was not affected significantly, but we had every...all of the females...all the treated females had severe lesions in...of ovarian and histopathology. They had atretic corporal lutean follicles, and so...and they also had a 50 percent reduction in uterine weight. So, it definitely had a positive effect but not on vaginal opening.

If you look at the...the other steroid synthesis affecting chemicals, prochloraz and fenarimol inhibit fungal and os sterol synthesis, but their profiles in the mammalian assays and in the fish assays are...are quite different, and, you know, they were...they really had multiple modes of action that are displayed in vivo. Prochloraz is an androgen receptor antagonist, and we don't...we don't...we don't know. It hasn't been run in the pubertal female. It's positive in the pubertal male, primarily for testosterone synthesis. It was very positive in the fish assay, very diagnostic there in the hormone and

reproductive endpoints in the fathead minnow assay, and it's very positive in nutria.

Fenarimol you might think was the...would be similar. It's the weaker aromatase inhibitor, but it's been reported to be an androgen receptor antagonist, to be an estrogen. You know, it does all things to all people except that it was negative in the pubertal female assay up to a point where there is a significant reduction in body weight beyond the M.D., and it was very positive in the fish screen. So here, the fathead minnow assays results were very important.

And then, here...here's chemicals we didn't think much about when we were at EDSTAC, because when we designed the initial battery, we came up with a battery that would have missed the phthalate, and...and EDSP has corrected that omission, but the phthalate inhibit steroid hormone synthesis through unknown mechanisms, and the only assay in which it would be detected would be the pubertal male assay.

And so, if you think about on a...take the pubertal male assay out, you're going to miss any of the phthalate that have that activity.

And then, this is...I have one chemical that our branch has worked on...on...that affects the

hypothalamic-pituitary-gonadal axis, and you can...can see that it's...it's going to be negative in vitro in the uterotrophic and Hershberger, and it's the pubertal male and female where that was detected, and it hasn't been run in the fish, but our expectation would be that it's positive. So, that's a hypothesis, and they...they're not always right.

I put thyroid hormone down there just to have an agonist, and as hard as we tried, the frog fell off the bottom. Sorry, Joe. We worked on this to get this on. I'm going to...so, the read for T4, the reason we put T4 on there was to show how it's tyro...this is...produces diagnostic responses to thyroid hormone agonists. If there are any in the environment, this is what's going to detect it, and that was the purpose when EDSTAC put the frog in. We care about frogs, but we really wanted something in there to compliment the mammalian assays for thyroid hormone agonist activity.

And our expectation would be that it would be positive in the pubertal assays. I'm sure somebody's run that, but we haven't.

The...we should have had one less beer, Gary.

What I have on the...there is a

polybrominated diphenyl ether. It's...it's an anti-thyroid chemical, and it's an androgen receptor antagonist, and it affects T4 in the pubertal male and delays preputial separation, and it's just a...I like it. You know, the pubertal male assay can detect weakly anti-thyroid chemicals.

And then, this is the chemical, diiodinase inhibitor. It's, again, the frog metamorphosis assay is going to give you the diagnostic response about the mechanism and...and detect this effect.

And I thought I'd...I'd end with this part on our toxic negative. This was hypothesized from the best available information to be a toxic negative, and it was run for that purpose, and it...it was pointed out and it's quite clear that it wasn't negative.

And, you know, that's what happens. It was a hypothesis, and we tested the hypothesis, and that's what happens with hypotheses.

And I think it really points to the value of the battery. Here's a chemical that, with the best available information, we thought was going to be negative, and it was clearly positive. And so, in 20 to 30 days' worth of evaluation, we learned something

important about an unknown chemical.

And whether you want to define that as an endocrine disruptor or not is sort of a battle. It clearly affects hormone synthesis indirectly by affecting the Sertoli cells and testis, and it's, I think, important to identify chemicals like that for further testing, whether you define it as an endocrine disruptor or not.

And so, I think that the point of that is it's...this is not a deficiency in the pubertal assays that it detects effects other than EAT, if you consider this other than EAT. I think it's...my opinion is it's a benefit. The assays detect...the battery detects EAT, and it also...it's going to pick up these gonadal toxicants. And it's not very difficult to interpret what's happening in that.

The last thing I'd like to address, Sue Marty raised some points about the pubertal assays yesterday, and...and I agree with about 99 percent of what Sue said, and what I wanted to do was...was to talk about the pubertal assays and...and highlight that what she said was that there...there are changes in ordinal weights, there's body weight is reduced, and so, there's a difficulty in interpretation in the specificity of the effects, and she said that

alternative statistical methods were needed to adjust for this.

And my point is that we have the data to develop those alternative statistical methods that will allow us to discriminate the effects of...direct effects of growth on the pubertal assays to do endocrine disrupting chemicals. And so, I was going to show some data from the food restriction studies.

These are data from Susan Laws' study on food restriction and what I have top...or this is Tammy's delay...right, Tammy?...delay in preputial separation in the male, and the food restriction study is the line along the axis. You can see that as body weight is reduced, we sort of have a dose response reduction in body weight out to the 20 percent and no effect on preputial separation, and all the little dots on that graph are...are dots that I plotted from different studies run in contract laboratories.

So, you can see you could easily put confidence limits around each of those points and know if it deviates significantly from that line, although here, there's no relationship, but we have data for all the other endpoints in the male and female.

And so, here's the data from the male on the effects of food restriction on the seminal vesicle

weights, and there is a decline there as you go out, and it's more or less linear. But you can see even on those points where you have, you know, an 8 percent reduction in seminal vesicle weight that you can easily discriminate those...those chemicals from the...the line. So, we have...we have the data, the...the relationship between these organ weights and body weight, that can be used to adjust the...and interpret the results of the assay, the pubertal male and female assays, even though there are body weight changes.

And then, this is the...just the...all of the reproductive organ weights in the male assay and how they changed with the food restriction. So, some are spared more than others, and each would have its own statistical methodology for adjusting, and I'm sure...there are members on the committee that know how to do this better than I do.

This is in the female. You can see there is a delay in preputial separ...in vaginal opening...excuse me...with food restriction out to 80 percent. It's...it's not that...it's about a day with a 10 percent reduction in growth.

What's remarkable, if you look way out to the...the right there, that little yellow-green dot is a PTU animal where they...they barely grew in the

assay from inhibition of thyroid function, and even there, vaginal opening is only delayed a day, so there's not a lot of confounding on this endpoint.

Other endpoints in the female...I just show a few are...there is more of a linear relationship decline in organ weight with...with body weight, but the...as I said, these data can be used to adjust and compare your treatment to. So, if you have a, you know, a 10 percent reduction in body weight, you can compare the response of your chemical to these data and see if it really appears to be specific or not.

And then, this is just a summary of the effects of those studies, growth retardation. Vaginal opening and preputial separation are relatively spared in the male. Epididymal and testis weights are relatively spared. Reproductive organ weights are...are reduced in the male when you get out to about 7 to 12 percent.

In the female, reproductive and non-reproductive organ weights do decline linearly with body weight, as Sue said, and...and the female organ weights seem more affected in small reductions growth than the male, but using the data from the Laws and Stover studies as a guide, direct reproductive effects can be discerned from those that may be associated with

reduced growth.

I thank you for your time.

DR. HEERINGA: Possibly before we move on, if there are any questions on this presentation? Dr. Chambers?

DR. CHAMBERS: Earl, on your summary charts there where you've got the pluses and minuses and so forth, were those at the high doses, or how did you discriminate amongst several doses in your summary?

DR. GRAY: I...I didn't. What I tried to do there was, like with methoxychlor, if the low dose effect in methoxychlor was vaginal opening, then I highlighted it in red to just show that was a very sensitive endpoint. On...on the ketoconazole, some of those effects...many of the effects were invoked dose groups, so the...the trenbolone is across all doses. But they didn't break...you know, most of those run in two or three doses, and I didn't break it down that way, but you could.

DR. CHAMBERS: The plus...plus on plus doesn't necessarily mean that...

DR. GRAY: Oh, I put that in...a plus, plus, plus meant it was a really robust response that was quite diagnostic, and then, a plus, some of those were kind of equivocal or small. And so, you know, if

you're looking for flags, if you saw...if you see something...if I put something in with multiple pluses, there was...there was no likelihood that that would be missed and misinterpreted.

But this...we did this...I wouldn't say hastily, but we, you know, we just...we did it since yesterday.

DR. CHAMBERS: One follow-up question. Do you have a negative control panel to run through these things?

DR. GRAY: Well, there's bisphenol A is negative in the pubertal and it's positive in vitro. So, on an assay by assay, there are negative chemicals. Fenarimol was negative in the pubertal female. Methoxychlor was negative in...in the...in the male.

And I think the...that points to an interesting...when you're looking at estrogens in the pubertal male assay, one of the things they affect the most is growth, because they interfere with food consumption in the brain. And so, when...you do get delays in preputial separation with methoxychlor in the male rat, but they're above the M.D. of 10 percent.

Does that answer your question?

DR. HEERINGA: Dr. Vandenbergh?

DR. VANDENBERGH: What...what were the

ages of the animals when...when they were dosed? Were these all adult studies that you're talking about here?

DR. GRAY: No, the pubertal studies are the pubertal...

DR. VANDENBERGH: They were...

DR. GRAY: Yeah.

DR. VANDENBERGH: Oh, pubertal.

DR. GRAY: Right? Those are just your...or it's unrestricted there in the pubertal assay as if it was...so it was 20...22 by 42 in the female and 22 to 40...53 in the male. So, I mean, they were directly comparable. They were run for that purpose.

DR. VANDENBERGH: Okay.

DR. HEERINGA: Dr. Bucher?

DR. BUCHER: Earl, do these relationships hold across strains, do you know?

DR. GRAY: Some of these pubertal assays are run in two rat strains. Early on, we ran them in the Long-Evans and in the Sprague-Dawley, and then, other...other laboratories that have run these assays have run other rat strains. I think that most of the ones run by EPA are...are now in the Sprague-Dawley, but I don't...Lianne? I'm sorry. Speak up back there. They know what they're talking about. Many of these are run in the Wistar 2, and I...I...we haven't really

seen any evidence of strain differences.

And...and, in fact, several...we ran five or six of the estrogenic chemicals back when we were comparing the Sprague-Dawley to the Long-Evans, ethyl estradiol and methoxychlor and tamoxifen, and there was no difference between the...the responses of the females to any of those chemicals at any dose.

DR. HEERINGA: Dr. Delclos?

DR. DELCLOS: You mentioned that if you threw out the male pubertal, you would miss the phthalate in the battery. Is that true if you substituted the male adult?

DR. GRAY: No, I don't think so. I think it's been known for 20, 30 years that the phthalate are much less effective in the adult animal, and you...so, I think there are some studies from 1985 where they go up to 2 g/kg and don't see anything. So, I think if you give it chronically to the male, you can see effects, and they're not as robust, but...

DR. DELCLOS: Decreased sensitivity is what you're saying.

DR. GRAY: Oh, in the phthalate, that's a...an excellent example of the sensitivity of the developing endocrine system in the pubertal and in the fetal as compared to the adult male.

DR. HEERINGA: Okay, we'll move on. Dr. Ankley?

DR. ANKLEY: I just wanted to take a couple minutes. I don't have any slides, but I wanted to take a couple minutes to talk about the OECD fish assay, and as you recall, yesterday, it was proposed that that could be substituted for the 21-day reproductive screen, and I had a couple perspectives on it.

First, the committee that Dr. Mahiatch referred to yesterday has been active for a number of years, and it's an OECD committee. Has multiple countries involved. I've chaired that committee since pretty close to its inception, so I have sort of a...a unique perspective in terms of...of seeing how things have developed there, and it's...I think, for those of you that have been involved in international activities of this type, you can relate to the fact that when you have lots of different stakeholders at the table, things don't necessarily evolve strictly along scientific lines.

And one of the challenges with the fish assays is that we're, as a rule, interested in having an assay that can be used for multiple small fish species. In the case of the OECD exercise, we're

trying to develop a system that would be amenable to fathead minnow, the zebra fish which is the preferred test species of several European countries, and medaka which the Japanese folks prefer in terms of fish testing.

So, there's some real biological challenges in terms of developing assay systems and assay designs that would accommodate what...for people who work with rats, a small fish maybe is a small fish, but there are some pretty unique differences with regards to their biology in terms of coming up with a...an approach that would enable you to collect robust endpoints.

And...and after some...some amount of testing, it became clear that if we wanted to move ahead quick...quickly, there were a couple of very robust endpoints, and, basically, most of the species that could be used for endocrine screening, and that was induction of vitellogenin in...in males which is a pretty specific and a pretty sensitive indicator of endogenous estrogens, and then also the production by androgens of male secondary sex characteristics in females, another robust, quite sensitive endpoint.

And so, the...the decision on the OECD side was that these were good endpoints to proceed

with.

The question was asked yesterday whether the 21-day assay, the screening assay as described for the EPA exercise, the ESP, and the OECD version of the test would produce the same indicators, and the response was yes. But I think the devil is in the details here. It's yes, sort of.

For very strong chemicals, that's certainly the case, that's certainly the case. If you're using estrogen, it doesn't really matter whether you use a 21-day design that is being used by OECD or the 21-day design that's being used by EPA. You produce vitellogenin and the same with trenbolone or methyltestosterone, a strong androgen. They will produce...you'll masculinize the females.

But you will miss a number of chemicals...a number of modes of action by using the design that OECD is currently looking at. What's happening with that design at present is that you don't consider fecundity in the assay, and you don't consider histopathology.

In not considering fecundity, the actual biology of the test is set up in such a manner that it's not optimized for reproduction in the animals, and that has some consequences when you get to testing the

weak chemicals.

For example, in the case of the fathead minnow, if you run a 21-day test using the OECD design, because it's not optimized for reproduction, what...what you have is a mixture of an even number of males and even number of females. These are group spawning animals that need a particular design if they're going to be affected in terms of reproduction.

So, what you're doing with the OECD design is essentially putting them into a test where you're disrupting endocrine function because of the nature of the test. And so, what you're going to do is you're going to miss subtle changes.

A couple of examples that I think are relevant to what Earl presented would include aromatase inhibitors and weak aromatase inhibitors. If you have reproductively active females that normally produce vitellogenin and if you depress steroid synthesis through any of a number of mechanisms, including depression...depressing aromatase, what you do is essentially decrease vitellogenin levels. It's pretty diagnostic. If you don't have the E2 layer to stimulate the estrogen receptor, you're going to depress VTG.

Now, what happens when you use the OECD

design is because the females aren't reproducing naturally, you'll increase, basically, the variability of that endpoint to such an extent that you can't detect the decrease. You have some females that have very high VTG levels, because they can't dump it into the eggs. Others are actually undergoing gonadal atresia, so they have very low VTG levels.

So, as a net result, if you use the OECD design where, basically, you aren't optimizing for fecundity, you would...you would miss a chemical like fenarimol, quite possibly prochloraz.

Another example, since the OECD design doesn't incorporate histology, you would miss a chemical like ketoconazole as well where, in a functioning reproductively active system, for example, in males, you'll see testicular changes that are consistent with the males trying to compensate for the depressed testosterone biosynthesis.

So, those are just a couple of examples where, although a 21-day fish test may look very similar on the surface, there really are some important differences, and I think that's one of the reasons, one of the critical reasons, why the slide that Les put up earlier that...that talked about why coming back to include these other endpoints is...is pretty important

in the overall scheme of things. And...and I think a lot of the folks involved in the OECD process, other countries, by now are...recognize this based on some recent peer reviews there.

So, that's all I wanted to say about that.

DR. HEERINGA: Thank you, Dr. Ankley. Any questions for Dr. Ankley? Do you have something on...

DR. ANKLEY: I apologize for...

DR. HEERINGA: No, that's...that's okay. This has been very useful, and, again, it's somewhat of an iterative process here, and there's been an excellent exchange of information, and I appreciate the way it's all been handled.

Earlier...Dr. Portier has just reminded me, too. Before we move on to the charge questions, I'm going to give each of the panel members a chance. Is there anything that, having thought about the proceedings of yesterday, last evening, that raise any additional questions that you would like to pose to the EPA scientific group? Yes, Dr. Zoeller and then Dr. Chambers.

DR. ZOELLER: So, this is kind of a general question. When I think about Tier 1, there

are...there are things that come before, and there are things that go after. And so, in order to...and I guess this, in part, goes to the concept of weight of evidence, but...but this...first of all, in concept, I remember at the end of EDSTAC, there was a lot of debate about how to prioritize chemicals.

Can we go into how these chemicals might be prioritized? To what...to what extent will previous information...there are some chemicals about which we know a lot. There are others about which we know almost nothing. What...what kind of front loading is occurring before it hits the tier?

And, secondly, once it goes through the tier, especially from the thyroid point of view, because there are very few...certainly, thyroid endpoints are not captured in the same degree to which estrogen and androgen endpoints in the...in the proposed tier are being reviewed, are being captured here. So...so, how is weight of evidence going to be used for that?

DR. TIMM: As you know from what we said yesterday, the...the 73 chemicals really have been put on the list with...without any review of existing information at all, and we...we have thought about doing so.

We looked...we conducted, actually, a pilot exercise looking at about 30 pesticide chemicals, and we found that as you got into really old information, that it...that it was not very helpful. And so, we...we went ahead and...and since we did not have the high throughput screens optimized to help us select, as EDSTAC had recommended, we just went ahead with the exposure approach.

Clearly, before...before testing begins, we will look at the information. People will...will be given the opportunities. One of the responses to the...the test order is to...to submit data that they believe satisfy the...the requirements. So, that's...that's one opportunity for...for looking at...at...at data.

In the future, however, there are a number of...of efforts going on. There's the Computox program where they're developing QSARs which would be helpful as priority setting tools, and we have not really thought much...we've been busy. We have not really thought much beyond the 73, and as you know, one of the things that before we plow ahead with...with the program, we...we will reflect upon what we have learned.

So, it's really premature to get into

speculating very much on...on how that will...will take place. The one thing we did say to...to people is that gee, we really wish you would have taken into account hazard information, toxicity information, in making your selections. We said clearly, we recognize that there is the desire to do that, and that would be done in the future, so we won't have a pure exposure list, in all probability, in the future. There will be other ways to...to prioritize.

DR. HEERINGA: Dr. Chambers?

DR. CHAMBERS: Two questions. I think one of them is a follow-on to Dr. Zoeller's. I'm still a little confused about the weight of evidence. So, are you saying that if...if there is existing information or QSARs predictions and all that exist before the screen is initiated, then that data could supply some of these lines of tests?

DR. TIMM: Q...QSAR information, I think, is proprietary. I don't see QSAR information, at this stage of the game, really substituting for any...any test results, but QSAR information would be useful for...for prioritizing in the future.

I think, though, we're looking at functionally equivalent information for...we obviously do not want people to repeat tests where...if it

already exists. I think that is a clear principle that the statute indicates, that...a principle EPA has...has embraced, and it's certainly one of the recommendations of EDSTAC.

DR. CHAMBERS: The other question is the bioassay that was presented yesterday, Lumey cell, was that considered, and if so, why was it rejected?

DR. TIMM: The Lumey cell assay was...was not considered and not rejected. It's...it's an ongoing exercise. I...I understand from the status of things that there...a lot of the...I think all the prevalidation work is...and correct me if I'm wrong in this...prevalidation work's been done. They're...they're going into the interlaboratory work on that.

But I believe that it is about a year away before they will complete that process and go through peer review. So, obviously, it's coming along too late, but that's where it would hook into our initiative with OECD to develop a generic test guideline for...for these kinds of assays.

And a generic guideline gets around a number of problems. One thing we...we really...OECD will not, regardless of the U.S. position, OECD will not allow a proprietary system to be required, so there

needs to be a generic approach with a proprietary system which would mean, basically, it's a performance-based test guideline, and so, probably using data from similar systems, the Staray system in Japan, the Lumey system, the assays that we've developed.

What we are going to propose is that an expert group get together and use those data to try to develop a performance-based test guideline that we could use the model for...for future such work.

DR. HEERINGA: Dr. Denver?

DR. DENVER: Yesterday, there was a lot of discussion of specificity and, you know, general effects, general toxicant effects, and clearly, the...the assays can...are intended to identify modes of action that are endocrine in nature, but it seems that they are...they're also going to identify, as was stated this morning, reproductive toxicants or general toxicants. And I'm curious how the EPA responds to those criticisms and whether it is, in fact, an intended goal to identify these more general toxicants or if it is an acceptable goal.

DR. TOUART: I get the palmer. This is Les Touart. I can provide a little bit of a response and...and Gary may have some...some follow-on.

But I think that the context...and,

again, the recommendations are that the...the EDSTAC were that...that we should stress more sensitivity than specificity. I mean, specificity is fine. It...it assists, but the...the goal of the Tier 1 isn't to confirm a mechanism of action. It's to be able to detect, you know, the potential for, you know, mechanisms to...to be involved.

In some cases, the more summary or apical, you know, endpoints would be downstream of...of other endocrine potentialities, but, you know, there are possibilities that...that these could be affected by...by non-endocrine, you know, bases, but some of these summary endpoints, in and of themselves, you know, on growth, development, or reproduction in particular, you know, these are endpoints of concern, you know, to the Agency. So, an assay, especially an in vivo assay that identifies that as a potential effect, whether it's endocrine or non-endocrine, we need to evaluate it in a longer-term more definitive study to understand, you know, what the adverse consequence is, but the more definitive endpoints or variety of endpoints will help understand whether it's really operating through endocrine or non-endocrine type mechanisms.

And...and part of the feedback on weight

of evidence...and I think Earl pointed to this fact in kind of just his little talk earlier...is the context of the assays themselves in play and the endpoints, you know, in play so that...that it helps us to kind of understand whether the...the strength of the information leads us to a conclusion that...that it's more likely an endocrine-active material than it's not, but we want to make sure that if...if an endocrine, you know, activity is present, that we have assays in place that would be able to detect, you know, that, you know, activity.

Whether we capture a couple of other things, you know, these are, you know, are true but a concept of what's really a false positive. If we have a positive, you know, reproductive active material or a developmental active, you know, material, you know, that's a positive, and it would be positive in the long-term, you know, test.

It just may be positive for other reasons, and that would be clarified in the Tier 2, and it's only after we've completed the Tier 2 would the Agency be in a position to say, you know, this compound is determined to be, you know, endocrine disruptive, you know, in...in nature, and so, it would be, you know, identified in...in that kind of context, but the

adverse consequence would be what the Agency would utilize in terms of risk management or risk assessment practice.

DR. HEERINGA: Dr. Delclos?

DR. DELCLOS: I just have one question. I guess about a legal definition. I may be the only person confused here, but representing assays as validated...and some of the public commentors are saying these assays were not validated...for instance, if you did not have a...a demonstration of a chemical which you would expect to be negative and it's not demonstrated to be negative in these pubertal assays, could you go forward with that program in August as you, as you plan, or do you have to stop and...and do that? Is that a legal requirement for the validation?

DR. TIMM: I think it's...it's clearly necessary to show that...and we wouldn't want everything to...to light up positive in the assays. I mean, as somebody mentioned the other day, you don't have an effective surrogate if everything's going to go through it. We don't think that that's the case.

Now, whether others are as convinced as we by the fact that when you look at the...the...some of the other modes of action and you find that you clearly have thyroid active chemicals, you clearly have

estrogen active and you have androgen active and they don't...they don't act by the other modalities, if...some people, obviously, are not persuaded. Some people would like to...and we would like to, actually...have had a clear negative. We...we...we didn't choose well.

I don't think that that means there isn't one out there. It just means we...we didn't make a very good choice.

So, we would...we would...we're now reflecting upon the peer review comments...we...we certainly may amend it...initial cut of peer review comments, but I suspect that...that...we're pretty convinced that we do understand how these assays work. I think that's the real test of validation, is do you understand the performance of the assay, the limitations of the assay, the trends of the assay.

DR. HEERINGA: Dr. Brown, do you have a question?

DR. BROWN: I had a question, I guess, related to the transferability of these various tests, and I...you know, I sit here, and I listen to Earl and Gary who are certainly experts, I mean, at the top of the field in these areas, and I wond...and...and I...I...I hear them expressing, you know, some little

thing that they found here or there, like Earl's Sertoli cell toxicity and Gary's concern about some of the...the fish reproduction assays. I just wonder whether, you know, the endpoints that we're really concerned about here are really going to be transferable from laboratories like theirs to the more routine laboratories that might be conducting these tests.

DR. GRAY: Let me...let me talk first to the Hershberger, because I...and Gary Timm was involved in the OECD validation of that. I mean, that's a...that's an old and fairly simple assay, and...and all of the endpoints are organ weights, and the only one that was really new to the tox community in general was the leather anions. So, I think there was no difficulty in transferring that technology to 17 or 18 laboratories.

I think, in the pubertal assays, there is not much new for endpoints in those. The assays, as they are constructed, are...are new, but the...even the vaginal opening and preputial separation are part of the 1998 multi-generational reproduction guidelines. So, I think the...some of the...much of those endpoints, there wouldn't be any difficulty in transferring.

I think you do run into different...different problems with some of the in vitro assays, clearly, and there's where we talk about having very strict performance criteria to make sure that a laboratory can run the assay and sort of self-validate before they run it, so we can validate an in vitro assay in ten laboratories and...and give it to somebody and they just can't do it. So, we...I think there needs to be that.

You want to talk about something?

DR. ANKLEY: Yeah, I think that's a...a very fair question, and in the field of ecotoxicology, primarily what's been used over the years are whole organism tests and whole organism endpoints, and so, it's not a lot of concern to me whether a lab's going to be capable of rearing frogs or fish and doing an exposure, but some of the endpoints are endpoints that have traditionally not been used in the types of consulting situations any way that these sorts of tests would be used in.

A good example here is vitellogenin measurement which is typically done within ELIZA, and ten years ago when...ten years seems like a long time now, but when we first started all this, there weren't a lot of labs that were really proficient in measuring,

for example, vitellogenin in the fathead minnow. Now, over that time, commercial kits have become available. People have become familiar, more familiar, with the concepts of ELIZAs, and labs, a lot more labs now, can do that.

And so, there's going to be a learning curve for some of these endpoints just because the assays, by the nature of looking past apical endpoints to some of the more mechanistic responses, a lot of labs haven't been familiar with these, but in the case of vitellogenin, for example, there's been a fairly rapid evolution, and, you know, quite frankly, one of the reasons for that is people see the potential to profit from doing this, and so, it's really spurred the competition, for example, to develop commercial ELIZA kits. There really wasn't anything years ago, and there's three or four now that could be used.

DR. GRAY: Let me...let me comment on what I said a little bit. I mean, I'm not...I'm not a specialist in the thyroid, so I don't think of that. I think those are...in the assays, those are probably the newer endpoints, and some of them are more difficult than others, and there just...there will be need...need to be more guidance there, I think, but I think that the people like Tammy or Susan who have worked on this

or Tom Zoeller could address the difficulty in transferring those endpoints.

But I wanted to clarify what I said, mainly on TS agents not routinely measured, and the histo...thyroid histo path is going to require some help.

DR. HEERINGA: Dr. Denver?

DR. DENVER: You know, a very important endocrine axis that has not been mentioned here is the hypothalamic-pituitary-adrenal axis and corticosteroids, and I'm just curious why and whether...I mean, you must have thought about these...these hormones and...and the fact that they could cause or influence many of the endpoints in these assays if they are disrupted.

Is there any thought among the EPA of including down-the-road analyses of corticosteroids in...in these assays, levels of stress that are...I mean, many of these toxicants can alter, you know, activity of the stress axis that could then lead to many of the effects that we see in the assay.

DR. HEERINGA: Dr. Touart?

DR. TOUART: This is Les Touart. I'll try to respond a...a little bit.

I think yeah, there have been

considerations in terms of what other assays and...and things to have considered, but we were, you know, following more of the...the EDSTAC recommendations in terms of...of considerations that they have, and, again, at the time, it was felt that estrogens, androgens, thyroids have the...the broader availability of...of assays and specific, you know, endpoints that would be, you know, relevant. And so, we kind of focused more on them in terms of identifying assays that would work and in going through validation, you know, processes.

In context of the in vivo apical assays, you know, clearly, HPA would be a component of the pubertals, you know, fish and even in frogs in terms of...of the axis, you know, being there, how much it would contribute. We just don't have specific endpoints that are...that are also being measured.

In terms of something like the fish assay, there's a limitation in...in the number of things that one might be able to look at in terms of sera. You know, you might be able to...like vitellogenin, you may be able to do, you know, an androgen or an estrogen in...in the sera, but to add other component, you know, parts would be difficult, for instance, if your...your sample would have been

completely utilized.

So, those are, you know, considerations. I think if...if there are some endpoints to...to be considered and if these could be done without disrupting the, you know, other core endpoints, I think we'd be interested in...in those, and there may be technologies that exist that others might be aware of of some of these.

DR. GRAY: You know, adrenal weights are included in these, and that seems rather primitive, but they are actually remarkably responsive to some of these chemicals, actually, quite a few of these chemicals, and...like ketoconazole tripled adrenal weight in the male and female in the 20 and 30 ages based study without any obvious toxicity in the animals.

The fungicides like vinclozolin increase adrenal weights, and I think the modes of action of those are completely undefined and definitely not the same. So, I think it's...it's interesting.

DR. DENVER: I mean, measuring plasma cortisol is a fairly straightforward thing to do that could potentially be included in these assays, but I...actually, I was more interested in whether these effects were being recognized or, at least, appreciated

and then considered down the road, you know, beyond August, 2008, because I think it's...it's not just another endocrine axis. It's a very central, critical axis that could influence all of these other endpoints, you know.

DR. HEERINGA: At this point, I...oh, Mr. Gray...Dr. Gray?

DR. GRAY: I agree, and it's unexplored, and I think there's a fairly large database on things that affect adrenal, but it wasn't...it hasn't been...it's not part of the program now, as are many other endocrine modes of action that may be very important.

DR. HEERINGA: Okay. At this point, I'd like to take a 15-minute break, and when we return, we will turn to the charge questions, the first charge question. At 10:25, we'll reconvene.

(**WHEREUPON**, a brief recess was taken.)

DR. HEERINGA: Okay, welcome back, everybody, to the second half of our second day morning session on the meeting of the FIFRA Science Advisory Panel on the topic of the Endocrine Disruptor Screening Program proposed Tier 1 Screening Battery.

At this point, we have gone through our series of presentations, had clarifying questions, and

also heard public comment, and we are about to enter the period where the panel will formally respond to the charge questions posed to it. So, I'd like to ask Dr. Touart to...to read the first charge question into the record, and I think Dr. Belcher is going to read all of the subpoints, but then, we can organize our response point by point.

DR. TOUART: Okay, I thank the chair. And the first charge question directed to the panel is: Please comment on the ability of the proposed Tier 1 Screening Batter to provide sufficient information to determine whether or not a substance potentially interacts with the estrogen, androgen, and thyroid hormonal systems based on the modes of action covered within the battery.

And we have seven modalities that are...that are listed in subsets. I don't know if you want me to read those, too, or...

DR. HEERINGA: You just read the modalities, yes. I don't think you have to read the descriptive part.

DR. TOUART: Okay. The modalities being estrogenicity, anti-estrogenicity, the androgenicity, anti-androgenicity, steroidogenesis effects, the hypothalamic-pituitary-gonadal effects, and

hypothalamic-pituitary-thyroid effects.

DR. HEERINGA: Our lead discussant on this will be Dr. Belcher, and I think that you've indicated that you would like to have a discussion sort of mode of action.

DR. BELCHER: Generally, what I'd like to do is to take each of the sub-components, the mode of actions, and address each of those in order as listed. The first mode of action will be estrogenicity, and there are five assays proposed that address estrogenicity. They include the ER binding assay, the human ER alpha transcriptional activation assay, the uterotrophic assay, the pubertal female assay, and the fish screen.

At this point, based on the diverse amount of data that goes into each one of these, I would like to open up for comment to the SAP to carry on and discuss each of these mode of actions individually and how they fit into the battery to address estrogenicity.

DR. HEERINGA: I think what I would like to do is to go through the associate discussants for their specific. Do you have anything yourself that you would like to...

DR. BELCHER: General comments based on

this is that there is strength in the multiple assays that inform on estrogenicity. Many of these assays are quite robust and reproducible.

One of the major comments that I might want to come back more on is the potential for ER beta in playing a role. As a major role, there is focus on ER alpha-mediated mechanisms, and the potential for the combined effects of ER alpha and ER beta to produce false negatives in this assay is apparent to me, and if...it's difficult to see how, with the important in vivo assays, how this may be resolved in a general...as a general comment.

DR. HEERINGA: Dr. Denver, associate discussant, your comments on the estrogenicity mode of action charge question.

DR. DENVER: Well, clearly, this...this mode of action is, I think, best addressed by the...by the Tier 1 screen, and this could be, in part, due to the historical reasons, that is, estrogenic compounds in the environment were recognized very early, and so, there was a lot of focus placed on means to detect them.

I think that the...there are five different assays, and each one...each of the assays has their strengths and their, you know, their weaknesses,

but taken together, they provide a very overall powerful test of the hypothesis that a compound has an estrogenic action.

So...and I would also add that, you know, even the amphibian metamorphosis assay would potentially find an estrogenic compound, although it's not designed to do that, because estrogens can, in fact, slow or block metamorphosis.

So, those are all general comments. I'm going to defer to the other panel members who are more expert on estrogenic modes of action to comment specifically on the assays.

DR. HEERINGA: Dr. Delclos is the next associate discussant on estrogen...estrogenicity as a mode of action.

DR. DELCLOS: Barry Delclos. I agree with what Bob just said in terms of this being probably the strongest of endpoints. There's certainly a very good strength in the battery for detecting ER alpha nuclear receptor mediated effects with the uterotrophic assay, the binding assay very strong. The other assays are comparable to that.

I think the one case...I don't know if maybe Scott, later in his comments, will discuss what might be appropriate in the in vivo assays for picking

up the ER beta ag...agonist, but there's also...there could be a number of effects, other effects, and I think this would be argument for...there's certainly arguments against including apical endpoints.

I mean, apical...inclusion of apical endpoints really has to be done in order to pick up some of these other mechanisms that might...that we know there are and may come up later. It's going to be difficult to...to adjust the battery to adapt to each new mechanism that might be identified in...in the molecular biology labs.

I have a few comments. There was some discussion with the uterotrophic assay here and the...and in the Hershberger later, there's a choice given between the route of administration. We discussed that a little bit, I think, yesterday, the questions, but I think it's...the EPA's approach of using, at least for compounds in which there is sparse information on metabolism and so forth, there...I agree with using the subcutaneous injection for...for one of the in vivo assays and oral for another would be valuable in those cases.

For example, in the bisphenol A case, there's clear, very clear difference in the...in the activity with route of administration, and so, while

using the...suggestion of using the relevant route to human exposure is a good one, any time...is necessary any time you're going to do a risk assessment, these data are not being used for risk assessment. They're being used to identify potential for action. So, I think that was a...a good point.

I really think that's about all I had to say at this point.

DR. HEERINGA: Dr. Cooke, the next discussant.

DR. COOKE: In terms of estrogenicity, we would pretty much agree that there's a sufficient number of tests to be able to say whether something was estrogenic or it wasn't estrogenic or maybe it's estrogenic for both the in vitro and the in vivo approaches. That's...that's a good thing in one aspect.

Maybe in a more general discussion, we can discuss whether you need all of those. It's designed to give you a maximum amount of information.

So, in common with the other people on our panel, that's pretty much all I would have to say.

DR. HEERINGA: Thank you very much, Dr. Cooke. I think the issue of complementarity and that potentially redundancy is...charge question 2 covers

that as well. So, we'll certainly get to that.

Dr. Furlow?

DR. FURLOW: Thank you. So, again, in thinking about these charge questions, often, I tend to drift into thinking about the second one in my comments, so I'll try to be careful about that.

DR. HEERINGA: Well, there's no need. I think we're just going to make sure that what...the point is that something gets covered, not necessarily when it gets covered.

DR. FURLOW: Absolutely, absolutely, because I was actually going to make a comment about VPA, for example.

Again I think, as Dr. Denver pointed out, the estrogen...estrogenicity assays are...are the most mature. They're the best validated, in...in my opinion. There's a combination of biochemical, molecular, genetic in terms of transactivation, although we haven't seen the validation yet. Right? That hasn't been fully completed, but...but I'm optimistic that will be a good online assay, and the uterotrophic assay is...is...is quite...is quite nice.

I'm also happy to see the fish screen included, not just because they're fish, but I think there are things that, in terms of the low background

of vitellogenin synthesis in the male is a particularly nice, sensitive screen.

The...the...the...well, the coverage and multiplicity of different assays in covering estrogenicity I do think is...is, in fact, also important. I was thinking in terms especially about bisphenol A. If you just looked...if you just, say, used the pubertal assay, you might say okay, it's a general toxicant, it's not an endocrine disrupter.

There has been...I was also concerned about...and this has been addressed a little bit about strains, but this is something maybe we can talk about in the next charge question, but I think that if the argument that's been made that Sprague-Dawley rats are, say, relatively insensitive to bisphenol A...this has been something that's been talked about a lot, actually...the fact that you have the fathead minnow assay, I think, helps.

You'd say, you know, by weight of evidence, you'd say okay, well, it didn't show up in the pubertal assay. That was a Sprague-Dawley. But you've got these other assays, estro receptor binding, transactivation, and the fathead minnow, to...to help cover that and...and something...but the, you know, extreme differences, I think, still is something that's

extremely important, should not be ignored by the EPA, but that my concerns are somewhat mollified by the redundancy and the different mode of actions incorporated in the estrogenicity assays.

So, I guess, those are my...those are my main comments. So, I'll stop there.

DR. HEERINGA: I'd like to turn now to other members of the panel on the estrogenicity of mode of action and the Tier 1 battery. Yes, Dr. Vandenbergh?

DR. VANDENBERGH: John Vandenbergh. I had some concern about the assay, the female puberty assay, in the sense that if one is looking for a measure of female puberty, the vaginal opening is, obviously, a good one, and the onset of first estrus is, but it goes on and measures cyclicity, and there are some real problems with...associated with that. Some have been brought up by the public comments that we've had, written and oral, that a lot of other factors can influence that cyclicity, the social conditions of the animal, nutrition, and on and on.

So, I just wonder how essential it is to...to do that part of it. You can get the puberty information by measuring vaginal opening and then smearing for a week or ten days, at most, and you

should get the first estrus cycle.

DR. HEERINGA: Other contributions from panel members on this mode of action? Again, we can return at any point in time if you have something else that comes up, but I think that covers. I'll turn back to Dr. Belcher. You want to wrap this up or move on to the next mode of action, please?

DR. BELCHER: If there's no other comments, we can go ahead and move forward to anti-estrogenicity. That is covered through the estrogen receptor binding assay, the human ER alpha transcriptional activation assay.

However, their using this as a reporter for inhibition has not been validated to this point. There is some information from the pubertal female and the fish screen. My feelings in...with this are...are in line what was presented by the EPA in the technical document, is that this is actually a rather weak...weak coverage of this mode of action, and, essentially, the strongest component is through the ER...the ER binding. However, there is no information beyond that a compound is a binder.

There are some...some potential information from the pubertal female assay and the fish screen. I'm not in a position to comment on how

increased vitellogenin in female is that robust of an endpoint for detecting these sorts of changes.

DR. HEERINGA: Dr. Denver?

DR. DENVER: I would just concur that this mode of action, the assays are not well developed to detect that, and that was stated by the EPA. And, obviously, we need to encourage further validation and development of the ability to detect that mode of action.

DR. HEERINGA: Dr. Delclos?

DR. DELCLOS: Well, I agree with what the previous commenters and with the EPA in saying that this is one of the weaker modes of action, really, in the battery. And, again, as with the...the estrogenicity assay, this focuses more on...specifically on ER alpha, and at the other end, at the binding assay, I'm...I'm not competent to comment on fish assays at all, but other than the ER binding, I think that the...there's weak coverage.

I was wondering if it could be considered to...to the relevance in value with the uterotrophic assay, consider adding in, the anti-estrogen component of that into that battery to...to complete things. That's all I had to say.

DR. HEERINGA: Dr. Cooke?

DR. COOKE: I don't really have anything to add to them.

DR. HEERINGA: Dr. Furlow?

DR. FURLOW: Yes, I want to actually amplify on Dr. Delclos' comments. I do think that there...there can be robustness in the uterotrophic assay and some of the transactivation assay in terms of developing and validating an anti-estrogen screen. I think...I think there's real potential there, and we've talked about how that has been done, and if it...if it's validated to EPA's satisfaction, I think those can be very powerful assays.

Vitellogenesis in the fish is the issue. One issue is that yes, it's a quite specific to estradiol or estrogens in the fish, and so, it can interfere with that by making aromatase or something but also anti-estrogenic activity at the receptor can actually serve as a...as a good assay for anti-estrogenicity.

That said, interference with thyroid hormone actually, you know, also interferes with vitellogenesis by unknown mechanisms, but you need thyroid hormone, adequate levels of thyroid hormone in order for estrogen to induce vitellogenesis. So, if we're...if we're concerns about the precise mechanism

of action, there may be some softness there anyway in the...in the fish vitellogenin assay, although I still, again, think that can be a good assay for estrogenicity...anti-estrogenicity, but, again, that's another opportunity. If you have the male fish, give a low dose of estradiol, and then introduce a battery of...screen for various anti-estrogens

Again, in parallel with the transactivation assay and the uterotrophic assay, I think you can develop a good validated anti-estrogenic assays, but I...but I...the discussion in the presentation made it feel like we're really not there yet, but we should get there, and I think we can get there.

Just one other point. The...the point has been made about the pubertal assay, then, is serving as one of the endpoints, and I...I guess I...I'm more comfortable with accelerated vaginal opening as an estrogenic endpoint.

With delayed vaginal opening, I'm less comfortable with as an in vivo anti-estrogenic endpoint just due to the specificity of the assay that's been brought up by a number of speakers, that you can delay and interfere with the activation of the HPG axis in a number of different ways. So, as a...as an anti-estrogenic assay per se, I don't...I don't think it

serves that purpose.

DR. HEERINGA: Thank you very much, Dr. Furlow. Comments from any of the other panel members on the anti-estrogen? Dr. Brown?

DR. BROWN: Well, I may be speaking a little bit from my bias as a research bias, but...

SPEAKER: That's appropriate.

DR. BROWN: It just seems to me that the maximum effort and the maximum gain would be from developing the estrogen receptor transcriptional activation assay as...as kind of the premier or the primary in vitro assay. And I say that because it's a system where I think you have much more control over the conditions, and it's also a...it's an assay that even though it's not indicated for antagonist screening, it has...obviously, has the potential to screen out...to screen for antagonists also. And it also can measure estrogen receptor binding for these agents.

So, it has the potential both for measuring estrogen binding to the estrogen receptor and, in this case, the estrogen receptor alpha, but it also has the ability to...to look at...at activation of...of...of...at a target gene. And the potential there is also that it could be modulating gene

expression through other means in addition just to direct activation through the estrogen receptor genomic traditional DNA binding modalities.

So, I'd like to kind of put in a plug for...for that assay, you know, rising up the...up the level of...of priorities, and, I mean, it also then addresses a number of things that came up yesterday, and that is, you know, it takes care of the three Rs, too.

And there's also the aspect of developing an assay that could be high throughput, in a way. Also could be developed potentially as a non-radioactive assay that does away with the...the radioactive waste and...and exposure potential, too.

So, I think I'd like to see that kind of put up as a higher priority maybe.

DR. LASLEY: Yeah, I'd like to amplify on that point. Clearly, the transduction assay is already...has already been shown to be useful in discovery in finding new types of endocrine disruptors. So, I think this is not only a good screening assay for what we know, I think it's going to be a good assay for what we want to learn.

And in addition to that, I think it has promise across some of the other categories of...of

endocrine disruption. I can see that thyroid and other hormones would benefit from...from this technology. So, I think that's one that will satisfy a lot of needs.

DR. HEERINGA: Thank you, Dr. Lasley. Dr. Bucher?

DR. BUCHER: Yeah, I would agree with...with those comments, but I think you have to remember that it goes in tandem with the problem of metabolism. So, one might want to consider, in some instances, when data come in from Tier 1 screenings that don't make sense that, in that regard, there be some thought given to testing major metabolites in...in these in vitro assays.

DR. HEERINGA: Yes, Dr. Kullman?

DR. KULLMAN: I want to go back to vitellogenin for a second, as I think it's well agreed that vitellogenin induction is a...an excellent mechanism for looking at estrogenicity. Vitellogenin or lack of induction or a decrease in vitellogenin has some inherent problems when looking at generalized toxicity. Certainly, a compound that is a general anti-toxicant will significantly reduce vitellogenin activity.

And so, it's like, you know, it may be a

decent indicant. I don't know that it's as robust as the vitellogenin.

DR. HEERINGA: Yes, Dr. Eldridge?

DR. ELDRIDGE: Yes, at the risk of sounding over redundant, the endocrine system is, by definition, a signaling system. It's...it's endogenous chemicals that make things happen.

So, looking for antagonism of that using a generalized in vivo model is inherently much more difficult than looking for an appearance of an effect. So, it...it puts much further emphasis on having specific kinds of tests looking for an antagonist, for a true antagonist of a system, and it's...there's a lot of risk of what we might call false positives from a chemical causing something to disappear which is completely unrelated to the hormone action.

DR. HEERINGA: Thank you, Dr. Eldridge. What I'd like to do, since we have general comments from the panel on both estrogenic and anti-estrogenic activities is maybe turn to Gary Timm and Dr. Touart here to see if...if there are any confusions or clarifications, anything that comes to your mind from the panel's comments. There's no need, but if you feel satisfied with what you've heard, then...

DR. TIMM: Yeah, I've been taking notes

vigorously, and I think I understand.

DR. HEERINGA: So, I think we'll try to check back, because, obviously, we want to make sure that you feel comfortable that you understand what you heard, whether you agree with it or not. I guess that's...so, okay, let's...let's move on.

Dr. Belcher?

DR. BELCHER: Moving on to the androgenicity mode of action there are one in vitro assay, the AR binding assay from rat prostate cytosol, the Hershberger assay, the pubertal male, and the fish assay.

In general, my comments are limited to that the AR...the AR binding study is, again, a reasonable and classic approach to finding...binding. The Hershberger assay is...was quite impressive and...and is an important assay for this component of it.

And, in general, my feelings were that this was a...a...had good predictive abilities, and I thought that the pubertal male assay with the oral administration and the...the ability to discern metabolic activation was also a good component of this.

DR. HEERINGA: Dr. Denver?

DR. DENVER: So, I think that this is

the second most robust or complete set of assays following the estrogenicity assay, and among these assays, the...the androgen receptor binding assay is probably one of the best developed of...of the assays in the tier, although it does...it does have some issues. It can't distinguish between agonist and antagonist, but that was recognized.

There was also issues related to the performance of the assay, that is, the generation of rat ventral prostate cytosol among different labs, and there was a lot of variability there, and so, that was a concern among reviewers, and the thing I would say to that is I would recommend considering, at least, moving to a recombinant AR binding assay as soon as possible to avoid some of those issues.

The Hershberger assay is strong, reliable. It's a validated assay. It's been around for a long time. It can detect AR-dependent processes.

It may be worthwhile to develop an AR transactivation assay to...to compliment this to...to round out the set of assays, at least in the future, similar to the estrogenicity assays.

The fish short-term reproduction assay, as an in vitro assay, is a...is a strong assay in terms of male secondary sex characteristics. Those are

robust endpoints that can be monitored readily.

And, finally, the pubertal male assay is...is necessary. An in vivo rodent assay is a necessary assay to detect androgenicity. However, I...I don't think I'm the one to comment on whether it's a better assay than the adult male assay, and so, I'd like to hear comments from my colleagues on the SAP on that matter.

DR. HEERINGA: Dr. Delclos?

DR. DELCLOS: In general, I agree with the previous comments. It's a strong...strong coverage of the AR receptor binding, and the Hershberger assay also, with the...as with the estrogen receptor, a transactivation assay might be useful.

I...I think, for this particular endpoint, I don't know that the pubertal male is a...has the strength relative to the Hershberger assay. I might be wrong, but I think the pubertal male still has...has advantages down the road for the HPG axis, certainly.

DR. HEERINGA: Dr. Cooke?

DR. COOKE: Yes, the...it's the opinion of most that development of a transactivation assay for the androgen receptor would...would be beneficial. In terms of answering the...the question, does the rat

prostate cytosol test tell you whether it's likely to interact, then I guess it does. It's just probably not the best system to use.

In terms of the others, obviously, the Hershberger, the animals are castrated so in...in terms of telling you from the point of view of potentiating androgen action, and maybe it comes up into...into the next one more so.

Pubertal male, that should...that should give you some indications of potentiation with respect to preputial separation, and the fish reproduction, I would defer to Dr. Furlow. He's much better acquainted with that system than I am.

DR. HEERINGA: Dr. Furlow?

DR. FURLOW: Well, I've seen fish, but I haven't worked with fish, so I...I teach about fish, remarkably even though I don't...I don't work with them.

So, again, while not, I think, quite up to the standard of the estrogen ass...estrogenicity assays, androgenicity assays have some value. I would echo the recommendation to get away from the rat ventral prostate cytosol and to get to a recombinant system quickly, because that...again, that would spare animals when I think we...we do need animals for the in

vivo tests. If you can get away from them in the beginning, I think that would actually be quite beneficial but also much more reproducible.

Transactivation assays, I, again, I think they would help quite a bit, almost more, maybe, for the anti-androgenicity than the androgenicity, in a sense, because the Hershberger assay does appear to be quite robust. Despite having to cut off the tissues I don't get to see very often and...and weighing them, that's the assay. It's remarkably robust.

And regarding the secondary sex characteristics in fish, I also think has an impact, quite...quite additive value.

The...again, the pubertal male, accelerating puberty, I guess, has more value than delaying it in the sense of general toxicity. You can be a little bit more...a little bit more certain about something being an androgen if it accelerates some of the pubertal endpoints, but, again, that's a...that's a tougher deal.

I think in terms of just essentially raw androgenicity, if you will, the binding transactivation, if it can be developed...and I would urge that the Hershberger and the fish have...have good coverage.

The question was just brought up about potentiation. I do think that's an issue, and the pubertal male could help you with that or the 15-day male could help you with uncovering hormones that could potentiate the actions of androgen...of androgens. That's something that is becoming much more apparent. Maybe Dr. Lasley can talk a little bit about that, too, but I guess in...well, we'll talk about the pubertal male and the pubertal female in a moment, but I...I think those are a little bit less well validated.

So, in general, just for straight androgenicity, I think sticking with the binding and developing transactivation, the Hershberger, and the fish assays are actually quite...quite useful.

DR. HEERINGA: Okay, at this point, I'd like to open it up to other panel members who'd like to contribute on the androgen...androgenicity. Yes, Dr. Lasley?

DR. LASLEY: I...I think the transactivational assay for the androgens, particularly for the anti-androgens, is...is not only feasible, I think that we really should talk about it in terms of validation, because there's at least four or five stable transvective cell lines that are in the literature that work, and I think every...everybody

appreciates that...that those are on line in a number of labs and...and could be validated and probably will be validated, and that will fill an important niche in...in this area of screening.

DR. HEERINGA: Dr. Lasley, I would...not to do any advertising, but we will cite...can we cite those examples in our minutes? Dr. Vandenbergh, yes.

DR. VANDENBERGH: Maybe I'll just comment a minute about the male pubertal assay in comparison to the 15-day adult. It does concern me that the only developmental period that we're dealing with in this is puberty. We're not dealing with the intrauterine or the early post-natal, because that's been eliminated by another study, unfortunately.

And so, I think it is important to maintain that. The other thing is the 15-day male doesn't tell you about female puberty, and so, you would still have to do the female puberty, and it would seem much more reasonable to, if you're going to study puberty, to study it in two sexes. I mean, both of them need to go through that experience. So, we need to stay with that.

DR. HEERINGA: Thank you, Dr. Vandenbergh. Additional comments on androgenicity? Yes, Dr. Cooke?

DR. COOKE: In...in response to Dr. Lasley's comment about the different labs, we, in fact, produced one several years ago. Please don't include it in the list. It doesn't work anymore.

DR. HEERINGA: Obviously, not a self-promoter. Yes, Dr. Brown?

DR. BROWN: Yeah, I'll speak to that transactivational assay, the in vitro assay, too. I think there's too little appreciation, probably, for the intricacy of handling the rat prostate cytosol preparations.

I mean, I think it was partially demonstrated in the validation proc...peer review that was done, but the prostate is nothing but a bag of enzymes, proteolytic enzymes. When you homogenize that tissue and try to make a prep and stable...stabilize the androgen receptor in that prep, you find all kinds of things that can affect it, pH, buffer, temperature specifically...particularly, and a number of other things. It's a very crude preparation, and it really needs to be handled with a lot of care and a lot of knowledge of what the...that the potential problems are in handling it.

And I think that led to some of the...the validation issues in...in the peer review, is

that, you know, for instance, one of the labs that was commissioned to do this couldn't make a...a viable rat prostate cytosol prep, and other labs, I think, had probably issues in relation to the stability of it, and it's an overnight incubation assay which isn't really a nice, quick assay which you can actually do in these transactivation systems.

You can do those relatively quickly, and you can also measure androgen receptor binding in those. You have a lot more control over the system, I think.

DR. HEERINGA: Thank you, Dr. Brown. Dr. Belcher, want to wrap up or move on to anti-androgenicity?

DR. BELCHER: I have no further comments on it, so moving on to anti-androgenicity, it is informed on the...the same four assays, the AR binding assay, the Hershberger assay, the pubertal male, and the fish screen.

To make a general comment about the binding assays and the transactivational assays, I would recommend that there would be additional effort of integration of the design and development of these lines. There are, in many cases, both for the ER and the AR assays, the ability to use similar initial cell

lines and to be swapping out using a standardized sort of...of cell lines, and if there could be future consideration and thought put into designing that, it may streamline the ability to look at different configurations and different variants of these receptors for...for the future.

The...in the anti-androgenicity, what has been brought up previously is there is a little bit of weakness because of, for example, looking in the pubertal male assay, looking at delays of puberty, and in the fish screen, the attenuation of male secondary characteristics was identified as being a rather weak endpoint, so there is some loss of the ability to detect effects relative to the...the androgenicity battery as a...as a whole.

DR. HEERINGA: Dr. Denver?

DR. DENVER: So, the same issues hold here regarding the andro...anti-estrogenicity assays, that is, the ability to identify antagonists, and that is a weakness in the...the overall panel of assays that we have.

So, the only thing I would add is to advocate for developing the AR transactivation assay's potential to screen better for antagonists.

Currently, I guess the...the best...the

best that we have is the...the AR binding where something is binding to the AR, because that could potentially be an antagonist. So, if that were then to move on to a transactivation assay, that might help to clarify that.

DR. HEERINGA: Dr. Delclos?

DR. DELCLOS: Well, I think this endpoint is covered a little better in the anti-estrogen series. I think the Hershberger is a very sensitive and effective measure, together with the androgen receptor binding and...and transactivation assay that's added eventually.

And also, there seems to be coverage with the pubertal male as well. So, I think this endpoint is...is better covered than the anti-estrogens.

DR. HEERINGA: Dr. Cooke?

DR. COOKE: Yeah, I haven't anything to add to it at this point.

DR. HEERINGA: Dr. Furlow?

DR. FURLOW: I would tag...echo again the transactivation assay being extremely useful for anti-androgenicity, and the use of the Hershberger for anti-androgenicity appears to have merit and, therefore, you know, the uterotrophic assay could be

used the same way for anti-estrogens.

Delays in the onset of puberty, et cetera, again, we'll...I will talk more about that in the HPG axis, I think, but as a...as a pure assay independently of other assays of anti-androgenicity, I don't think it has great value, but I think it's important to look at.

Other than that, I...I don't have further questions...further comments.

DR. HEERINGA: Additional comments from panel members on the anti-androgenicity? Dr. Lasley?

DR. LASLEY: Parading the transactivation assays as much as we are, I think it needs to be pointed out that although there are several in the literature, they're all slightly different in their format, and...and I think we...we have to be aware that these differences lead to performance differences, and before we jump on the train, I think we need to make sure we're headed to the right station in terms of which format we're going to choose, and this is something that, I think, needs to be considered early in the development and validation of these.

DR. HEERINGA: Maybe just to stimulate a little further discussion, with regard to both androgenicity and anti-androgenicity, several people,

Dr. Vandenbergh's touched on the pubertal assays and their potential role there. We touched on the fish assay with regard to androgenicity. Is there a little more discussion about the pubertal assays with regard to the anti-androgenicity mode of action or the fish assay? Something that you'd like to defer?

DR. FURLOW: I guess I would ask Dr. Vandenbergh about that, I mean, maybe...maybe in a greater discussion about the pubertal assay versus the adult male assay. So, in terms of anti-androgenicity, the 15-day male would tell you general things about how androgens are working, but if it...if it is true that the pubertal male, that...that period, pubertal period, is, in fact, more sensitive, then that is something we ought to...we ought to consider, and maybe as part of the limitations, et cetera, talk about whether or not the specificity is...is there.

But I guess...I guess related to that, something we didn't talk about in the estrogen assays as well...and maybe Dr. Vandenbergh can have some particular input on this...would be, say, behavioral endpoints, so organizational versus activational effects of...of estrogens or androgens. And these are not at all addressed in any of the assays.

And so, either in utero or peripubertal

exposure to these...to these hormones and maybe behavior effects is...could be something that they ought...that the EPA ought to consider. Maybe you can address that one.

DR. VANDENBERGH: Let me address the behavioral aspect first. I didn't bring it up, although I have talked to people at the EPA about it, because there are behavioral measures that are quite reliable. Lordosis in the rat, for example, is an excellent...is well worked out, and...but we're not here to add more procedures to the program. So, that's something that could be considered in the future.

The other thing about the behavioral is that they're truly an apical test, because behavior summarizes a whole lot of things that occurred before that time, and so, you can detect differences. The hard part is identifying which of those variables that we know affect behavior is the one that's important here, and you can control for those, but it's difficult. So, I didn't go into the behavior aspects.

Your other point about organizational versus activational effect is absolutely right. I don't see anything in here that really deals with the organizational at all. It's almost all activational.

That's why I said the only developmental

aspect that we have at hand here is the puberty, and we don't think of puberty so much or that period of puberty as being the time when act...when organizational events occur. It's usually much earlier, often in utero.

But there is evidence that, I think, there are differences in the response of the animals to testosterone when they're pre and early puberty than they are when they're old and gray like...like I am. So, there are changes over time, and that's why I think it's essential to keep at least some semblance of a developmental inquiry in this battery of tests.

DR. HEERINGA: And the role of the fish reproductive screen? Maybe Dr. Furlow can pick it up.

DR. FURLOW: That's fine, and he should. So, the...most of that's focused on the...on the secondary sexual characteristics, and I think...I think there is some value. I think, like the estrogen assays, though, if you have...it might be a cleaner system in some sense if we decide on how we want to do a transactivation assay and then titrate in inhibitors. The same principles, perhaps, ought to be employed in the fish or the Hershberger assays, and I think they are complimentary if you have either a pre-pubertal fish and add androgen and then take it away. I think

this may be a more controllable system to try to understand anti-androgens, say, than just taking the intact animal and then titrating and hoping something happens.

And...and so, that's...that's the idea with the Hershberger, is that you have a castrate system, and then you put in a little androgen and then try to...try to inhibit its action. That same principle, either in a...in a...in a mature fish or even a castrate fish might...might...might be useful as well.

Thank you.

DR. HEERINGA: Additional contributions on androgenicity or anti-androgenicity? Dr. Brown?

DR. BROWN: I'd just like to make a comment on the...on the assays of the hormones themselves with measuring testosterone, estradiol, LH, FSH, that are incorporated into some of these assays. I think this is really not necessarily a trivial matter, either.

I know we broached the subject yesterday of, you know, whether there should be a...a standardized lab that would conduct these tests or not, and, you know, obviously, that's not what you want to do, is condone, you know, a commercial lab to...to do

all these tests, but I think there's been a lot of discussion in the literature lately that all these kits that measure these...these various hormones are not created equal, and you've got a lot of variability depending upon which kit you use and, particularly, I think, in terms of the sensitivity of some of these...these kits in actually detecting the hormones.

And then, the other matter is, you know, measuring these hormones at a very distinct time of day under very controlled conditions, because stress adds a lot of influence to the...to these hormones in vivo.

In addition, they...they vary over the...over the diurnal variation of...of the daytime so that it's...it's critical to really make the measurement of these very much a standardized condition for the collection of the blood, it's processing, and then also in...in relation to the acts...actual assays that are used to...to perform the measurements.

DR. HEERINGA: Dr. Zoeller?

DR. ZOELLER: So, to follow up on that a little bit, it seems to me that clinical labs that do...that do radioamino assay or other hormone assays have, for a long time, figured out ways to standardize across labs across the country, and there are...there are a lot of, I think, good ideas or good ways of

ensuring that a...a laboratory that's performing a specific radioamino assay or hormone measurement in one contract lab is getting the same answer that another contract lab might get.

And these assays have been around for 30 or 40 years, and ways of...you've got ways of standardizing results across clinical labs seems fairly well worked out, and those could easily be integrated into this program without compromising principles of making recommendations for specific kinds of assays, et cetera.

DR. HEERINGA: Maybe I can turn back to Gary Timm and Dr. Touart to see if you have any questions. Is there anything in this...yes, Dr. Touart?

DR. TOUART: Just on...a clarification just for your information purposes based upon some of the comments Dr. Furlow made in terms of the fish assay and anti-androgen...anti-androgenicity and some of the considerations that are going on in terms of how to improve or better address, if I could call Gary Ankley up and if he can take a little bit of time to talk about some of the efforts and how the assay is being adapted for that.

DR. HEERINGA: I think that's

appropriate at this point.

DR. ANKLEY: This is a great segue, because I'm doing...a paper I'm just finishing talks very much about an assay along the lines that you describe. Essentially, what you do is you take female fish. We've run trenbolone which is an androgen, quite latinal, so you can induce tubercles in fish, and what you can use...what you can do is block the production of tubercles in the fish by treating them concurrently with anti-androgens.

And so far, we've tested vinclozolin and flutamide with Soperton acetate in that system, and it's a very effective way of picking up anti-androgens.

Now, it's probably a...a little far off to try to imagine that would be ready for an August time frame, but just to follow on to your suggestion, we do...we are thinking very much along those lines and...and we do have some promising data that...that would enable the fish to...the fish system to actually handle that particular mode of action.

DR. HEERINGA: Additional? There will be opportunities later, too, but I just want to make sure that as we go through these modes of action systematically, there's a chance for you to make sure that if there's anything that's confusing or needs to

be developed...yes?

DR. TIMM: One point. When we were talking about the transcriptional activation assays and the constructs, when you write your report, if you could give us more detail on the...the types that you think would be most profitable, that would be helpful.

DR. HEERINGA: We'll definitely do that.

So, Dr. Belcher, you want to continue on with steroidogenesis effects?

DR. BELCHER: Sure, let me go down to steroidogenesis. There are five...five assays that were in the tier, the H295R cell line, the aromatase assay, the pubertal male, pubertal female, and the fish screen.

My comments are going to be primarily on the in vitro assays with the H295R assay.

There...there really isn't enough information to be able to comment on the utility of this assay, and the endpoints that are being measured, the estradiol and the testosterone content in the cell supernate would be a, actually, a very good assay, although I think premature at this time as...as suggested in the technical document to...to replace the aromatase assay or any of the other steroidogenic type assays.

The strength, to me, in the battery does come from the in vivo assays and the abilities to detect, in a general way, the...the influence on steroidogenesis. The aromatase assay is...is a straightforward and validated assay that does have utility in this, and I tend to be rather strong in supporting the inclusion of the aromatase assay through the indirect impact of aromatase on some of the earlier developmental effects that may be occurring through aromatasation that are...aren't really directly addressed by any of the...the sensitive period of not being...having the in utero or the earlier components in there.

DR. HEERINGA: Dr. Denver?

DR. DENVER: This mode of action, while important, is...is relatively weak right now in terms of assays that can address it. The...the strongest is the aromatase assay which, admittedly, addresses only one point in the steroidogenic pathway.

It...it appears to be well designed and...and could be a robust assay. It cannot account for compounds that will change aromatase expression which is another potential...potentially important mode of action.

The H295R cell line based assay is

potentially a valuable addition to this in that it could address multiple points in the steroidogenic pathway. However, this has not been peer reviewed, so we can't really evaluate that fully at this time.

The in vivo assays, the pubertal and fish assays, although they are potentially capable of picking up disruption of steroidogenesis and could potentially signal this as a mode of action, they...they really cannot define this as a mode of action at this point, and it would be difficult to decipher that type of action in...in those assays.

DR. HEERINGA: Dr. Delclos?

DR. DELCLOS: I have nothing much to add to the previous comment except to say that on the last point that in the technical review document, there's a lot of focus on the use of the cell line data, the, that's undergoing validation in interpreting the changes in the in vivo responses as far as their effect on the HPG axis for steroidogeneis, but I think that it really may not be that easy because of the lack of metabolic capability of that cell line. And so, that's just something to keep in mind.

DR. HEERINGA: Dr. Cooke?

DR. COOKE: Yeah, I have a...a few things. I was sort of glad to see that the minced

testis idea was being put aside, because I can attest to the variability of that, but then, that does leave us with the...the cells and the recombinant aromatase.

One of the criticisms of using the recombinant aromatase was that you couldn't see potentiation in terms of expression which was already mentioned, but presumably, it could pick up any other steroic activation of the enzyme activity, and I...I haven't come across a chemical that...environmental chemical that does that, but I presume it would. And, obviously, it would be good for inhibition.

To look at the...the cells, I have questions. I don't really have comments; I have questions. I'm presuming that the cells, according to the...the literature I read, are very happily producing testosterone and estradiol, and then, in fact, the suggestion to use the cells as an aromatase assay substitute would be a possibility. I...I'd like to see that developed a lot more, because how...how efficient is the steroidogenic process in those cells in comparison to a normal aromatase activation...activity?

The...the other question...maybe two other questions relating to the cells. One, I don't...I don't see any mention of 5-alpha reductase which is a quite important enzyme, and I don't see

how...how the cells, if they have 5-alpha reductase, could then be used as an aromatase assay, because you would have two draws on the...on the testosterone and the estradiol in the precursor.

The...the...the other two comments that I have are related to the comment that the...the cells would be good, because then you could look at the transcription of the steroidogenic enzymes. So, my question there is when you're doing the validation or the peer review, one of the questions they asked was are the enzymes in these cells regulated by steroidogenic factor 1, because that would be sort of the normal process that would regulate the transcription of those enzymes?

And then, on the other side, some of the criticisms of the assay, some of the assays were that they would not detect chemicals that promote the degradation of the proteins, and I would imagine that the cells, you would be able to, at least at the expression, although maybe at the Midwestern blocks, find whether the...the effect on the steroidogenic pathway was at the level of protein degradation.

And my last comment refers...and I would defer to the fish experts on this...is that the androgens in the fish, as I understand it, are 11-keto

derivatives. They're not the same androgens as would be found in a human or a rat.

And so, from the point of view of using the fish assay as a mechanism...mode of action leading to this chemical affects steroidogenesis, I'd...I'd like to get some feedback on how...how you would determine that in relation to the rodent or the human mode of steroidogenesis when the...the active androgen is not testosterone or dihydrotestosterone.

Thank you.

DR. HEERINGA: Thank you, Dr. Cooke. Yes, okay, let's do that. Dr. Ankley, please, and then we'll...Dr. Ankley and Dr. Furlow after that.

DR. ANKLEY: It's correct that in the fish, the...an active androgen is 11-ketotestosterone which is derived from testosterone, but up...up to that point in the steroidogenic cascade, all biosynthetic enzymes are the same. In fact, both males and females, have fairly...fairly high level of testosterone, and what you see in males is a...a correlation between testosterone and ketotestosterone.

The ketotestosterone is only found in the males, not in the females, and the biosynthetic pathway in the female fish is exactly the same as other vertebrates. So, really, what you have is an

additional biosynthetic step where before that, it's essentially the same set of enzymes.

DR. HEERINGA: Dr. Furlow? Thank you, Dr. Ankley.

DR. FURLOW: So, before Dr. Ankley gets away too for, so how...I guess one question to follow up, if I may.

DR. HEERINGA: Sure.

DR. FURLOW: How low is the enzyme between the 11-ketotestosterone? It's a liase or it's a blood or whatever it is, and how well is that characterized in terms of inhibitor spectra and that sort of thing? I mean, has anybody...are there...are there known inhibitors of that testosterone, 11-keto conversion?

DR. ANKLEY: We haven't found any in our studies. What we have seen consistently, both in control fish and in fish that have been treated with all the sort of chemicals we've used to characterize the system, is this continued correlation between T and KT. The actual KT levels, abs...on an absolute basis, are higher than T about four-fold, but the results seem to be correlated.

So, we haven't found a specific inhibitor. That's not to say that they wouldn't exist,

and so, what we have been doing in the assay is measuring both T and KT in the plasma of the fish.

It would be very interesting to find an inhibitor, but we haven't see it yet.

DR. FURLOW: Okay. All right.

DR. HEERINGA: Dr. Kullman?

DR. KULLMAN: I think you probably know this, is the...the potency of the 11-keto versus the testosterone would be AR.

DR. ANKLEY: The relative binding affinity?

DR. KULLMAN: Right.

DR. ANKLEY: They're actually pretty similar. In fact, that's why one of the...I...I don't want to get too far afield, but it's been quite a challenge in the area of fish endocrinology to try to sort out exactly how KT works, what it's key roles are, partly because you have both androgens there at the same time, and they both have affinity to the receptor. There doesn't appear to be a KT receptor. It appears to be a common androgen receptor.

DR. HEERINGA: Thank you, Dr. Ankley. Dr. Furlow, your general comments on the steroidogenesis?

DR. FURLOW: Okay. So, just in...in

general, then, yeah, I...I agree that the...the steps in the fish, the steps up to 11-keto are identical, and they...and...so, the effects of ketoconazole, et cetera, should be...should be expected. There may be something that...that comes up, and that would be of great interest to the field if there was, in fact, an 11-keto inhibitor.

Just to actually, then, in terms of steroidogenesis and the effects of potential endocrine disrupting chemicals on that, to echo Dr. Brown's comments on standardized tests, I mean, you know, standardized radioamino assays or ELIZAs, I mean, these really have to be reproducible, reliable, and if...and if I was...you know, if you can't, do what I said which is do it yourselves or...or have one designated contract lab that...I still want to echo Dr. Brown's point. That has to be very tightly controlled so we can...we can make some...some kind of guess about what might be going on if you do see an effect.

Otherwise, in terms of the appropriate....the appropriatess...appropriateness of the assays, et cetera, I'll defer to Dr. Cooke's comments on that.

DR. HEERINGA: Comments from other members of the panel on steroidogenesis mode of action?

(No response.)

DR. HEERINGA: Before we move on to the endocrine pathways, Gary or...you're okay? Again, we can revisit this again if questions do come to mind.

Dr. Belcher?

DR. BELCHER: Next mode of action is interference with the hypothalamic-pituitary-gonadal system, and that's covered by the pubertal male, pubertal female, and the fish screen. I am going to defer my comments to my colleagues at this point because of being a little bit further afield from my area of expertise.

DR. HEERINGA: We'll turn to Dr. Denver.

DR. DENVER: And I'm going...I'm going to make, really, just a few general comments.

I think that, together, these assays have...have the ability to identify disruption of the HPG axis. Individually, they have weaknesses. For example, there are concerns about quality control, repeatability among laboratories, you know, time...timing of puberty, things like that that...that could...could represent some real concerns in terms of evaluating the data.

The in vivo assays suffer from issues of specificity, and that's been raised a number of times.

However, I recognize that such apical endpoints are really necessary to identify compounds that can disrupt hormone-dependent processes such as reproduction and growth.

The challenge is going to be to understand the modes of action and to distinguish endocrine modes of action from general reproductive toxicity which, presumably, is going to be addressed further in the Tier 2 assays, but I think that's going to be the real difficulty at this level at these apical endpoints.

DR. HEERINGA: Dr. Delclos?

DR. DELCLOS: I think that the EPA has made a strong case with their experience over the years that these assays in term...to detect effects on the HPG axis, and, again, there is the problem of specificity which was mentioned by the last commenter.

I think that there...there were some problems with validation studies with transferability between labs, differences in endpoints, but the...the conclusions came out to be the same even though there were...there were some differences, and...and the validation issue that I asked about earlier is...is still a concern to me, but that's really, I think, out of our...our purview and up to the EPA to make a

decision on that.

DR. HEERINGA: Dr. Cooke?

DR. COOKE: Yes, thank you. I just have a...a couple of small comments to make.

The...the aspect of steroidogenesis should...should also give you some indication as to whether your chemical of interest is likely to affect the HPG axis. If you...if you know what inhibitor of androgen production or estrogen production, then you...you've got a good...you've got a likely candidate for affecting feedback loops and things like that. So, in...interpretation of the steroidogenic data could help you with that.

The other thing re...refers to the...getting back to the question of whether you're going to get a yes or no answer regarding the production of hormones in gametes, and the pubertal assay seems to be...both pubertal assays seem to be the more contentious assays around the room, if I could say that.

So, because they're pubertal, you...you don't have the...the gamete question answered to...to the maximum, because while you're measuring vaginal opening and...and preputial separation and you can measure the hormones, admittedly at a single time point

probably, the...the gamete question, I don't...I don't see that being answered as best as it could.

DR. HEERINGA: Dr. Furlow?

DR. FURLOW: So, I guess my comments, again, are more general, and...and, actually, my comments on...on these assays are...are clearly more relevant to the second charge question in terms of are they...are they appropriate, are they sensitive enough, et cetera.

Do...do these assays detect changes in the HPG axis? Clearly, they can. Right? So, I think that's...that's clear.

How they do it is...is another story, and whether they're endocrine related or not is another story, and whether or not that's the purview of this...of this screen is something that...that we should...we should take up. I...I personally believe that if something is, in fact, active in the HPG axis that isn't specifically endocrine related, that that is important, however. Right?

If reproductive tox..toxicity is something that the EPA ought to be concerned about, then regardless if we know exactly how it works, if we know exactly it's inhibiting trnH release or...or changing sensitivity at the...at the pituitary, et

cetera. So, that's my...that's one general comment.

So, the other...well, and that relates to the question of not having a negative control in the...in the pubertal female assay. I mean, it certainly doesn't look good. Right? So, it certainly doesn't give...give one confidence that the assay can be specific enough.

But, again, I actually do tend to agree with what was presented this morning, that that...that may not be a terrible thing, that that may, in fact, have some utility. It just may not specifically tell us what...what hormones may be...may be being affected.

The other...the other issue was if, of course, they stood alone, they wouldn't be completely satisfactory, and I point again to the BPA issue, that it would not necessarily have been scored as an endocrine disrupting chemical in the pubertal female assay, and that may be strain dependent. That may be just how it works in that particular assay. I don't know, but...but as a...as part of a battery, it may have utility, but, of course...and we're not considering it by itself.

Whether or not, again, we need them relative...the relative utility versus the 15-day male assay, I think, we'll take up again.

The issue...I guess I'm unclear as to whether or not statistics can really help you in terms of body weights and tissue weights and if they're endocrine related or not. That, to me, isn't convincing, and I think the fact that drops in body weights can, then, affect the HPG axis, that...that's even a more general toxicity that...that...that you'll have to...you'll have to resolve.

I...I guess I don't...maybe some of the statisticians can weigh in on that. I don't...I don't know if the al...these alternative statistical methods are appropriate and can be used to sort out what might be going on there.

And then, finally, I guess I would urge, although alcohol does seem to be negative in the 15-day assay, maybe you ought to run that through the...the pubertal assays and see if that gives a negative result, and I think that would strengthen EPA's position that these are, in fact, useful assays for determining effects in the HPG axis.

In addition, I guess I also agree with Dr. Vandenbergh's comments earlier. I mean, we...we don't have an assay other than the...the metamorphosis assay that deals with earlier development, a particularly sensitive developmental time point, since

the inter...intrauterine to lactational is out.

And so, despite a lot of the concerns about the sensitivity and specificity of the HPG or the pubertal assays which are...which are really mostly what are being used to test effects in the HPG axis, I guess my...my major comment is that if they could be tightened up, if they could be made more specific, maybe not use weights, per se, as the assay but...but have very tight...the vaginal opening and the PPS looks okay, to use those rather than the weights and to have a negative control, that these things would make me feel a lot better, and I think...I think they are important to include, actually, in...in a battery. We shouldn't just throw the baby out with the...with the bath water in that sense.

DR. HEERINGA: Other general comments on the HPG pathway and the effectiveness of this test battery to...

DR. VANDENBERGH: Right, I...I agree with Dr. Furlow's comments about a better negative substance to test. I think that...that would be very useful.

I had one brief clarification from Dr. Cooke about that the puberty assays don't answer the gamete question, and I think that's true. They don't,

because they...they're not being measured.

I think I, from what I hear from the EPA, this would be done in a second tier where you do multi-gen rather than in the first tier, because to add that to the first tier would make it a second tier. Right?

DR. HEERINGA: I think maybe between Dr. Portier and I, we can touch on the statistical question, I think, on the body weight versus organ weight relationships. Essentially, that winds up being a calibration problem in the design.

In some ways, if you were to design this, you would integrate...you'd essentially integrate the diet restriction component as an arm of the study and sort of capture interlaboratory and interexperiment variability, but you're...you're really throwing in another comparison group, and I can't do the numbers in my head, but I can tell you that if that is going to be a requirement to...to utilize this to determine effects, I think power considerations, now that you've got this calibration, even if it's brought in externally, it has variability. I think it's going to drive up...it's essentially going to drive up the number of subjects per arm in this particular assay. And so, I think that'll...that'll be a critical issue.

I know the people in ORD could certainly look at that issue for you and try to assess just how much that would affect, but you clearly will have to take, I think, what would amount to, if there is that calibration step, to determine effect in the presence of body weight reduction, I think you're going to have to be looking at increased size for each of these assays in terms of numbers of animals per dose, and I guess that's something we've been trying to avoid, but it's, I think, statistically, that's the general implication as I see it.

Dr. Portier, weigh in, too.

DR. PORTIER: I'm not often hesitant to jump in on these things, but I guess what I'm hesitating about is that from what I'm hearing, body weight is both a covariant and a response at the same time, and that's what...I hesitate to think about it that way, because you're basically saying the effect of the drug...the chemical might be to decrease body weight at the same time affecting the HPG axis.

So, is body weight being affected because HPG, or is HPG being affected because body weight? And I don't think you have the data to pull those two out.

So, adjusting for body weight may

actually weaken the strength of the test to determine HPG effects. I'm going to have to think more about this over lunch, but I...I think it's not a straightforward answer. It's not a just simple kind of thing because of that tight multi-connection that's going on here.

DR. HEERINGA: I think because of that, essentially, you're not sure whether this variable's on the right or the left-hand side of the equation. I think that introduces this uncertainty.

And I think my point that whatever happens is going to drive up the demands on the data in terms of sample sizes to either calibrate or to untangle this effect. So, we can work a little bit more on this, too, but I think it was an important issue that affects the general nature of what...what this will require for these relationships between body weight and organ weight that we saw demonstrated, in fact.

Additional comments on the HPG axis?

(No response.)

DR. HEERINGA: Dr. Belcher, if we could, turn to the HPT axis.

DR. BELCHER: Yes, turn to the HPT axis. The pubertal male, pubertal female, and the amphibian

metamorphosis assay are all informative on these points.

Generally, my comments will be limited to there is a strong reliance on the amphibian metamorphosis assay for integrating these components. That leaves this component with some weakness in the reliance, that it is relying on this one assay, and there's some concern with the actual portability of transfer of this assay to...to the...to the general spectrum of investigation.

DR. HEERINGA: Dr. Denver?

DR. DENVER: So, it appears that the only assay that was specifically designed to address thyroid is amphibian metamorphosis and that, you know, addition of thyroid measures in the rat pubertal assay is...is sort of an add on.

These assays have the least, in my opinion, have the least power in addressing specific modes of action, and that's due, in part, to the...the limitations that we have in the number of assays that are addressing this question. In the...the pubertal assay, the major measurements are serum thyroid hormone levels, TSH, and thyroid histology, and, you know, it was pointed out yesterday that there is...there is perhaps overlap or redundancy.

I wouldn't use the term redundancy in, you know, looking at thyroid dysfunction between the amphibian metamorphosis and the pubertal assay, but, really, the only overlap there is at the level of thyroid histology which is really only one measure of thyroid axis function and would indicate, you know, perhaps a...perhaps a direct action on the thyroid, perhaps disruption of thyroid hormone synthesis.

There's a well known syndrome in humans. It's called sick thyroid syndrome that results in...you know, it's...it's associated with non-thyroidal illness that results in changes in...in measures of thyroid function, and virtually every human illness results in...in some as...some changes to the thyroid system.

And it was pointed out yesterday that many of these compounds did, in fact, lower plasma T4. And so, without other assays to really look at mode of action, one concern is that you find lowered plasma R4 in many, many instances that are not directly related to disruption of thyroid function per se.

Another issue is, you know, when you get a lowering of plasma T4, you've got a change in THS, you know, what is the nature of that compound that is causing that...that change? And that's something that can't really be addressed from these assays.

One of the best known and best characterized mode...modes of action in thyroid disruption is the disruption of binding to serum transthyretin and, also, clearance of thyroid hormones, and that's not addressed at all in this assay. In fact, you know, in...in terms of going forward and developing other tests for modes of action, looking at binding to transthyretin, looking at thyroid hormone clearance, gluteoronidation, sulfation, things like that would be obvious points to...to...to follow up on.

In terms of the only assay that was developed to specifically look at thyroid function, the amphibian metamorphosis assay, that is the only assay that specifically addresses compounds that act as thyroid hormone mimics, that is, thyroid compounds that would accelerate metamorphosis perhaps.

So, that...that is a deficiency in the screen, that there...there's...there's a real limitation in being able to...to address the...the diversity of modes of action that are possible in disruption of the thyroid axis.

The amphibian metamorphosis assay is generally the only assay. It's actually...it's a fairly good assay for addressing thyroid mimetics. I think, given that, you know, the tadpoles depend, you

know, entirely on thyroid hormone for metamorphosis and it's well known that thyroid hormone will cause metamorphic changes, there are also limitations in that assay in that being able to distinguish acceleration of metamorphosis by scoring some of the endpoints, for example, to focus on hind limb development as opposed to other measures of...of...of metamorphosis.

Staging can be complicated among laboratories, so transferring the...the assay among laboratories could be a challenge, as was mentioned earlier.

The most sensitive part of that assay is the thyroid histology, as was pointed out in the peer review, and, you know, that can detect very low dose effects of glutrogenic compounds on the thyroid, but it, you know, it's remarkable that those effects were not seen at the higher levels, the apical endpoints.

And, really, what that...what that indicates is just the biol...the underlying biology which is that you really just need very little thyroid hormone to move this process forward. And so, in order to use it as some....as a screen for compounds that would decrease thyroid activity, you really do need...need to rely on the...the endpoint which is the thyroid histology.

So...so, I think it's fairly strong as a...an assay for thyroid mimetic in terms of looking at the apical endpoints. It's fairly strong as a...an assay for disruption of the thyroid axis and its inhibition if one looks at the thyroid histology.

The other point that was made is it could potentially signal disruption of diiodinases through asynchronous development, and this may be true. However, this could...so, this is based largely on the studies with IOP, iopanoic acid.

Now, there are three different diiodinases that come into play during metamorphosis, potentially three, at least two, that can convert thyroid hormones to an active form or inactivate it, and so, using asynchronous development could be an indication of disruption of diiodinase or something else. So, at this point, I'm not convinced that that is going to be an definitive test for disruption of thyroid function.

But, you know, as I said, it's the only assay in the screen that is directed specifically at a thyroid hormone act...thyroid hormone action.

DR. HEERINGA: Thank you, Dr. Denver. Dr. Delclos?

DR. DELCLOS: I have nothing to add on

this.

DR. HEERINGA: Dr. Cooke?

DR. COOKE: I don't really have much to add except because it is the only one and it should give you a yes or no answer to...to your question, it's sort of a fait accompli, you know, at this present time, but from the point of view of developing other assays that could detect thyroid interference, like an in vitro test, histocyto, those sort of things, thyroid effects on Sertoli cell number in the testis, just that are ideas, but other than that, I don't have anything to add to Dr. Denver's points.

DR. HEERINGA: Dr. Furlow?

DR. FURLOW: So, there were comments yesterday about, say, redundancy and that the amphibian metamorphosis assay, what it gives you is covered in the pubertal assays, and I do disagree with that.

The metamorphosis assay, as Dr. Denver said is...and Dr. Zoeller actually mentioned yesterday...is the only one that can look at disruption of thyroid hormone in peripheral tissues, and everything else, both the pubertal and then even a central aspect of the metamorphosis assay, really has looked at thyroid...has really focused on thyroid histology. And then, at least in the pubertals, is TSH

and T4 levels but not...but not directly at action.

And because metamorphosis is such...is...is...is completely dependent on thyroid hormone for progression and the assay is started before there are circulating thyroid hormones in the animal but there are already expressed thyroid hormone receptors, it is, in fact, an assay for looking at thyroid mimetic compounds and has been...has been very useful for that.

You know, that said, but it...that said, with that background, it puts quite a lot of pressure, actually, on the amphibian metamorphosis assay then, because, you know, I look at iopanoic acid, and, really, there wasn't a good response in the pubertals. Right? There was only a response in the metamorphosis assay.

Now, to me, who really likes and loves dearly metamorphosis, I wonder if things that are weakly active in the metamorphosis assay but not at all active in the pubertal assays, whether or not that would trigger your weight of evidence response. I mean, that's something that...that...that can be addressed, because, again, there's...there's nothing in the pubertals that addresses action at all or, in fact, the...the activity of the diiodinases per se.

So, I think, for the future...I don't think this will happen before August, but I think for the future, it would be incredibly desirable to have both binding assays for thyroid hormone receptors. I know in the EPA's analysis, there was no evidence for direct binding, but, in fact, there is, and Dr. Zoeller and I can...can provide references on that and enter them into the record. So, there is evidence for direct binding of...of various compounds with the thyroid hormone receptors.

A. ..a transactivation assay would also be extremely useful, both for agonists and antagonists.

And there are other improvements to the...to the metamorphosis assay that I...that I will actually wait until we discuss the limitations and...and improvements in...in the next...the next part of this.

So, again, in the absence of any other assay to detect peripheral effects, I think you have to include the metamorphosis assay, and I have to say I'm actually quite impressed at the, at least within, within lab, progression through metamorphosis. I think that the labs in Duluth and in Germany that...that really put, to...to my knowledge, the lion's share of...of work into developing this assay really are to be commended, because before they got started, we were

all over the board in terms of trying to have reproducible progression through metamorphosis as...as a...as an endpoint.

And so, so that part of it is...I think should be particularly noted.

Finally, just some general comments. It's the only amphibian assay, and I think the EPA is to be...is to be commended on including amphibians in...in their assays. And it is now, without the intrauterine to lactation assay, the only one that looks as development, as has been noted, at least morphological development or...or development through organogenesis, and I think that is, in fact, extremely important, an underappreciated aspect of the endocrine system.

There are limitations in terms of interlab variability, practicality, these things, cost, length of time. These things have been brought up, and...and we will...I think we will revisit many of them.

There...I think there can be some improvements to the assay, and I will...I will address those then, but...but I think that just relying on the pubertals for effects on...on thyroid hormone system in general is...is clearly not sufficient. If there are ways of really improving the metamorphosis assay and...and allow it to achieve its full potential, I

would...I would certainly strongly...strongly encourage that.

DR. HEERINGA: Comments from other members of the panel on the HPT axis? Yes, Dr. Zoeller, please.

DR. ZOELLER: Just one small comment is that there are known instances where gender differences in metabolism of compounds can greatly influence the ability of those compounds to impact the thyroid system. So, if you were to replace the pubertals with a different assay or get rid of one of the pubertals, you would really miss chemicals or potentially miss chemicals that...in which there are...there are significant gender differences in metabolism.

DR. HEERINGA: Turn to Gary Timm and Dr. Touart to see if there's any questions of clarification.

DR. TOUART: This is Dr. Touart, and it's not necessarily a question, more of a comment back on...on some of the points that Dr. Furlow had raised, and this goes back to some earlier, you know, points in terms of the context of HPG or even HPT axes, you know, being affected and the...the generalized nature or these non...non-specific context that that would...would have and whether we should get concerned

about that or not.

And I think that, in general, the endocrine systems, it is built on...on homeostasis and whether we have a disruption on homeostatis or, you know, just a compound that overwhelms, the ability of the...of the endocrine system to compensate, and these would be of concern, so that's the perspective that they would be, you know, utilized in determining, you know, the nature of an effect.

It wouldn't...wouldn't necessarily move on to, you know, to want to be able to compensate, but I think I would...would urge the in common considerations and...and I think Dr. Furlow alluded that there may be some suggestions in terms of improvements in...in, you know, in particular endpoints or endpoints that could be added that would assist in the specificity, you know.

You know, I think the Agency's perspective, you know, the battery overall and part of the strength of the battery is to help us focus on a variety of endpoints that we...that we consider reasonably valid to include and approach, but then the consideration of...of what more we might be able, because as was pointed out, in some things, especially like maybe amphibian metamorphosis, you know, it's

maybe weak in terms of...of compounds that are weakly active in terms of how we're going to be able to interpret if that's the only assay or the only endpoint that might have been affected. So, any help in...in trying to buttress or, you know, assist in...in improving that ability to...to resolve as well as to detect and identify would be helpful and appreciated.

DR. HEERINGA: Comments?

(No response.)

DR. HEERINGA: Okay. What I would...5 after 12:00. What I would like to do is I'd like to adjourn for lunch, but we're going to start a little later than we normally would. We'll start again at 2:00 p.m., and the reason there is that the group that's responsible for charge question 2 which has some very important issues, I think has...needs a little more time to prepare some explanatory materials and power point. So, we'll provide a little extra time and start again at 2:00 p.m.

And, again, Dr. Portier will...will serve as chair when you reconvene. So, see everybody at 2:00 p.m.

(**WHEREUPON**, a luncheon recess was taken.)

DR. PORTIER: Okay, we'll continue with the panel discussion. Sounds like the panel has had a

productive lunch. They produced their slides, and they're ready to begin the discussion on the second question. So, I'll have...ask the EPA to go ahead and read the second question.

DR. TOUART: Jerry, I...this is Les Touart again, and I'll...I'll go ahead and...and read for the record the...the second charge question that we'll be discussing this...this afternoon, and it reads: EPA proposed a Tier 1 screening battery that includes many assays that are complimentary in nature and that coverage of the estrogen, androgen, thyroid hormonal systems. The strengths of one assay offset the limitations of another, albeit by different taxa, life stages, endpoints, exposure, and use of in vitro and in vivo methods executed at different levels of biological organization. Example, cytosolic receptor binding, cell-based assays, whole organisms.

The subparts to the question, a) please comment on how well the proposed battery minimizes the potential for false negatives and false positives;

b) are there any unnecessary redundancies for mode of actions across the battery; and

c) please comment on whether a different combination of validated assays would be more effective in achieve...achieving the purpose of the battery than

that proposed by EPA.

DR. PORTIER: Dr. Lasley?

DR. LASLEY: Yes, I'd like to introduce this part of our response with three caveats. First of all, we recognize that the conversation here represents the work that is in progress, and we really now need what is a stop frame on a moving target.

And we understand, the second caveat, that some, perhaps many, of these assays will be extended. There will be additional transactivation assays and assays that will see different mechanisms. Clearly, there will be new endocrine disruptors revealed in the near future, and these new endocrine disruptors or...or discovered endocrine disruptors will require additional assays to be developed and validated.

Our approach to addressing charge 2 is we're going to divide the work up into three groups, and questions 1 and 2 are going to be answered by way of a grid which is going to allow us to first talk about false positives, false negatives by looking at the redundancy of the assay as applied. In that same grid, we'll look at complimentary assays to see if we can see if there's any additional requirements or what the benefits are of...of the complimentary assays.

Then, finally, in the third category, we'll...we'll go back to our whole group and talk about combinations that might do better or as well.

But first, I want to start with Dr. Kullman and Dr. Eldridge who are going to talk about the estrogen assays and how they feel. So, with the first slide, Dr. Eldridge will introduce.

DR. ELDRIDGE: What we did here was to try some linear thinking to try to break down the system in terms of its different components in a somewhat more linear fashion, that is to say, starting with overall control of...of gonadal function, either gonad, and then moving to the gonadal products and what they do to produce their responses, and what we've done...where's the list of the nine...of the eleven...those numbers...

DR. PORTIER: Go to the next slide for a minute and then come back.

DR. ELDRIDGE: Yeah, I think so. So, those numbers represent 11...the 11 basic assay types, and you can see them. Right? Where it says key. And what is does is start from the most direct basic. 1 and 2 are hormone receptor binding on up to hormone synthesis, fundamentally, to the Hershberger uterotrophic being tests, essentially, of hormone

expression, and...and then, 8 and 9 are...are whole system integrity tests, and then, 10 and 11 are the amphibian and fish assays.

And so, what we then tried to do was to take each of the tests in the battery and see which of these components they apply to, and that would give us an indication of how much redundancy there is, whether there are significant gaps and holes, and, you know, what do we think about it from there. Okay.

DR. KULLMAN: Seth Kullman. Since we're on the grid, we'll...we'll kind of go through it step by step just to give you an idea of how these break down. If you look at the x axis, you can see that we've listed a number of the components that we think represent different types of mechanisms of actions and different responses of an organism or systems. Along the...the top there, you can see the numbering, 1 through 11. Those are referring to the different types of assays that have been included in the Tier 1 battery.

And so, the way we worked this out was we went, basically, from column C down through the different types of responses that we're going to see. You can see in column C there that the estrogen receptor binding interacts specifically with the

target, and it really does not involve signaling or cell response or any of the other components. In this particular instance, let me reiterate that we're...we're looking at an estrogen response, and thyroid and androgen will be reviewed subsequently.

So, number 2 is AR binding. We have no response for AR binding in any of our assays when we're looking at these estrogens.

Number 3 is the signal transduction such as the transcriptional activation assay, and we're going to assume here that that will interact both with the target binding and interact with cell signaling such as transcriptional activation.

Row 4, aromatase. Aromatase, at this point, is providing information regarding enzyme activity alone.

Row 5, or column 5, sorry about that, is the H295R cells. At this point in time, we put down enzyme activity. We do understand, however, that these cells provide a fair amount of additional information, but no data has been validated at this point in time, and I think we're still awaiting peer review of that particular assay, but we've all seen this cell line as providing a significant mode of assays and opportunities to assess estrogenic activities here in

addition to looking at steroid and estrogen production.

6 is the Hershberger estrogenic activity. Would be null there.

7 in uterotrophic assay. We have, for both receptor and target binding and cell signaling A's in these position, A standing for an assumed response. We're not measuring receptor binding, and we're not measuring receptor transactivation in these assays, but this is assumed.

However, the caveat to that is that with new mechanisms of action for various endocrine-acting compounds, such as membrane receptors, G proteins, tyrosine kinase receptors is such that there may be alternative mechanisms besides estrogen receptor binding that may elicit a similar type of response, and this is why we put in the target binding here as well.

So, in this, we are looking at both cellular responses, organ level responses, and female system integrity responses.

As we continue on, 8 is the male pubertal assay. We don't expect any activity with that with the estrogenic compounds.

9 is the male pubertal assay. Again, we have our A is assumed ligand binding and assume...receptor binding and transactivation, but

also, we have here cell responses, organ responses, female system integrity responses, and complimentary system integrity where we can use these for complete HPG and/or partial HPG and make comparisons to the other assays in the battery that do have HPG components.

With the last two being the fish and the frog, you can see that we have positives in the organ system response through the comparative system integrity.

The take-home for...essentially, for all of this is that we're able to then tally up the number of redundancies for the different types of processes that we see and also the number of compliments that we have for different types of assays. So, if we look at redundancies for receptor target binding, we can see that we have three additional redundancies that include 7 and 9 in this.

And then, the same as far as you go down the chart here. For cell signaling, two redundancies. Cell response, one redundancy. Enzyme activity, one. Organ response, three. And so on and so forth.

On the bottom row is the number of compliments that we...you can see, and the first thing I'll point out is that the compliments are heavy on the

right-hand side which showed the in vivo assays, and they're rather light on the left-hand side which are the in vitro assays. We believe that making this type of tally provides us with a mechanism to ask the questions about both the redundancy and the possibility of creating false positives and false negatives.

When we look at the compliments again, the in vivo,...in vitro assays provide probably a greater degree of specificity than some of the in vitro assays, but when you begin to look at the balance of them, I think we...we begin to extract for a balance between both specificity and sensitivity which the battery of assays may provide.

With that, we'll move on to the next.

DR. PORTIER: I would like to open it up for discussion on this part, and then we'll go to the androgen component and then, finally, to the thyroid component. Any discussion? Comments?

(No response.)

DR. PORTIER: Dr. Lasley.

DR. LASLEY: All right, then, I'd like to ask Dr. Brown to do pretty much the same thing with the next slide which is...is the testosterone grid, and it follows the same format. It's a...Terry?

DR. BROWN: Okay, we're using the same

format here, so the slide that's up there now just show where the different components that Seth just described that we divided this up into, y on the...on the...on the integrated axis that includes the...the hypothalamus, pituitary, and gonad, producing the...the steroids in terms of either estrogen or androgen and...I thought it was going to be...

Okay. So...so, what we're dealing here with is, you know, the CNS, the hypothalamus, pituitary, labeled the HPG axis, including the gonad where the steroids are produced. The steroids then, either androgen or estrogen in our cases, affect the target tissue, and they do so either through a combination of receptor binding signal transduction and cellular response. So, the numbers that are shown on here are the numbers that...that Seth just referred to in terms of the...the different assay components that comprise the different responses that are en...entailed in...in looking at this full complete axis of...of activities. So...

Yeah, slide 3. Okay, so as Seth did for estrogen, we took the same approach for androgen, using...using testosterone as our...as our potential test substance. Okay? So, that's shown here.

Here are the different levels of

responses that we could get at the receptor target binding, the cell signaling level, cell response, enzyme activity measures, organ response measures, whether...and then, whether the assay includes, you know, looking at the integrity of the male system, the female system, the...the entire axis, whether it looks at development related to thyroid or to the compar...in the amphibian system or the comparative system integrity such as with the...shown by the fish assay.

So, those are all represented down here. So, then across the top, we have the...the 11 different assays that are envisioned here, and then we have the different types of...of measures that we will get from...from those 11 different assays. So, if we're dealing with the androgens and testosterone, obviously, column 1 which looks at estrogen receptor binding, we don't really have anything there. That's going to be essentially a...a non-complimentary assay. But looking at receptor target binding in the androgen receptor binding assay under column number 2, the androgen receptor binding assay would be the...the single component here.

In column number 3, we're looking at AR signal transduction, again, obviously, that doesn't apply to androgens.

In column 4 where we're looking at aromatase, effects on aromatase, the...this would be a compon...the component here would be to affect enzyme activity.

And, again, in the H295R cell line, again, in column number 5, we'd be looking essentially strictly at enzyme activity.

Then we go on to column...in the in vivo assays, captured by...first by the Hershberger assay, and that's represented in column 6 where, if we have an androgenic substance, chemical, we would assume that we would have activity at the receptor target binding level through cell signaling, through cell response, potentially an enzyme activity, and we would really actually be measuring in these...in the Hershberger assay an organ response and also the overall integrity of the...of the male system.

And if we have an androgenic response in the Hershberger assay, we would essentially expect that that would be reflected in the...in the comparative fish system, that we would also have an androgenic response in...in that assay also.

Obviously, in the uterotrophic assay, we wouldn't expect any response to an androgenic substance.

In column 8 where we have the male puberty assay, again, we would assume that an androgen would be acting at one or more steps through receptor target binding, cell signaling, cell response, effects on enzyme activity, certainly, at the organ response level where we would be looking at effects on various organs and also on the integrity of the...of the HPG axis.

If we go to the female puberty, obvious...here we would expect that an androgen might have an effect on the female system in its integrity through some other...some mechanism not reflected here.

In the frog metamorphosis ass...amphibian metamorphosis assay, if we have an androgenic substance, we would expect that it would also be reflected in the developmental aspects of...of amphibian mor...metamorphosis through responses at the organ and system level and...and throughout development and...and also would be seen in the comparative system integrity.

And then, in the fish comparative system, again, if we have an androgen, we would expect to see these responses.

Okay, so then, if we...if we address the question of do these systems have...assays have

redundancy, we've summed those up over here on...in this...on the right-hand side of this slide where, obviously, if we have...if we have responses in three different assays at the receptor target binding level, then there would be a redundancy of two. If we have responses in cell signaling at...in two of the assays, we would have a redundancy of one, and so on down through the...the various assays.

And then, the complimentary, the assays that would be complimentary to each other are summed up down on the bottom. Again, as Seth mentioned, we see that the in vivo assays contribute to complementarity between the various responses in the test systems, whereas the...the in vitro assays over here are kind of stand-alone assays where we have a single readout and, therefore, there's really no complimentary assays within the...within the battery that actually can lend credence to these.

So, in this case, we're...we have specificity and we have a...a...a single response readout, whereas in the in vivo assays, we have complementarity combined with redundancy, and, therefore, this is where weight of evidence would...would probably enter into the...into the judgment of...of evaluating these assays more...much

more so than on the side of the...the in vitro assays.

DR. PORTIER: Any additional comments? Does everybody agree with this? Okay, Dr. Furlow.

DR. FURLOW: Yes, I just had one clarification, I guess. Right? So, Dr. Denver mentioned earlier that estrogen can affect metamorphosis. Right? And that...so, you could have an outcome there.

I guess I'm interested why you have...I guess maybe I'm not understanding this correctly. So, you think that metamorphosis could be a scorer for androgenic properties because...so...so, gonads aren't being looked in that...at in that assay. Actually, one of the reviewers pointed that out. So, sexual differentiation, yeah, it's just kind of progressing through metamorphosis.

So...so, there might be something on the estrogen side, but on the androgen side, I'm not aware of any interactions. Maybe Dr. Denver might know. But I guess I want a clarification of why that was in there.

DR. BROWN: Okay, this may...

DR. PORTIER: Dr. Brown?

DR. BROWN: This may have been our naivete on one...on one side of things. On the other

side of things, we...we were...we looked at this as...as these ass...both the...the amphibian metamorphosis assay and the...and the fish assay included both sexes in...in...in the assays. They weren't...they weren't defined by male or...and/or female separately, and, therefore, if we had an estrogen or if we had an androgen in the system, we...we thought maybe that would in some way affect the development, but that may be a naive assumption on our part. I don't know.

DR. PORTIER: Don't...don't forget to identify yourselves when you make comments. Dr. Lasley?

DR. LASLEY: Yeah, Bill Lasley. Yeah, we're being as optimistic as possible here, because the question is what is the possibility of false positives and false negatives, is the first question, and any information you get would guard against getting the false information. So, if there is the possibility of seeing it would...a...a redundant assay, that it might not be there, it...it might prevent accepting a false...a false positive. It might avoid a false negative. So, we were being as optimistic as possible, but I agree it's not necessarily going to...to have a lot of weight.

DR. FURLOW: Yeah, David Furlow. Yeah, I just wanted some clarification. I mean, I guess the other point is that it is truly the only developmental assay. Right? So, there is a lot of weight there, but it...it's a...it's an aspect of development, not global development.

DR. PORTIER: Since we don't have printouts of this in front of us, would it be possible to slide this sheet down so we could look at both of them from here? Maybe one of you can slip over and see if you can do that. Minimize this one. Yeah, the second...that...the other one, the next box. Not minimize but shrink it and try to show both at the same time, so we can see...there you go. Click on that and drag that one down so we can see both of them at the same time.

So, that's the estrogen one. If you can put the...the testosterone one at the bottom, then we can kind of...you don't have to cut and paste. Charlie, you know what I want. And just drag that down. You don't even have to split it. Just drag it down. Just drag that one down a little bit. Now you can see both of them.

There you go. Yeah, so you can see most of it. You can just see the...I just wanted to look at

the...the complementarity of the two. Look on the front one. There you go. Good. There we go.

So, why don't we have cell signaling for the testosterone?

DR. LASLEY: That's the transactivation assay.

DR. PORTIER: Oh, that's the TM. Okay, any further comments?

(No response.)

DR. PORTIER: Dr. Lasley, continue.

DR. LASLEY: I'd like to have Dr. Zoeller now to present a similar approach with the thyroid.

DR. ZOELLER: Okay, so I'm going to start out with a similar construct and point to or kind of illustrate the similarities and differences between the thyroid axis and the HPG axis.

So, again, we're running out here. Like...like the APG axis, this axis is neural endocrine axis with the hypothalamus controlling pituitary, pituitary controlling thyroid gland. Thyroid hormones are secreted, travel through the blood bound to various proteins, and can act on target glands. Also, there's the negative feedback effect of thyroid hormones on the hypothalamus.

Now, there are also some important differences here that aren't maybe so well illustrated in this diagram, but one is that thyroid hormones themselves have to be taken up selectively in the cells and tissues which is a little bit different. And this is a point of likely interference.

Also, thyroid hormones have to be metabolized. So, a T4 has to be converted to T3, and the enzymes that do this are selectively expressed in different tissues, like the type 2 diiodinase is expressed in the pituitary gland, and if you knock it out or if you inhibit it, thyroid hormone levels change, because they do not...they don't effectively exert a negative feedback as well.

Can I go to the next one? Now, there's an issue that I want to talk about very specifically, and that is one of the main mechanisms by which thyroid hormones can be suppressed by chemicals, and that's by...can we go back one? And that's by activating enzymes in the liver to cause clearance of thyroid hormone, and this is...this is a mechanism, for example, of phenobarbital, and phenobarbital was used as the positive control in a couple of these assays.

So, in this case, these enzymes can be activated in the liver. Thyroid hormone level...or

thyroid hormone clearance is increased, so the half-life is decreased. As a result, T4 levels decline. In this...in this example, TSH is increased. Cell proliferation in the thyroid gland is increased, and thyroid tumors can be...can occur also.

And this is what the histopathology would capture. You'd be able to see those histological changes in the thyroid gland that would be secondary to THS increase that would be secondary to thyroid hormone reduction.

Can we have the next slide?

So, here's an example from Kurt Klausen's lab that was published in 2001, and what he's looking at is the number of these microsomal enzyme inducers that have similar effects on activation of these enzymes in the liver and can all cause a reduction in T4, but they don't all increase circulating levels of TSH.

So, if you look on the left-hand side here...I'm not sure that this is working, but if you look on the left-hand column, you can see free T4, free T3, and TSH, and along the bottom are phenobarbital, and there's two other enzyme inducers in PCBEs.

You can see that T4 levels are reduced by all of these microsomal enzyme inducers, but TSH is

not increased by all of them. On the right-hand side, he's looking at labeling index. So, this is kind of a marker of cell proliferation in the thyroid gland.

He's also got thyroid histopathology in his paper as well, and you can see that thyroid histopathology is altered by these enzyme inducers that increase TSH. So, there's link between TSH and changes in...in thyroid histopathology.

Next slide.

Okay, so now, if we look at the assays, and, in fact, the thyroid gland isn't represented here. Can we fit this...so the point is...the point from this...from this slide is going to be I've changed these...these points at which chemicals can interfere, in principle, with thyroid hormone action at the hypothalamus-pituitary-thyroid hormone synthesis and release, and so, this is kind of a direct pituitary effect.

Circulating levels of thyroid hormone is going to be central to all...to most or all of these modes of action, serum binding proteins, tissue responses, liver metabolism.

That's not the entire...we need 8, 9, 10, 11. Well, that's...this kind of illustrates a good point about the Tier. Yeah, so...

So, there are essentially three assays that...that touches on thyroid disruption or measures thyroid hormone, and that is the two pubertal assays, male and female pubertal assays, and...that's not very visible...and the frog metamorphosis assay.

Now, in this case, if you look at the...the measures that are being taken is, in the mammals, there's T4, TSH, and thyroid histopathology. And one of the...one of the concerns that I have is that serum T4 is affected by many chemicals, and it's probably being affected through liver metabolism. A concern that I have, though, is that if phenobarbital is the positive control and it acts by activating the liver, we can't rule out...another example would be linuron that is being presumed to increase liver metabolism, but it doesn't increase TSH. So, T4 levels go down to a significant degree, not trivial, in a dose dependent manner, but TSH isn't increased.

This profile looks exactly like PCBs. So, PCB exposure decreases T4, doesn't change TSH, doesn't activate the thyroid gland, but we know from many different studies that it can affect thyroid hormone signaling in the developing brain, in the liver and heart and other tissues.

So...so, we can't rule out the

possibility that T4 decline, in the absence of an increase in TSH, is informative. And so, we can't...we can't rule that out.

The amphibian assay is important in this regard, because it captures endpoints of thyroid hormone action. There are differences...there may be differences in metabolism between amphibians and...and mammals that...that...that I'm not sure, as I think about it, complementarity and redundancy, I'm not sure that it's entirely...it is complementary. There's no doubt about that, because the amphibian metamorphosis assay captures endpoints of thyroid hormone action, but I...but I don't think we can say that it's redundant entirely, because metabolic differences may exist that may be important.

So, for just final points here, serum T4 in the pubertal assays is a reasonable measure of the impact of a chemical on the HPT axis, but failure of TSH to respond to lower T4 I don't think can be assumed to be benign. So...so, we can't ignore that whether the amphibian...whether the amphibian assay reveals an effect of that chemical or not.

So, the pubertal assays don't have a measure of thyroid hormone action, and that's a weakness, but I don't think that's a weakness that can

be solved immediately, because I...I can't think of anything that's a validated assay at this point that would...that would be able to stand in there.

The amphibian metamorphosis assay captures measures of thyroid hormone action but can't necessarily be assumed to be directly related to mammals because of these cases of metabolism.

I think the more we know about it, the better we're going to feel about the relationship between those two assays.

Next slide.

So, I don't need to...you...you want to have a conversation about the general presentation before we move into the last part of these questions, or...okay.

So, in terms of minimizing false positives and false negatives, this...I think at this point, it's difficult to estimate, because we don't really know. I think that it's a valid point, for example, that Dr. Denver pointed out earlier that there...there is the possibility that...that chemicals can have a generalized effect that would be similar to this non-thyroidal illness that we see in humans, and that's a genuine possibility.

And I don't know...I don't think

that...that we know enough yet to be able to estimate how many of these chemicals that cause a reduction in T4 with no increase in TSH are false positives or in that case. So...so, it's going to be difficult to estimate that.

On the other hand, we do know a very...you know, clearly, of examples where T4 declines, TSH doesn't increase, but there are adverse consequences down stream.

Okay, the next slide.

Are there unnecessary redundancies for the MOAs here? I think the answer is just no. I mean, I'm not sure how many redundancies there are, but it would be, certainly, not too many.

Okay, one more.

Different combinations of validated assays would be more effective. This can be interpreted two ways. One is currently validated assays or two, gee, wouldn't it be nice. And in terms of current validated assays, I'm not sure that there are any that can be just immediately added on to supplement weaknesses here, but capturing some measures of thyroid hormone action in pubertal assays would be important. I think simple ideas are...serum cholesterol levels, heart and cardiovascular functions.

In vitro assays, I think, might also be important for thyroid hormone receptor binding. Because the TR-alpha and TR-beta mediate different kinds of effects on the thyroid system, it's important to separate these two things out. For example, l, the beta receptor mediates negative feedback. An effect on the alpha receptor that's selective wouldn't be captured, even in...in serum TSH levels. And, also, the transactivational assays would be important.

Okay, that...that concludes the presentation on our part for question 1 and question 2. We...we believe that we can point to a good number of redundancies, particularly with the in vivo assays.

And...and same with compliments, a good number of redundancies in...or...or compliments in the in vivo assays. The in vitro assays are the place that probably there is less. The question is, is there enough. We don't know. Is there too many? I don't think so. I don't think we show that there is...there's really too many in...in any of the categories.

But I think we might want to discuss now questions 1 and 2 together. They're...they're connected.

DR. PORTIER: Does anybody want to jump

in? Okay, Dr. Denver.

DR. DENVER: I just want to follow up on a point that Dr. Zoeller made about potential differences in metabolism across species, and...and we really don't know very much about that.

One thing we do know, though, and I...and I was referring to one of the most common modes of action, thyroid disruption is competition for binding to transthyretin, the serum binding protein, well, you know, a well characterized mode of action, at least for PCBs and...and related compounds.

And it's important to note that the...the specificity of transthyretin across species is actually quite different. In mammals, it's for T4, and in amphibia and, actually, most non-mammalian vertebrates, it's for T3.

And so, what that suggests is that you may have very different compounds that would cause thyroid disruption through that mode of action in amphibia and would be picked up in amphibia and other wildlife that wouldn't show up in mammals. And, on the other hand, you would have compounds that would disrupt thyroid function in mammals that wouldn't show up in amphibia.

DR. PORTIER: I think I want to push it

a little bit more on the false positive/false negative. Jan? That's the question Jan was going to ask, too. Okay. I want to get a little bit clearer on...on the feeling of the panel on...on this especially...let's..let's take the issue of false negatives.

DR. LASLEY: I think the safeguard to false negatives is the redundancy. If...if you have redundant systems and they're sensitive...and I think we...we have selected the more sensitive assays that are validated...then I think that's the only safeguard you can really have.

DR. PORTIER: Yes?

DR. KULLMAN: Dr. Kullman, Seth Kullman. I think also represented are a range of both specificities and sensitivities within the assays across the board, that where some may not provide both, I think there...there's a number of different ways that chemicals may interact with either complete specificity or incomplete specificity versus the sensitivity, and I think we saw some...some good examples of that when we were given the presentation this morning on a variety of different compounds, both strong and weak agonists and antagonists to several of these systems.

Not all the systems lit up the same way

or as well or were activated or...or depressed the same way, but, certainly, there would seem to be the ability to capture a range of both positive, negative...well, maybe not so negative...but...but strong and...and weaker agonists and antagonists.

DR. PORTIER: Dr. Belcher, you look like you're ready to make a comment. Nope? Dr. Zoeller.

DR. ZOELLER: So, for the thyroid system, one thing that's important, I think, to...to...at least for me when I think about it, is the Tier...the goal of Tier 1 is to just see if some...if there's some chemical that might interfere with, in the case of thyroid hormone, thyroid hormones signaling at any point in time. So, it's just a...it's a...it's a very quick, simple assay that would give a broader picture.

In the case of this issue of thyroid hormone decline in the absence of a TSH increase, in a pregnant female, the first trimester and, you know, the first 7.5...what am I thinking...16 days in a rat is...the fetus can't make thyroid hormone. And so, really, in terms of the mother, it doesn't really matter what's happening. As long as T4 levels decline, whether TSH goes up or not is irrelevant, and the fetus is going to be deprived of thyroid hormone regardless

of the mechanism by which T4 declines.

So, I think that...that if the tier is to be viewed as an indicator of what could be really an important endocrine disruption event that would have to be explored more fully in a Tier 2, and that's where you would get hold of this kind of issue, it's important not to ignore a decrease in T4 regardless of the other kinds of events that you see happening in that assay.

DR. PORTIER: Dr. Eldridge?

DR. ELDRIDGE: Eldridge, yes. With respect to the reproductive parameters, our...our little group with this charge question felt pretty comfortable that...that the redundancy does help obviate false negatives with two important things. One is that it's...it's perhaps more important to avoid false negatives than anything else. In other words, it's...it's necessary to not let an active material go unidentified.

And the other thing is, of course, you can test lots of little mechanisms, and we were thinking of many little mechanisms that are not picked up here, but the...the group of assays that are identified at this time before us seem to be providing a lot of redundancy on most of the important effects.

The...the problem of false positives is a different kind of issue. The...unfortunately, the false positives are most likely to happen with the large-scale, extensive, complicated in vivo testing, because that's where more known specific actions can take place.

And so, the Agency, then, is...is on tightropes to make a call on things, because repeating a binding assay is fairly simple if you're not sure if that worked, but repeating a...an in vivo study because of...of uncertainty of indirect actions, it's a lot more difficult.

So, this...this causes us to caution the Agency to be very careful and very willing to look at the entire spectrum of results as it decides how to proceed once a positive happens, especially in these large-scale, difficult to perform in vivo tests.

DR. PORTIER: Dr. Lasley? Any more discussion on false positives? Because as you...as you mentioned, I mean, that's where the potential for expense, animal use can build up. Right?

DR. ELDRIDGE: Yes, Eldridge. And once is expensive, but...but I think most of us have experience with larger-scale animal testing versus smaller-scale specific testing like a receptor binding,

and...and we all know how difficult it is to get a very precise answer, to get it to happen the same way every time in...in a case like that. So, it...it's a much more complicated system to get definitive answers, and if you're going to start testing chemicals at or near the MTD, you're almost begging for some effects to happen.

DR. FURLOW: So...David Furlow, UC Davis. So, a couple things. We touched on some of these points earlier. Maybe that...that's why it's not so fleshed out this afternoon, but I guess the MTD issue is something that still concerns me.

So, is there a possibility that if you're working at the MTD and then half below that, then you may get general toxicity, but if you went to lower doses, you may reveal endocrine disrupting effects? I think...I don't think that's really be talked a lot about here, and I...I am actually quite concerned about that. Right?

So, if you throw something out because it's generally toxic at the dose where it's causing 10 percent of the body weight to drop, and you say well, okay, there's no endocrine disrupting effect, I mean, maybe some other folks can weigh in on this, but I guess I'm a little bit worried that you may be masking

something by working at those high concentrations. I don't think we've really talked about that maybe enough.

DR. LASLEY: Lasley. No, we didn't...we didn't consider that. We took the question with the assumption that these were the assays, the validated assays would be employed as they stand, and we would accept that information, but we didn't really consider what would be or should be improved with individual assays.

DR. ZOELLER: I...I would at least want to...this is Tom Zoeller...to echo that concern. I think it's...I think there are a number of important published cases where this is true. And so, I worry that, you know, I mean, it's a tough call, because if you're only going to use a couple of doses, right in the beginning, we don't know where you are unless you do a full range testing. On the other hand, you have to be aware that this kind of situation is going to crop up.

DR. PORTIER: On the redundancy issue, we...you demonstrated that we have redundancies on the in vivo but very little redundancy on the in vitro testing. Does that bother you? Does that...I mean, it seemed to indicate that that was okay, but I'd like us

to discuss that maybe a little bit more.

DR. LASLEY: Lasley here. I...I was surprised, actually, when we broke the numbers down, because I thought it would be perhaps even the other way, but I think the reason is before that is the fact that the in vitro assays are more mature, they're easy to handle, they're more specific, and it's possible that you just don't need as many of them to get the same kind of information.

So, I think that's possible. On the other hand, just with the previous conversation, where we have concerns about levels and doses and what's happening, I think that's the area where improvements could be made by adding more...more efficient, more redundancy in the in vitro assays.

DR. PORTIER: Dr. Brown?

DR. BROWN: Brown. I'm not sure that we even have all the assays that we need, yet alone redundancies. So, I mean, in the ideal world that Dr. Zoeller referred to, what would we like to have? Well, we probably would like to have some more in vitro type assays that would span a wider spectrum of...of endpoints, because those tend to be, as Dr. Lasley said, the more specific, the more sensitive assays that really target a specific endpoint that's actually, you

know, absolutely measurable as opposed to the in vivo assays where we're left with redundancies but I think, in fact, we need those redundancies, because we're not as sure of what those endpoints are really telling us.

DR. PORTIER: Okay, before we go on to the last component, I just wanted to check with EPA. Did you get what you needed out of this conversation?

DR. TOUART: Just a clarification point in terms of MTD effects. I think part of the comment that Dr. Furlow made, and that's if we see an effect at the high concentration which may exceed an MTD and we have toxic effect, that we might discount what would be seen at a lower concentration. I think that, in general, the interpretation would be at the lower concentration. If there are no indications of...of toxicity at that level, you would like to be able to interpret any. That's part of the...the purpose of having more than one concentration in the...in the in vivo screens, in case the...the high dose is washed out.

In those cases where all doses have some, you know, overt or systemic toxicity, I think that the indication would be that we might need to...to repeat that. The intent is that...that we...we will have tested up to, you know, a...a limit dose or a

maximum, you know, level to make sure that we've covered, but the context is we don't want to exceed a...a...a maximum tolerated dose.

DR. PORTIER: Dr. Chambers?

DR. CHAMBERS: Okay, but most remain concerned about this MTD approach, though, because I understand what the rationale is to try to get...to get a worst case scenario so that you can identify effects. However, all of the animal's defenses or many of the animal's defenses are compromised at that particular point, and that's why you start seeing some toxicity.

And so, any kind of positive effect in the endocrine parameters here could just reflect the overall toxicity. That's been mentioned by others, too, and if the only positive you get is at the MTD dose, what are you going to do? Are you going to look to the lower doses to...to claim that it's a positive effect? Because I don't know that you can if the only positive effect is at the MTD dose, reflect the overall toxicity.

DR. TOUART: This is Les Touart again. Respond to that. I think, again, I think the concept is we want to be, you know, below one, an MTD, but we want to be approaching it in...in that context. If...if we do see, you know, toxicities and at that

level is the only one where an endocrine response is occurring but the next lower level it's not, then that's a little bit of a...of a...of a quandary of sorts, you know, for us.

So, I think any advice in terms of...of how to step down from that, whether we might need an additional, you know, level in something like the pubertals where there are only two concentrations, you know, those are some things, you know, to consider, but any advice in a better approach, then, in trying to...to use an MTD in a screening, you know, level mode or in interpreting that, I think, would be helpful.

DR. PORTIER: David?

DR. FURLOW: I mean, I don't know if this is the point for give and take sort of thing, but if I...I mean, so, I guess, not being a toxicologist by training, thinking of the MTD and just going...I guess the ra...I guess I don't understand the rationale for just going and say a half a concentration below. I mean, in...I understand we want to limit the number of animals. I am, certainly, actually am sympathetic to that, but, I mean, 10-fold below or 100-fold below, but...or just half below, how does at least a toxicologist come to setting up the minimal dose response curve, and how did you come up with just one-

half? Is that true for just the pubertals? Is that how it goes? It's MTD and then one-half the MTD?

That was my understanding, but I may be...I may be missing something there.

DR. TIMM: I think that the protocol has used a quarter of MTD or a half of MTD, and I think maybe looking at the data, that generally shows the right sort of spread, but, again, it depends upon the dose response curve with the compound and a lot of other factors. So, it's, you know, it's a rule of thumb that...that seems like it works okay, but you're right, to do those levels is...is difficult. You have to...you have to peg it pretty well.

DR. PORTIER: Dr. Bucher?

DR. BUCHER: So, just to carry this conversation on a little bit further, what kind of information do you expect that there will exist on these 73 chemicals in addition to the...the...the success or failure of this tiered approach is that it depends heavily on the 73 chemicals, and you picked the 73, because they have infor...well, because there's high exposure. So, presumably, they'll have a range of other types of toxicity information available.

How are you going to figure that into the...to...to determining whether the pubertal assays

are...are going to do the job for you or not given the fact that, you know, the information that may exist for these may allow very good dose selection in the male and the female pubertal or it may not? I mean, do you have any sense of...of what your 73 chemicals look like in that regard?

DR. TOUART: This is Les Touart in response. The majority of the compounds are pesticides, and most of those are FUGIs pesticides where there is a fair amount of mammalian data in terms of acute oral toxicities, other, you know, feeding studies, 9-day feeding study type...type...type data. So, there's a fair amount of information to help...to help focus, you know, where one might be maximum, but, again, there's always going to be, I think, some difficulties if you're trying to approach to a, you know, a level that's at the threshold of...of toxicity but below it, because the context under the conditions that one study and life stage that may have been tested versus the other, you have that situation.

On the...in the aquatic, you know, studies, it's a...it's a little bit different, you know, approach, and we do have more than the two dose levels. We're generally using three concentrations. We also have a limit which we don't test above 100 ppm,

because that's a very high concentration, and we don't feel that for compounds, it's, you know, you're not going to go above that, you know, approach anything.

And, also, we're not going to test above the...the...the limits of solubility for the...for the material in terms of what the organism would be able to be exposed or what we could maintain concentrations for, but we still would...would try to...to use levels that are below toxicity that would have been manifest in, you know, fish acute toxicity studies. Or for the frogs, we would usually be, you know, utilizing whatever information we had on...on the aquatic organisms, or we would have to do some level of range finding in terms of identifying toxicity going into the...into the screen proper.

DR. BUCHER: And are you going to be putting out guidance with regard to that last point?

DR. TOUART: This is Les Touart again. In response to the...for the fish and the frog in the...in the...the test method, there is some guidance associated establishing that the...the dose levels and...and...to use. I think that the caveat with that in terms of interpretation. Again, you know, it's a...it's a fairly, you know, narrow window that we might...might be looking at, and if we do see

toxicities of what might be perceived as toxicities through more systemic, you know, routes, you know, that isn't going to have to weigh into the interpretations of...of what those effects in general.

If...if...if the consideration is that...that we're dealing at...at...you know, in general toxic levels, we will try and discount those levels and...and move to the levels where we...we don't believe that's occurring to determine what other activities might be going on.

DR. CHAMBERS: To follow on Dr. Bucher's earlier point, if...if these are mostly chemicals that are very well characterized, because they're registered pesticides already, and you have the databases, are the doses for these particular endocrine disruption tests, are those using the information from all that database?

DR. TOUART: Les Touart. I...I think the...the general situation is...is we would like the...interpret those...those data and utilize those data, but, again, the...the life stages, you know, may be different in terms of...of the toxicities in terms of pubertal. I'm not as familiar with all the...the full data set that...that we have on...on even the mammalian, you know, set to determine if we have comparable, you know, information in terms of toxicity

for the...for the pubertal life stage or in terms of the...the stages that would be done at Hershberger and uterotrophic, you know, kind of context.

On the aquatic side, you know, we would, you know, have fish, you know, toxicity data. We may have fishery life stage, you know, data to...to also, you know, utilize in trying to establish the normal.

I think, you know, another thing in terms of the intent for...for endocrine screening is we...we're looking at...at evaluating levels of...of toxicity, you know, that are going to be in a...in a range below what our traditional toxicity tests and stuff are already identifying, you know, as...as concern levels and stuff, but in...in a screening, we do want to test at...at as...the highest levels as would be appropriate or practical within...within these assays to determine if we're seeing some level of...of...of a response in a screening, and then we're, you know, the intent is to use Tier 2 to actually do the...the dose responses and find out what the...the...the lower bounds of that are.

But if...if we miss it in the screening, then...then that would be, you know, a miss and...and become a false negative in the context.

DR. CHAMBERS: I do appreciate what

you're saying, but what concerns me is that if...if some of the pesticide modes of action are really well characterized already, say, they're neurotoxicants, and you know the levels at which neurotoxicity occurs, and you force the levels for these endocrine tests much, much higher than that in order to find that, is that going to happen? You're shaking your head. No?

DR. TIMM: Yeah, this is Gary Timm. No, I mean, clearly, if...there is a large database on...on the pesticides, and it would include 90-day synchronics, it would include two-generation tests for the presence of pesticides, two-generation mammalian assays. But they may be old. They may not have endocrine-sensitive endpoints in them, so things may have been missed. Probably developmental tox studies as...as well.

So, there's a lot of data that would...would pertain to this life stage, I think, for setting dose levels in this particular case. In some cases, they are known neurotoxicants and you're right. I mean, you would not force...your MTD would be set by whatever your...your database looks like. No, you're not going to run up above a dose level that has been seen to be an effect level in...in, for instance, a neurotox study.

When you get to the more beta poor chemicals, then, obviously, setting a dose level becomes a much more difficult sort of thing, and there may have to be some range finding studies of some sort to be done as just part of a general...general tox study design.

And for the...for the large volume inerts, there is a program, a voluntary testing program, that has been...it's both domestic and in conjunction with OECD where they are getting a minimum data set. And so, some of that information could be used, though they're not starting from scratch on those, either. So, we would hope that that would be taken into account when industry sets those levels, and I'm sure it would be.

DR. PORTIER: I think we'll go on to part C. Dr. Lasley?

DR. LASLEY: Lasley. Short answer is yes. I think we've already heard a very long list of...of...wish list of things that people would like to see: perhaps more specific assays for puberty, trans...transaction assay for androgens, perhaps improved steroidogenic cells for steroidogenesis, perhaps adding some development or organizational tests, certainly including more or better negative

controls, and certainly specific thyroid assays if they can be found.

But in general, I think, there's room for increased, more specific in vitro assays and certainly, I think, perhaps improved and more specific in vitro assays to replace some that are there that are a little difficult to interpret.

So, yes, there's room for improvement. I don't think anyone thought that this was a...a final list of assays or a final tier at this particular date.

DR. ELDRIDGE: Eldridge. I'd like to add to that just to feel that...that the SAP process seems to work pretty well and that I'd recommend that the Agency continue using the SAP as...as new tests and new parameters come along as a way to assemble a group of people to render advice, because this is a...a regularly constituted panel that can be called again and again, and it would be fairly simple to arrange it this way.

So, we would hope that this whole process would be ready to evolve and able to evolve as new technology, especially as new technology comes out and also after you begin to get some results on the 73 compounds that are on your list. I think there would be a lot of times when you'd want to come back to this

panel for more advice.

DR. KULLMAN: Seth Kullman. I don't have any comments on specific assays to add, but I would like comment on the fact of looking at the standardization of the assays that are actually currently on the books, and it appears from some of the discussions that we've had that there's some...some possibility to standardize some of these assays to a greater degree, make them more amenable to interlaboratory comparisons, and really provide a mechanism to tighten up on how these assays are run, depending on the...the labs and agencies that are going to be performing them.

DR. PORTIER: Anybody else want to comment on this? Dr. Furlow?

DR. FURLOW: Since hepatic toxicity could be one of the reasons why many of these hormone systems are affected, I wonder if it's possible to include a...a panel, just a general hepatic toxicity screen, either gene expression of p450...is there...is there a sort of a panel of genes and enzyme products from the liver that could be included to...to look at what might be going on? I mean, Dr. Zoeller already...already showed you enzymes affected indirectly that could then affect thyroid homeostasis.

And so, that might be something to add.

And the other thing, too, to reduce the number of animals or to tighten up some of the data, I mean, certainly about gene expression studies, quantitative PCR has, you know, sort of been alluded to. Maybe in some of the...some of the proposals, that's something that might be coming on line, but certainly, in the amphibian system, there are...there are some very nice, very strongly induced, and very specifically thyroid hormone lead and cell thyroid hormone induced genes that could be looked at with quantitative PCR that may make it more sensitive and actually tighten up the data and give you a better, say, okay, this is where we are in response to thyroid hormone signaling.

So, you know, those are my biases, because I work on gene expression, but I...but I think they actually do have some value, value added. Those are my main things.

I guess the...the...the major thing I, additionally, that I would also recommend in...in trying to get around sort of the false positive question we've had over and over again in the pubertal would be if you can find the negative. Right? So, the al...alcohol maybe has been suggested, but I...I guess

I would...I'd feel a lot more comfortable if you did have some sort of negative in...in the...in the pubertals before going forward.

DR. PORTIER: Dr. Cooke?

DR. COOKE: Gerard Cooke, yes. When considering a weight of evidence approach, there are some other things that you...you could incorporate which...Dr. Chambers' suggestion that there's a lot of data available on the 73 chemicals that you're going to choose such as exposure data and tissue distribution data and bioavailability data which you could then run, when you're running through the testing, on a weight of evidence approach, you could say well, okay, we...we've got all these yes and noes and...and maybes that might trigger to...to test which may have been done and may have already been proved negative, some of the endpoints, and then you can go back and look at the tissue distribution data and the bioavailability data and say these would have prevented us from having to go to...to...to a test. I mean, that gives you credence for your Tier 1 tests.

DR. PORTIER: Dr. Vandenbergh?

DR. VANDENBERGH: Yes, Vandenbergh. I'd like to really emphasize something that Bill Lasley said a moment ago about the...the effects of hormones

do occur both as an organizational phenomenon very early in life and then activational, and what we're dealing with here is almost all activational. And I think that somehow...and I can't come up with a nice simple little assay for you, because it's a complicated question...but it needs to be addressed that we're going to see long-term effects in the animal and probably in the human population as a result of some relatively minor changes during fetal development.

And those effects include things like the brain is organized between masculine and feminine areas. It includes a variety of different organ systems that are affected by this.

I know that's a very complicated story. It's probably going to end up being in your care, too, but I think it's...it's something that is well worth exploring.

DR. PORTIER: Dr. Furlow?

DR. FURLOW: Some of these developmental things get me going. So, in...in five words...no, five minutes or less, I wasn't here for the presentation, so that...that may have been covered, but the intrauterine to lactational assay was not accepted, and I haven't read through all the reviews, but, you know, what, beyond amphibian metamorphosis assay, then, what other

kinds of developmental assays is the EPA considering or...or not considering?

I mean, is your experience with the intrauterine to lactational so disheartening that you don't want to go...go beyond? I...I think it's incredibly important, personally. So...and...and to rely totally on the amphibian assay as the only developmental one and...and a very specific one, thyroid hormone, at that...may...may...may not be a good idea.

DR. TIMM: Yeah, this is Gary Timm. We did bring the...the whole question of whether the inutero/lactational assay should be in Tier 1 or not before the SAP about a year ago, and, you know, it was, I think, the consensus probably of EPA staff and the consensus of the panel that gee, it would be great to do it, but it is so long, so expensive, so complicated that it really did not fit the definition of a Tier 1 screen, and we...we felt that a better design of Tier 2 than what we have was where we should put our energy, and that...in fact, that's what we're doing.

We're looking at a...a one-generation assay that utilizes more animals than...utilizes virtually all the animals to increase the...the representation of the...the litter so you characterize

the...the effects within litters much better than...that what the current two-gen does. So, it's a, I think, a more effective test and adds more endocrine sensitive endpoints, and it's a shorter-term test than the current two-gen.

So, that's what we're doing. And it...the philosophy clear back in the EDSTAC days...and I...I don't think anyone in the, you know, research labs or...or the literature has shown that we would miss things.

I mean, what we're looking for in Tier 1 is a signal that there is a problem with the endocrine system, that a chemical is causing some perturbation of the endocrine system. Identifying the adverse effects and getting a dose response relationship between the...the chemical and the adverse effects are what we do in Tier 2.

So, we're...we're happy if we can get the signal in Tier 1 that we have a problem, and we're' even happier if it gives us some ideas about how to...how to proceed in Tier 2, but...but...but that's maybe more wishful thinking than always the case.

Obviously, we want Tir 1 to be an effective filter and keep the things that are...are not a problem out of Tier 2 and flag the ones that are a

problem to us so that they can go on to Tier 2.

DR. TOUART: Just to talk about some other assays that are kind of in development, you know, but we really haven't considered them as far as...as...as Tier 1 just because of the...the length of time, and I think any time you start dealing with organizational type studies, you generally have to carry these out long enough for individuals to...to...to mature and...and the like, and that's been the...the big limitation in the context of our Tier 2s are established to do that.

But you want an assay that's being developed at the OECD level. There's a fish sexual development, you know, test. And...and that assay looks at...at the fish in the EN through early development, and with species like the medaka and zebrafish, we do have genetic sex monitors so that we can indicate, you know, the difference between the genotypic and phenotypic, you know, sex changes.

And that's in development, but these are 60-day, you know, plus type...type tasks and really extensions of...of something like the existing early life stage test which is a...a traditional test.

In...in the development or pursuit of the Avian 2-generation test, we investigated an egg

injection, you know, method as a means of doing kind of a range finding for...for looking at...at that, but to do the egg injection, you...you're doing...letting these birds, you know, hatch and then reach maturity before you...you can collect the markers to indicate that you had some effect, you know, by the...the, you know, hormonal disruption that was in the embryo.

So...so these are some...some context, but, again, the...the difficulties with those have been, you know, we can't find something that...that, you know, would really be in, you know, the kinds of time frame that some of our other assays are which are still, you know, in...in a relative sense, not...not short assays, I mean, when you're dealing with 21 days or...or longer.

So, you know, that's the context. If there are some suggestions of...of assays that might exist or might, you know, be on the horizon or areas that might be worth pursuing, I think that's something to consider, but, again, the problem right now is...is we don't really have a viable, you know, candidate that probably can move forward with in this case.

DR. VANDENBERGH: I'm going to make one more comment along that line, the...the Tier 2 test, and that is it's been since '96 that we've been working

on getting the Tier 1s organized, and it looks like it's getting closer and closer. I'm sure that the public and, as you mentioned, the Congress is a little concerned about how long it's been taking to do that.

Is there a plan afoot now so that you've got a schedule of what needs to be done to set up one or more Tier 2 tests? Because I assume that maybe before too long, you'll have Tier 1 data coming in that there were some effects.

DR. TOUART: Yes, it's, you know, some of the advancements and evolutions, improvements of...of the rodent 2-generation, you know, assay, the generation test, you know, this exists, and we consider that very valid. There are some additional endpoints that have been added, and there are some others that are maybe still under consideration, and...and if those are perceived to be valid, those would be added into that method.

On the...for the other, you know, toxic groups, we do have projects ongoing in developing fish 2-generation, an...an avian 2-generation, you know, assay. We're looking at an amphibian, what we're calling a growth reproduction assay, because we haven't figured out a...a viable paradigm for...for doing a full life cycle and having the frog reproduce, and

we're...we're using a...a...a Xenopus species, you know, in the pursuit of...of that which we have been able to...to spawn within the laboratory, at least on a consistent basis.

The anticipation, you know, for these...and we also have a...an invertebrate that we're...we...we're using in Tier 2 to determine, since there is ability for vertebrate active, you know, materials that interfere with the invertebrate, you know, hormonal system, so we felt that anything that tested positive we wanted to evaluate in...in that context, so we do have a 2-generation mysid, you know, test that we're...we're looking at in developing.

In developing the eco, you know, assays, we're really, first of all, looking at value added of the 2-generation test in terms of what more it gives us than existing methods that...that guidelines exist for avian reproduction and fish, you know, life cycle testing and the mysid life cycle testing. On the frog side, we really have no full chronic, so that has to...has to be developed.

The time lines that...that we have right now is anticipating trying to have these studies through in a laboratory, you know, testing by 2010. That's optimistic that everything looks right with the

first time.

We do have some initial, you know, trials that have been completed or on their way in terms of establishing a standardized method that could then go into interlaboratory testing.

We have partnered with some international colleagues. Especially, Japan has been interested and have been very helpful in pursuit of the...the fish 2-generation test and, you know, are ramping up to assist in the pursuit of the amphibian growth reproduction, you know, assay.

The avian 2-generation is the one that's the...the...the most limited, because the...the U.S., you know, and our program is the only one that's really putting forth the...the resources, but we have projects associated with some other partners, including the Department of Army and the USGS who have, you know, their own in...interest in...in assisting in developing these longer-term type tests in avian species.

DR. BROWN: Brown. I guess I'd like to go back to the rationale for the...the 73 chemicals that have been selected, and I...I just do not...don't know exactly what the rationale was for the selection of these compounds.

I mean, obviously, they're...they're

pesticide, heavy on pesticides which are probably also in the environment, considerable amounts in the environment, but as I recall, going back to some of the...a couple of the other groups in which I participated in...in this overall process, the emphasis seemed to be on selecting compounds from a wide diverse range of different chemical classes rather than kind of focusing on...on...on single classes where I would assume the pesticides, in general, tend to fall in similar chemical classes, a few chemical classes rather than a wider range.

DR. TIMM: Gary Timm. Well, first of all, one has to remember that the...what we're required to do by law, and we are required to screen pesticides. So, that was...was one of the things that we would have foremost in our minds, that, you know, if you don't do anything else, do what the law tells you to do.

And we had originally attempted to use a high sequence screening that...to help us sort out through some other...other candidates, as...as I mentioned in my remarks yesterday. That didn't work terribly well.

We took, really, off-the-shelf methods that the pharmaceutical industry found satisfactory, and, of course, they're looking for...they're looking

for very powerful compounds. They're looking for the ethanol estradiols in the world. They don't want a birth control pill that's the size of a football.

So, they're not looking for...for weak stuff, but the stuff that...that we have is several orders of magnitude less potent, typically, than...than your pharmaceutical levels, so it's that we had to...to optimize those assays, and, you know, at the time we were making decisions about picking chemicals, that hadn't happened yet.

So, we didn't...didn't rely on that technology. We moved to a strictly exposure-based system, because we also did this pilot study that I mentioned where we looked at existing data and we said, you know, that stuff isn't...the payoff that we would get from looking at existing data as...as to hits that...of chemicals that we should proceed with wasn't worth going through the data. We, you know, on the 30 chemicals, there was a lot of old stuff there. There was nothing that would really tell us to move forward, and this was...mainly, this was all pesticide actives.

So, we said we...we should focus on pesticide active. We should also use high production volume inerts, because they're pesticide chemicals under the law as well, and we will use just strictly an

exposure base for this first 50 to 100. We started off with slightly over 100 and...and we winnowed it down, looking at these exposure databases, to 73.

And that...we proposed the methodology, got comments on the methodology. People were not wild about it, because everybody really would like to have had a hazard basis but said, you know, given everything else, this is a reasonable approach.

We...we've done it. We put out a preliminary list. We're...we've taken comments on that list, and so, a final list will come out along with the...the orders for testing and the...the final battery. And that's, you know, the target for all that stuff is...is August of this year.

DR. PORTIER: Well, this has been fun discussion, but we need to kind of come back and finalize our program here. I think we've covered all of the questions that EPA has...has asked. I just want us to take this one last opportunity to go through the panel and see if there's any topic that we haven't touched on that you'd like to make sure we discuss and include in our...our report.

And...and yesterday, if I heard right, EPA did open the door that we can make recommendations on where we think short-term research might want to be

directed. Clearly, the in vitro methodology is an area that's going to show up in our report, but I just wondered if there were other things that haven't been discussed yet that we should put on the table and discuss in these final minutes.

Dr. Lasley?

DR. LASLEY: Well, I'm not sure that these things weren't brought up or discussed, but I...I think there's clearly some avenues that are opening up. I mean, there are new endocrine disruptors being described in the literature, and these are going to go beyond estrogen, androgen, and testosterone. Specific are...are thyroid and, specifically, the glucocorticoid assays.

And I think the technology in signal transduction assays is...is growing by leaps and bounds, and I think this will serve well to...to fill in the area of in vitro assays and...and take some pressure off some of the less specific in vivo assays.

So, I think these are two areas that, you know, are definitely going to come up.

DR. PORTIER: Yes, Dr. Zoeller?

DR. ZOELLER: You know, I...I've said this before, but I guess I'll say it again within in this context, but I do think that...that the thyroid

system is...is certainly the least represented in the tier, and, you know, maybe depending on your research bias, it's either the more important axis or maybe the less important axis, but, certainly, in terms of public health, there's a lot of good reason to believe that thyroid function is important.

And having...having specific and sensitive measures of thyroid hormone disruption at the...at the receptor tissue response would be a really valuable thing to have. I don't think anybody would dispute that, but I think that there are probably a few ideas that are...that are at least manageable within the context of Tier 1 that would be good to develop.

It's clearly not there, but just in terms of development, if there...if there are specific research avenues that EPA were going to take, it seems to me that that's something that really needs to be built up into the tier.

DR. PORTIER: Dr. Timm?

DR. TIMM: Gary Timm. I'm aware that the Japanese have developed a transductional activation assay for TR-alpha and TR-beta. I...I don't know how far along it is in terms of, I mean, they've run a bunch of chemicals through it, but validation of such a system would be difficult if you don't have a number of

chemicals that are...are flagged as...as interfering with the receptor.

So, I don't think it's progressed very far, but...but, at least, a start on that technology exists.

DR. PORTIER: You were asked. Last chance for comments. Dr. Furlow? I knew I could count on you.

DR. FURLOW: Yeah, well. It's...this has been touched on, but I guess one other concern I have. It's impacted my...the research I do in my own lab, and that is...and that is the somewhat controversial of...of strain differences, and I...I do understand that in...in your experience, the experience of the EPA group, this is...this hasn't made a big difference, but I guess for future research, I notice in discussions of...of strain differences and...and sensitivity of these different compounds, though, that when that was discussed in either the...the ISRs or...or answers to the peer reviewers, basically, the...the answer was well, you know, yes, there are strain differences, and it would take a really long time to figure out the basis of those strain differences and whether or not, you know, what strain is...is appropriate for what...what assay.

And so, I understand the...the time and convenience and the time pressure and...and the money pressure you guys are on, but I guess, for future down the road, I'd like to...I'd like to put in a plug for trying to understand what those genetic differences are if there...there's research that can be done, either supported by the EPA or other agencies to try to figure out what...what is it that makes one strain more sensitive to chemical X than others.

Is it simply...is it simply the liver, or is it any number of things, receptors, et cetera? And so, I'd just kind like to put in a plug for that and...and also just make sure that just, again, to tell the EPA I think you guys need to keep paying attention to that, because I...I'd hate...that is a place where you could have false negatives.

I know in my...my own research, we study glucocorticoids, and looking at muscle mass loss in C5756s or, essentially, we're almost a factory to dexamethazone. We give it to 5Cs, and bang, within a week, their muscles are shrinking like crazy. So, you know, why is that? We're trying to figure that out, and, actually, if we figure out the genetics of that, it might be very interesting from a basic science question.

But I also...also think it's a very important question in terms of toxicity testing broadly, even beyond endocrine disruption. So, as a plug for future research, I mean, you...you could even criticize, easily, the Xenopus labis assay from that standpoint as well. I mean, this is not a North American species.

This is an animal that's adapted that's completely aquatic, et cetera, and even if you...you know, I know there were some...some discussion in looking at Xenopus labis versus Tropicales, Tropicales is harder to raise and et cetera, but, you know, you could...you could criticize any of these assays from that standpoint. I...I understand that, but I think it's something that if...I know it can't be done in the short term, but I think it's something that, as a priority in toxicology research, I think it's something that we ought to think about moving forward.

DR. TIMM: May we interject? Ralph Cooper as a...an observation that is, I think, pertinent to some of the discussion we've been having, so I would like to turn the mike over to him for a few minutes.

DR. COOPER: Ralph Cooper, EPA. There was two discussions about some things that might be

added, one of them, I think, in the thyroid axis with some of the maybe materials you could add a few endpoints that might be useful in interpreting thyroid mode of action. One of them was cholesterol.

And then there was the comment about looking at the liver as we're trying to evaluate liver function in this...in tier protocols, and I wanted to mention that the clinical chemistry panel has included those, and I think rather than just say that that's in there...and I can't list all the things that are included in it...but if you could give us some insight as to a), how to use those measurements when we evaluate the data, I think that that would be very useful.

So, that's part of that working protocol, whether or not that would help us out with that.

DR. PORTIER: So noted. Not seeing any enthusiastic hands to continue the discussion, I think I'm going to ask EPA if they got out of this...enough out of this last discussion. And we're certainly going to write. Hopefully, we're going to capture everything in our minutes, and...and you'll see that, but any final comments?

DR. TIMM: Gary Timm. I...I...I

think...I think we have a fairly clear sense of what the panel feels about...about the battery that we proposed, despite some misgivings, certainly, that we have heard from...from people yesterday.

I think there was certainly concern that the battery was too redundant and that it...it should...it was...it's costly, but what I heard today is that we would lose valuable components if we...if we did remove anything that...that we had proposed, and I guess that...that's a very, very useful message for us to...to take home.

And we also appreciate, once again, I...I think reminding us of some of the problems that...that still exist that we can work on, and we look forward to moving ahead and appreciate your...your input.

DR. PORTIER: Dr. Eldridge?

DR. ELDRIDGE: But I would follow that up by suggesting that, as time passes and technology improves and you also collect more information, that some redundancies that may appear to be candidates for omission. Unless you could find better tests, more specific tests, and...and other ways of getting at the questions, particularly with regard to specificity, and...but because there's always going to be this

concern about the large-scale in vivo testing, and...and so, finding ways to...to reduce that would...so, the suggestion is to keep alert for potential ways to reduce the redundancies if you can find better specific substitutes.

DR. PORTIER: Okay. I think, at that point, we're finished with our regular program and all the questions, and we've made the rounds. So, I'm going to turn it over to the Federally Designated Official to formally close the meeting.

MR. DOWNING: Thank you very much. Well, as we draw our day to a close, we find we've come to the conclusion of the meeting of the FIFRA SAP on the Endocrine Disruptor Screening Program Proposed Tier 1 Screening Battery.

I want to thank everyone for their participation in this meeting. I think it's been excellent, and we've had a lot of really excellent presentations as well as the wonderful exchange of ideas. I think the Endocrine Disruptor Screening Program folks have a good sense of the panel's thoughts about all that.

I would like to mention that the presentations and the slides, even those that we've seen this afternoon, will be available on the OPP

docket shortly. I'll say maybe tomorrow, as well as, of course, within 90 days, we will be preparing our final report, the meeting minutes, essentially, which will also be published.

Thank you. Good to know.

All the documents that were presented yesterday are now on the docket, she tells me, so we're getting better and better and quicker and quicker at this. So, anyway, that is available as well, as I say, the documents today will be up there shortly as well.

Well, with that, then, I will draw to a conclusion of this meeting of the FIFRA SAP. And, again, thanks to everyone and thanks to the audience for hanging in there with us as well, and we will be adjourned.

DR. PORTIER: And if the panel will meet in the break room in, say,10 minutes. Give you a chance to get your stuff together.

(**WHEREUPON**, the meeting was adjourned at 3:38 p.m.)

CAPTION

The foregoing matter was taken on the date, and at the time and place set out on the Title page hereof.

It was requested that the matter be taken by the reporter and that the same be reduced to typewritten form.

Further, as relates to depositions, it was agreed by and between counsel and the parties that the reading and signing of the transcript, be and the same is hereby waived.

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COMMONWEALTH OF VIRGINIA

AT LARGE:

I do hereby certify that the witness in the foregoing transcript was taken on the date, and at the time and place set out on the Title page hereof by me after first being duly sworn to testify the truth, the whole truth, and nothing but the truth; and that the said matter was recorded stenographically and mechanically by me and then reduced to typewritten form under my direction, and constitutes a true record of the transcript as taken, all to the best of my skill and ability.

I further certify that the inspection, reading and signing of said deposition were waived by counsel for the respective parties and by the witness.

I certify that I am not a relative or employee of either counsel, and that I am in no way interested financially, directly or indirectly, in this action.

MARK REIF, COURT REPORTER / NOTARY

SUBMITTED ON March 26, 2008

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